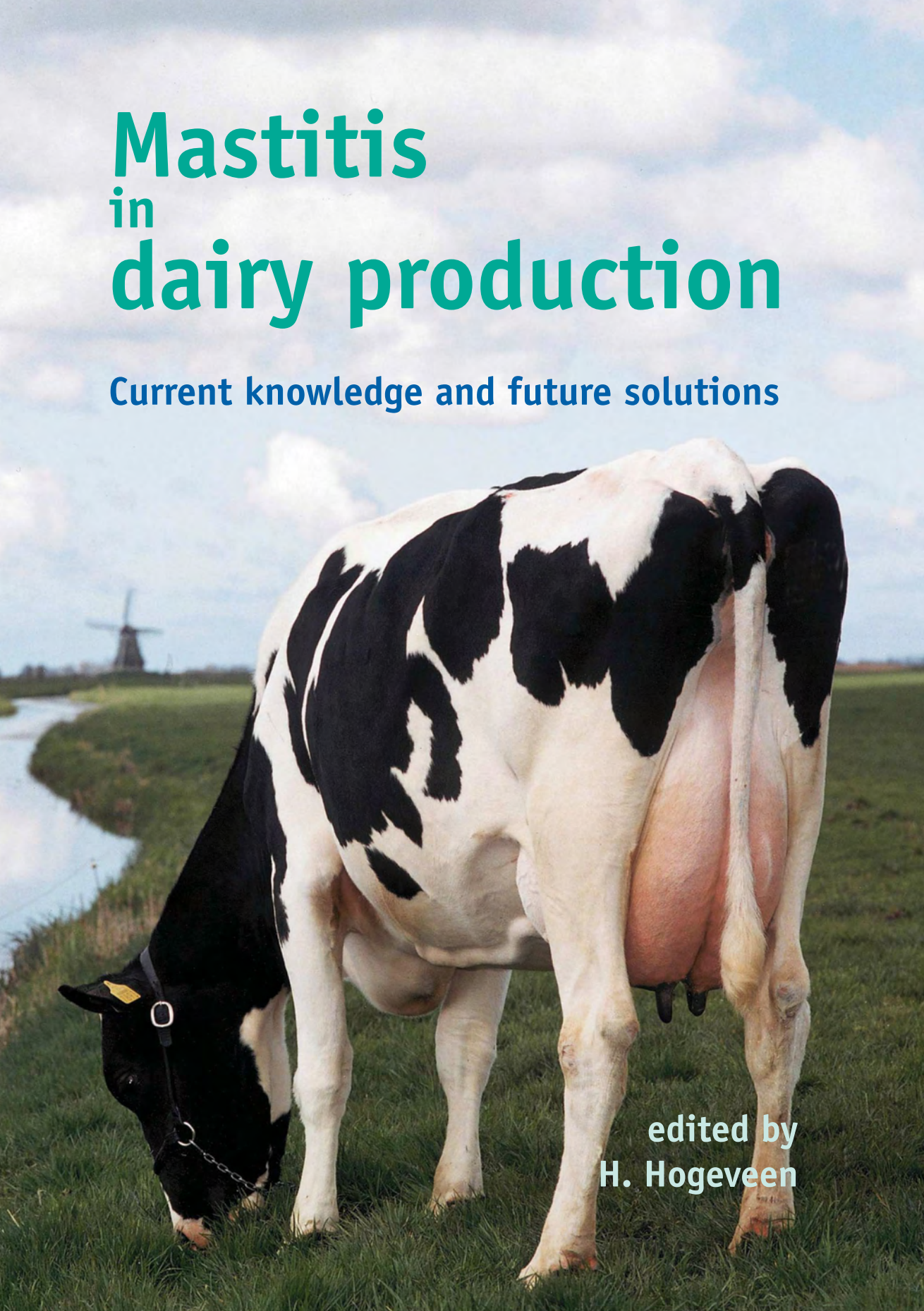
Mastitis in dairy production

Current knowledge and future solutions



edited by
H. Hogeveen

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Preface

For more than 100 years, the International Dairy Federation (IDF) is an active organization, providing knowledge to the dairy industry worldwide. Since many years IDF is providing expertise on mastitis, first through the activities from the working group A2, later this work was combined with other work on animal health in the Standing Committee Animal Health. The working group A2 took the initiative to organize a large international mastitis seminar in 1975. Some 150 delegates from all over the world met in Reading (United Kingdom) and a little more than 70 presentations were made, starting a great tradition. Throughout the years, the IDF Mastitis seminar was held in Kiel (Germany; 1985), Tel Aviv (Israel, 1995) and Maastricht (the Netherlands, 2005). For the last meeting, it was envisaged that the attendance would again increase and the name Mastitis seminar was changed to the 4th IDF International Mastitis Conference.

The Conference was organized by the Netherlands National Committee of the International Dairy Federation under the auspices of the International Dairy Federation and is supported by the Dutch Dairy Board, the World Organization for Animal Health (OIE) and the NMC. The organizing committee, consisting of people from all Dutch mastitis research organizations, put a lot of effort in the organization of the Conference and made the exchange of knowledge, views and ideas possible. The organization was financially supported by many sponsors, which are acknowledged for that.

The 4th IDF International Dairy Conference was attended by more than 500 full delegates. Some 400 persons attended one of the days. More than 350 presentations were given. This growth in numbers reflects the growth of interest in the field of mastitis. Although the research subjects changed over the years, one thing is clear: Mastitis is still one of the most important diseases in the dairy sector. Being a multifactorial disease, caused by multiple pathogens, control remains a difficult issue. Mastitis not only affects the health of milk-producing animals, having consequences for the profitability of dairy farms, it also affects the animal welfare. Moreover, mastitis negatively influences the milk quality having consequences for the dairy processing industry. In other words: mastitis affects a large part of the dairy production chain.

This book reflects the current knowledge from all over the world on mastitis as it was presented during the 4th IDF International Mastitis Conference. Out of more than 350 submitted abstracts the scientific committee selected 98 oral presentations. The members of the scientific committee and the many people that reviewed submitted abstracts are gratefully acknowledged for their efforts. The papers of the selected oral presentations and of 13 keynote presentations are combined in this book. It is my belief that this book does not only reflect the **current knowledge** of mastitis control but is also providing ideas for **future solutions** for control measures.

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editor

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Inhoud

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Keynotes

Mastitis research and control: Where do we come from and where are we going?

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Abstract

The history of the IDF International Mastitis Seminars is reviewed. The seminars held in 1975, 1985, and 1995 are summarized and some of the highlights noted. On the basis of number of papers presented and the number of attendees, there is obviously continuing interest in bovine mastitis control in dairy herds. Since the first seminar in 1975, there has been a decrease in the discussions of definitions and proper techniques for the diagnosis of intramammary infection. My opinion would be that there is need to continually address these issues by an international group of mastitis experts as they do impact the quality of research submitted for publication in peer reviewed journals. Such experts currently exist in the Mastitis Action Team of IDF and the NMC.

Introduction

We are gathered in Maastricht, The Netherlands for the fourth in a series of international mastitis seminars sponsored by the International Dairy Federation. The first three were products of the A2 Group of Mastitis Experts of IDF. With rearrangement of the Program of Work and the structure of IDF, the A2 Group of Experts was abandon in approximately 1999. The work of the former A2 Group was continued under the new Standing Committee for Animal Health and more specifically, the Mastitis Action Team of that Committee. It is the Mastitis Action Team that is responsible for the current seminar.

In all cases, the seminars have produced a truly international group of presenters and attendees. In the case of the first three seminars, there was a progressive increase in attendance and papers published in the proceedings. The proceedings from all three have become valuable resource material on mastitis the disease and control of mastitis in dairy herds.

The purpose of the present paper is to provide the attendees of the Maastricht Seminar with a brief perspective of past seminars and hopefully provide a sense of where we have been in terms of our understanding of mastitis and mastitis control. Hopefully, this information will provide a basis for assessing the progress or lack thereof that has been achieved.

The 1975 Seminar

The first IDF international mastitis seminar was held in Reading, England and was organized by a United Kingdom working committee under the chairmanship of Dr. F.H. Dodd with several recognizable names serving on the committee, ie. A.J. Bramley, J.M. Booth, T.K. Griffin, R.G. Kingwell and C.D. Wilson. There were approximately 150 delegates from

24 different countries attending the seminar and a total 69 papers were published in the proceedings.

Six scientific sessions were organized around the following topics:

- Diagnosis of Mastitis and Intramammary Infection (6 papers);
- Somatic Cell Counting (10 papers);
- Prevention of Infection (15 papers);
- Elimination of Infections (10 papers);
- Special Aspects of Mastitis Control (8 papers);
- Implementation of Control Techniques (10 papers).

In addition there was an introductory paper by Prof. A. Tolle, Kiel, Germany and A Review of the Seminar by Dr. F.H. Dodd (UK). Prof. Tolle elaborated on what he termed "9 statements" (Tolle 1975a) and these statements shed light on the prevailing knowledge and ideas being considered important in 1975.

Statement 1 was; "The defining of mastitis by a Group of Experts of IDF was, without doubt, an essential step forward in uniform and comparative quantification of diagnosis in the sub-clinical range; it must, however, be improved and include physiological factors such as breed and age of the animals as well as the dynamics of the cytological process". "Definitions such as "latent infection" and "nonspecific mastitis" should be reconsidered." The definitions of various mastitis conditions and what was and was not considered to be an infection of the mammary gland were much debated within the A2 Group of Mastitis Experts during this period of time.

Statement 2 was; "Many factors contribute to mastitis and infectious agents set the character of this disease; nevertheless it is essentially influenced by management. The comparatively small success achieved in mastitis control appears to be due to the fact that scientific interest has been mainly concerned with the infectious agents and not with the conditions of infection". His contention was that we needed a better understanding of the interaction of the cow, microbes and the environment.

Statement 3 was; "Though we have gained knowledge of the fundamentals of transmission of mastitis agents between and within animals during the past years, our knowledge of pathogenesis especially of the invasion of microbes into and penetration through the streak canal is still incomplete, though this is the crucial point of understanding the process of infection". An excellent point and in the year 2005 we still do not fully understand the exact mechanism(s) by which microorganisms penetrate the streak canal.

Statement 4 was; "Many mastitis agents - e.g. Group B streptococci and enterotoxin producing staphylococci - are pathogenic to humans, and therefore represent a danger for public health. Control of these agents with the framework of the overall concept should be given priority". The potential impact of mastitis on human health is still debated.

Statement 5 was; "Every microorganism may produce mastitis under suitable conditions. As regards pathogenicity, however, there is a clear order of merit. There is evidence for the assumption that, in cases where the usual pathogens are kept away from the mammary gland, other organisms come into prominence according to their ability to invade and their pathogenicity (Enterobacteriaceae, *Pseudomonas aeruginosa,* Mycoplasmas and others). Complete elimination of mastitis thus appears scarcely to be possible". Certainly many herds have seen a shift from problems with contagious pathogens to environmental pathogens and environmental mastitis is now a major focus of much research.

Statement 6 was; "Success in reduction of the rate of new infection (prophylaxis) does not appear to be very impressive, but is a safe way to solve the problem in the long run". I believe that many would now take issue with the first part of this statement. Many countries have implemented programs that significantly reduce the rate of new infection by contagious pathogens and alterations in housing and management can reduce the rate of new infection by the environmental pathogens.

Statement 7 was; "Chemotherapy and culling are the methods available to eliminate existing infections. Just as it is necessary by hygienic methods to prevent new infections so we must cull cows which do not respond to treatment. Antibiotics are very useful if they are properly applied, but dangerous if used in excess". Many of us would still agree with this statement.

Statement 8 was; "The efforts made in mastitis control will only succeed when the farmer is convinced that, with the same employment of land, capital and labor, he will gain much higher economic returns without mastitis. From current information on the state of health of his herd he should always know whether or not his profit is diminished by mastitis." Good advice then and good advice in 2005.

Statement 9 was; "As examples have shown from different countries or regions, the present state of knowledge allows the frequency and degree of mastitis to be reduced effectively. The question is: where is the optimum expenditure and control under the particular economic conditions?". Progress on mastitis control has been achieved in many countries and clearly not all countries have achieved control by applying the exact same measures. There is not one absolute way to achieve success.

In his review (Dodd 1975) of the seminar Dr. Dodd stressed that to control mastitis we must control intramammary infection and that for surveys, control schemes or large field experiments, diagnosis should be based on bacteriological tests alone and there was little need to also apply some measure of inflammation. He noted that electronic cell counting was making great progress and would certainly help implement mastitis control schemes. The general consensus was that bulk tank milk somatic cell counts would be useful indicators of the "... total effect of the mastitis in the herd". Dodd maintained that there was no absolute threshold cell count separating infected from uninfected quarters, although evidence was presented indicating that it was reasonable to assume that herds with a cell count of bulk milk exceeding 500,000 cells/ml had a mastitis problem. As for prevention of infection, post milking teat disinfection was seen as a very useful technique. It was recognized that the milking machine contributed to exposure to mastitis pathogens and to intramammary infection, however, modifications which would minimized the machines contribution were not entirely clear. Dodd commented that work on the natural defense mechanisms of the mammary gland were contributing to our understanding of the nature of the infection process in the gland but that to date "the principles have not been harnessed into techniques useful in control schemes". The role of antibiotics in elimination of infections was pointed out and it was noted that "many delegates were concerned about possible increases in antibiotic resistance of pathogens, antibiotic residues in milk, the greater chance of infection with primary pathogens if antibiotic therapy greatly reduced the infection with secondary pathogens, and the trend to lower cure rates for staphylococcal infection when drying off therapy is used for all cows". Many of these concerns are still with us in 2005. Finally Dr. Dodd worried that, "...so few (research) workers carry out research on control itself". "The way in which techniques are integrated to give the greatest degree

of control cannot be assumed, neither does a potential hazard always materialize." "There is no doubt that field research is expensive but unless this type of work is increased, the full value of the smaller scale basic work will never be realized."

The 1985 Seminar

the second IDF Seminar was held in Kiel, Germany in May of 1985 and Prof. A. Tolle and Prof. W. Heeschen were primarily responsible for arranging the Kiel seminar. A total of 87 papers were published as a special volume of the journal Kieler Milchwirtschaftliche Forschungsberichte.

There were 8 main sessions organized and the session topics were:

1. Current Control Methods, Effects of Mastitis on Processing and Human Health, and Economic Losses
2. Diagnosis and Definitions of Mastitis
3. New Developments in the Identification of the Diseased Udder
4. Practical Methods for the Control of Mastitis
5. Natural Defense Mechanisms and the Immunological Approach to Control
6. Genetic Approach to Mastitis Control
7. Antibiotic Therapy
8. Less Common Forms of Mastitis

The most striking difference in session titles when comparing to the titles used in the 1975 seminar, is the addition of sessions on Natural Defense Mechanisms and the Immunological Approach to Control and a session on Genetic Approach to Mastitis Control. As an interesting note there were only 6 papers addressing the subject of antibiotic therapy.

Dr. F.H. Dodd was again an important participant giving both the introductory paper (Dodd 1985a) and the review of the seminar (Dodd 1985b). Session 1 pointed out the growing importance of the relationships among mastitis, effects on milk processing and the possible effects on human health. Clearly this is still an issue of interest in 2005. Session two pointed out the importance of clean sampling procedures in diagnosing subclinical infectious mastitis. Dodd concluded that, "a standard bacteriological test based on a single milk sample will give results with small errors." "The inclusion of a cell count threshold will, under some circumstances, reduce the errors and in research when measuring new infections or the elimination of infections a diagnosis on two or three samples is necessary."

Session 3 produced many papers looking for markers of inflammation (mastitis) other than milk somatic cells. Papers reported on conductivity, lactose, NAGase, antitrypsin and serum albumin. Too quote Dodd, "none of these were superior in all respects, and the choice depends on the objective of the work". Many of the tests were being considered for continuous inline measurement of the parameter in cows as a means of screening for infected cows and particularly newly infected cows. We see similar interest in 2005 specifically in reference to the needs of robotic milking. Papers in session 4 emphasized the important differences in epidemiology for control of the various pathogens and the important progress that had been made with regard to milking machines by reducing bacterial transfer, damage to teat ducts and reduced penetration of the teat duct by pathogens.

There were a large number of papers presented in session 5 on Natural Defense Mechanisms and the Immunological Approach to Control. In the 1985 seminar a major new concept that was presented was that of the Intramammary Device (IMD) designed to

stimulate the cellular defenses against infection. Subsequent research revealed that negative aspects of the IMD could not be over come and IMD research ceased. This session also contained papers showing the importance of vitamin E and selenium in resistance to infection, effects primarily mediated through improved function of phagocytic cells.

Session 6 on Genetic Approach to Mastitis Control provided evidence that the advent of electronic cell counting and computerization of records now made possible the ability to breed cattle for resistance. How the records and the data would be used for this purpose was an area of much discussion. The Antibiotic Therapy session 7 was interesting from the fact that there were only 6 papers on the subject and there was little new information provided. Interestingly, the main papers in session 8 on Less Common Forms of Mastitis dealt with *Escherichia coli* mastitis, mycoplasma and the complex udder disease syndrome known as "summer mastitis". Papers suggested that control of E. coli mastitis centered around bedding and that fly control was essential to control of summer mastitis. An interesting summary statement by Dodd was that, "Somewhat surprisingly few papers were presented on either the epidemiology or therapy of mastitis, the areas which so far have been the most productive in developing control methods."

The 1995 Seminar

The third IDF Seminar was held in Tel Aviv, Israel and Dr. A Saran and Dr. S. Soback were largely responsible for the organization of the seminar. Attendees were from 33 countries and approximately 214 papers were presented. There were 8 sessions and they were:

1. Natural Defense Mechanisms of the Lactating and Dry Mammary Gland (24 papers or 11.2% of papers presented)
2. Identification of Mastitis Pathogens (26 papers or 12.1% of papers presented)
3. Mastitis, Public Health and Milk Quality (34 papers or 15.9% of papers presented)
4. Mastitis Control Systems (41 papers or 19.2% of papers presented)
5. Treatment of Mastitis (43 papers or 20.1% of papers presented)
6. Epidemiology of Mastitis (31 papers or 14.5% of papers presented)
7. Milking Machine and Udder Health (12 papers or 5.6% of papers presented)
8. Genetics and Mastitis Control (3 papers or 1.4% of papers presented)

Each session began with an invited review paper. The invited paper for session 1 was given by Prof. Burvenich (BE) (Burvenich *et al.* 1995). The review and the session papers presented showed that considerable progress had been achieved with regard to our understanding of the natural defense mechanisms including the role of immunity. There was clear evidence that our knowledge of the role of cytokines, T-lymphocyte subpopulations, and the arachidonic acid metabolites in the bovine mammary gland had made major progress in the previous 10 years. However, there was still no evidence that this knowledge was being incorporated into control schemes and resulting in reduced mastitis in dairy herds.

The review paper in session 2 was presented by Dr. U. Vecht (NL) (Vecht, 1995). Dr. Vecht discussed conventional techniques used for infection diagnosis as well as newer diagnostic techniques that were available. Many of the papers presented in this session involved evaluations of new bacteriological tests that might be applicable to field evaluation of intramammary infection. Prof W. Heeschen presented the overview paper in session 3 and his paper clearly demonstrated the changing view of mastitis from that of being a production limiting disease to a disease with significant impact on milk processing

properties and human health ramifications. The session had several papers dealing with residues from both antibiotics and teat dip compounds.

Session 4 dealt with mastitis control systems and Mr. J. Booth (UK) presented the overview paper (Booth 1995). He concluded his comments by stating that there was great difficulty when trying to determine progress in control of mastitis since the 1985 Seminar. The difficulties were associated with a lack of accurate data from most countries with the notable exception being the Scandinavian countries. He concluded that, "there is little doubt that herd milk cell counts have been reduced, but there are no indications at present of a parallel reduction in either subclinical or clinical mastitis during the last decade". My opinion would be that the situation is not much changed in 2005. One feature of this session was the several papers reporting on the use of Gram-negative core antigen vaccines to help control clinical coliform mastitis in dairy herds. The majority of these reports were from North America and represent the first successful use of a vaccine in mastitis control and the first reports of success in an IDF Mastitis Seminar. This session also contained a number of papers looking at mathematical modeling as a means of understanding or controlling mastitis in dairy herds.

The largest session at the seminar was that of Treatment of Mastitis and accounted for 20.1% of the papers presented at the 1995 seminar. This seems to suggest a marked increase in activity over the past 10 years, as there were only 6 papers dealing with this subject at the 1985 Keil Seminar. Dr. G. Ziv presented the overview paper (Ziv 1995) and while he expressed displeasure at the progress made over the past 10 years with regard to the basic problems associated with antimicrobial treatment of mastitis, he commented that there was a clearer understanding and appreciation of the place and limitations of antibiotics in control programs for subclinical mastitis and the role antibiotics can play in reducing economic losses due to clinical mastitis. There were several papers reporting on the efficacy of "new" antibiotics, combining antibiotic therapy with the use of immunomodulators, and the use of non-antibiotic treatments for mastitis therapy.

The overview paper in session 6 was given by K.L. Smith (US). General conclusions (Smith, 1995) were that mastitis control was dependent on a thorough understanding of the epidemiology of the various pathogens; control of environmental pathogens was becoming more and more important and that greater control of coagulase negative staphylococci in dairy herds will be needed as the legal limits for bulk milk SCC continue to be lowered.

M. Woolford (NZ) presented the overview paper in session 7, Milking Machines and Udder Health (Woolford, 1995). Woolford noted that good progress had been made over the past 10 years due largely to the IDF A2D Group of mastitis Experts and concluded that, "the detail of key teat/machine interactions remains undefined". He elaborated that the specifics of teat canal penetration by pathogens are still not fully understood, l*et al*one the relative importance of the various proposed mechanisms. He also lamented about the lack of significant funding for mastitis research. The session was also one of the first in an international mastitis conference to address the subject of robotic milking.

The final session was on Genetics and Mastitis Control and the overview paper was given by Dr. K. Leslie (CA). The paper (Leslie, 1995) covered the major parameters that could be evaluated and thus used or incorporated into a genetic selection program. Because of the vast amount of data available somatic cell counts are an obvious choice and are being used in some countries to select against bulls with high somatic cell count genetic evaluations.

He also noted the concern on part of some regarding the wisdom of somatic cell count reduction to very low levels.

The uncertainty was whether the goal should be lowest possible concentration of somatic cells or a somatic cell count level that is quantitatively and functionally optimum. The other papers in the session reported on attempts to find additional markers for enhanced immune response and disease resistance in dairy cattle.

Conclusions

The first three International Mastitis Seminars have shown that there has been overall progress in the control of mastitis in dairy herds as demonstrated by the reduction in herd somatic cell counts. There has been less progress on reducing the amount of clinical mastitis and little improvement in our ability to treat clinical cases of mastitis and to reverse the damage done by the infection. There have been major advances in our understanding of the natural defense mechanism associated with the bovine mammary gland but little of this knowledge has been incorporated into mastitis control schemes. There has been a clear progress in the understanding of the important aspects of milking machines that can cause or contribute to mastitis in dairy herds but there is still a lack of knowledge on the exact mechanisms of teat canal penetration by mastitis pathogens. Over the 30 year period, studies have demonstrated that genetic selection can be a component of mastitis control in dairy herds. Since1975 there has been shift in the importance of mastitis as being strictly a production limiting disease to the fact that mastitis adversely affects the processing properties of milk, its suitability as a human food and does impact human health issues.

The progressive increase in number of papers presented and number of attendees in the three previous seminars indicate there is a continued interest in mastitis research. I would suggest, as did Dr. A. Tolle in 1975, that the collaboration (Tolle, 1975b) between the Mastitis Action Team of IDF and the NMC has been a productive relationship and I would further suggest that if the interest in mastitis and its effects on milk quality continue to decline within IDF that NMC should play a greater role in sponsoring future international mastitis seminars.

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Pathogenesis of mastitis and mammary gland immunity: Where we are and where we should go

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Abstract

The large availability of new molecular techniques and, more generally, the advances in research tools could induce a deeper knowledge on very specific aspects, but also increase the risk of missing the overall picture. Therefore, there is a strong need for an increased integration of the researches on the modulation of mammary gland immunity. The development of DNA vaccines to prevent *S. aureus* mastitis is presented as an example on how an integration of available knowledge and a broader cooperation among research groups would improve the efficiency of the development process. The development of vaccine based on plasmid DNA could increase the efficiency of vaccine production, reducing costs; they are easy to store, handle, administer; they can induce persistent cell-mediated immunity without the need for adjuvants; they can include multiple antigens. The future research should be directed to collect more data to identify antigens closely related to bacteria pathogenicity; to identify the proper molecules to increase antibody response; to identify molecules that could enhance non-specific (cellular) immune response; to identify effective and easy way of administration; to develop in vivo model to assess the activity of these substances, taking in account the variability of cow physiological and immunological status.

Keywords: pathogenesis, immunity, vaccine, molecular biology

Introduction

IDF mastitis symposiums are organized every 10 years and they represent an opportunity to revise the progress on different aspects of mastitis epidemiology. I introduced the keywords immunity, pathogenesis and bovine mastitis in CAB® database for the years 1995-2005, retrieving about 250 papers. Applying *ad hoc* software, it is possible to identify the correlations among the major common "keywords" of these papers and to group them in main clusters (Figure 1). The figure shows a large cluster including pathogenic, genetic, proteomic and vaccines aspects of *S. aureus*. Five smaller clusters include papers on pathogens adhesion such as *Str.uberis* and *E.coli*; cow and udder immune defences, epidemiology of mastitis (in relation to pathogenic factors) and a sparse cluster including research prospects.

These results resemble quite well the programme of the session on Pathogenesis and Immunity of this Symposium. Instead of summarizing the knowledge on these topics, I would like to stress a concept already introduced in the IDF Ruminant Mammary Gland Immunity Symposium (Zecconi and Smith, 2003): the need of an increased and deeper integration of the researches to develop useful tools to modulate mammary gland immunity. Indeed, the large availability of new molecular techniques and, more generally, the advances in research

tools could induce a deeper and deeper knowledge on very specific aspects, but also increase the risk of missing the overall picture. In other words, we need to reduce the distances between the different clusters showed in Figure 1, avoiding a centrifugal drift.

I would like to use the example of vaccines developed to prevent *S. aureus* mastitis to show how a deeper integration of available knowledge and a broader cooperation among research groups would improve the efficiency of the development process.

S. aureus vaccines

In the last 40 years many studies have aimed to develop a worldwide efficacious vaccine for *S. aureus* mastitis, but without success (Leitner *et al.*, 2003, Zecconi and Smith, 2003). The reasons for this failure are various, but mainly related to our incomplete knowledge of bacteria pathogenic factors, to the interference of milk components with antibodies and cellular immunity and, not least, to the way of delivering antigens into the udder. To overcame these problems, common to human and animal diseases, *S. aureus* third generation vaccine were developed with interesting results (Sharma and Khuller, 2001).

DNA vaccines

DNA vaccines are defined as an immunization method induced by a protein antigen expressed in vivo by the introduction of purified plasmid DNA encoding polypeptide sequences (Sharma and Khuller, 2001). These vaccines could deliver both specific antigens and immunomodulatory proteins, inducing both T lymphocytes activity and antibodies (Ulmer *et al.*, 1996; Sharma and Khuller, 2001).

The development of this type of vaccines offers several advantages:

- the development of vaccine based on plasmid DNA could increase the efficiency of vaccine production, reducing costs;

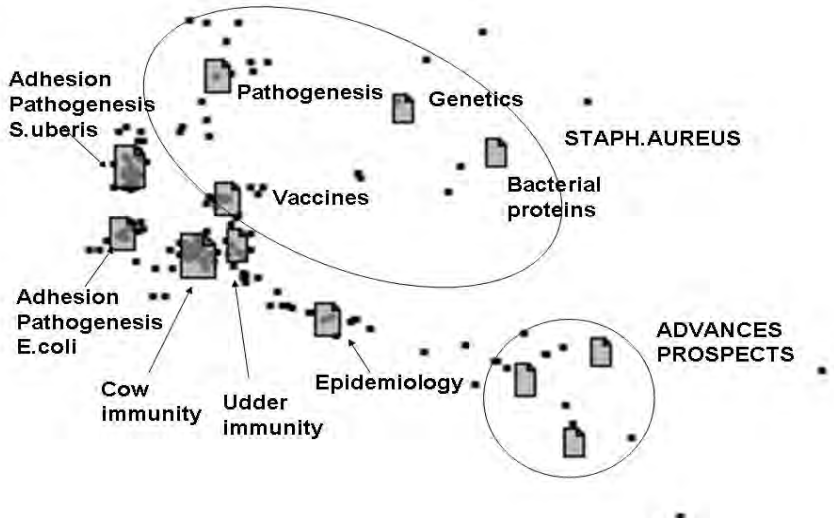

Figure 1. Distribution of available literature on mastitis pathogenesis and mammary gland immunity (cluster represent papers with common keywords).

- this vaccine could be easy to store, handle, administer;
- they can induce a persistent cell mediated immunity without the need for adjuvants;
- they can include multiple antigens;
- they showed to induce protective antibodies against several human diseases.

Most of the vaccines developed for human diseases are based on two major components: the antigen(s) plasmid(s) and the immunomodulating plasmid, encoding a cytokine (mainly GM-CSF) or a correspondent co-stimulatory molecules (Xiang and Ertl, 1995; Okada *et al.,* 1997). Mucosal DNA vaccines are an appealing approach to various infectious diseases in veterinary medicine, since they are easily administered to large number of animals using needleless devices, provide systemic and local immune responses and are quite inexpensive. Indeed, mucosal associated lymphoid tissue (MALT) is able to recirculate migrating to distant lymphoid organs, such as limphnodes, spleen, Peyer's patches, and to stimulate these immune cells through antigen presentation (Foxwell *et al.,* 2003).

DNA vaccines and mastitis

These vaccines are available in human medicine for several diseases (Davis *et al.,* 1993; Donnely *et al.,* 1997; Montgomery *et al.,* 1997). To apply them in veterinary medicine and, particularly, in mastitis prevention, we need to gather knowledge on:

- the antigens to be used
- the immunomodulating molecules to be elicited
- the interfering factors (cow physiology and immunological status)

Antigens

There are a number of potential antigens that could be considered. These antigens could be grouped in (Foster and Hook, 1998)**:**

- Capsules; these virulence factors are produced by many strains, i.e. 90% of clinical human strains showed a microcapsule.
- Adhesins; adhesion is one of the most important pathogenic factor for the development of IMI. *S. aureus* have different adhesins and their expression could justify the observed different pathogenic characteristics of the strains.
- Surface proteins; there are several cell wall bound proteins on *S. aureus* surface: i.eprotein A, fibronectin-binding proteins, clumping factor.
- Toxins; toxins are probably the best known pathogenic factors and also the most used antigens in commercially available vaccines.

Within the different putative molecules that can be used as an antigen for vaccine production, the extracellular matrix binding proteins (ECMBPs) are actually the most considered in human and veterinary medicine (Foster and Hook, 1998; Flock, 1999). There is many ECMBPs and the list of those currently identified is reported in Table 1.

These ECMBPs are particularly important for *S. aureus* mastitis. The bacteria initially must gain access to the mammary gland through the teat canal and then they have to avoid to be removed by the flushing of the milk during the milking process. Therefore the ability to adhere to the epithelial cells and extracellular matrix proteins is instrumental to colonize the gland and develop the pathologic process (Lammers *et al.,* 2001; Kerro Dego *et al.,* 2002; Brouillette *et al.,* 2003).

Table 1. Extracellular matrix binding proteins produced by S. aureus (Flock 1999).

Extracellular matrix binding proteins	Host counterpart
Surface proteins	
Fibronectin binding protein A (FnBPA)	Fibronectin
Fibronectin binding protein B (FnBPB)	Fibronectin
Collagen binding protein (Cna)	Collagen
Clumping factor A (ClfA)	Fibrinogen
Clumping factor B (ClfB)	Fibrinogen
Vitronectin binding protein (VnBP)	Vitronectin
Laminin binding protein (VnBP)	Laminin
Thrombospondin binding protein (TsBP)	Thrombospondin
Elastin binding protein (Ebp)	Elastin
MHCII analogous protein (Map)	Different proteins
Protein A	IgG
Ig binding protein (Sbi)	IgG and β-2 glycoprotein
Plasmin binding protein	Plasmin
Bone sialoprotein BP (BsBP)	Bone sialoprotein
Lactoferrin binding protein	Lactoferrin
Epidermolytic toxin (extracellular)	Fillagrin
Secreted proteins	
Extracellular fibrinogen binding protein (Efb ex Fib)	Fibrinogen
Extracellular adherence protein (Eap)	Fibrinogen. Prothrombin, fibronectin
Coagulase	Fibrinogen. Prothrombin
Streptokinase	Plasminogen

Thus, fibronectin binding protein A (FnBPA) mediates bacterial attachment to immobilized fibronectin and contributes to *S. aureus* adherence to udder epithelial cells, whereas collagen adhesin (Cna) induces bacterial adherence to collagen substrates and cartilage. A DNA vaccine based on a bicistronic plasmid encoding two sequences respectively for *S. aureus* FnBPA and ClfA, and a plasmid encoding GM-CSF stimulatory factor gene was developed (Shkreta *et al.,* 2004). The results of the challenge trial including 11 pregnant heifers showed some protection against infection, an improvement of cow physiological parameters and a lymphoprolipherative and humoral immune response. Previously, Carter and Kerr (Carter and Kerr, 2003) developed a DNA-vaccine based on a plasmid encoding for protein A. Even if the overall results were not as good as the previous one, they showed that an immune response could be raised through different ways of administration including intravulvamucosal jet injection. Our research group used multiple bacterial proteins involved in bacterial adhesion to host tissues as target antigens, Efb (Mamo *et al.,* 1995), FnBPA (Lammers *et al.,* 1999), ClfA (Brouillette *et al.,* 2002), and Cna (Visai *et al.,* 2000). Applying a mouse-mastitis model we observed a specific systemic and mucosal immune responses induced by DNA-vaccine coding for *S. aureus* adhesins. In addition, the immune response elicited by the different adhesins showed to be different. Indeed, a mixture of anti-adhesin antibody, but not the single adhesins, was effective to reduce *S. aureus* adhesion to mammary gland *in vivo* and *in vitro* (Castagliuolo *et al.,* 2005ab).

Immunomodulating molecules

Together with the development of vaccine eliciting a humoral and cellular immune response, it is important to manipulate the vaccine to direct this response towards the proper immune direction. Brown *et al.* (1998) summarised the factors involved in such modulations as: APC cells, cytokine environment during antigen priming, cytokine regulation of T cells, antigen dose and affinity with T cell receptor, the level and timing of co-stimulatory signals from APC-T cells interactions, the cessation of B and T cell activity.

Among these factors, cytokines represent probably the best candidate as molecules to be modulated in order to develop efficacious vaccine. Table 2 summarizes the different cytokines, their cell source and target and the availability of data on bovine and udder immunity. More information of these molecules were reviewed by Hamblin, (1993); Sordillo and Streicher, (2002); Alluwaimi, (2004);.

Among these cytokines, some seem to be more appropriate to be included in a DNA-vaccine. The use of GM-CSF has been already documented (Shkreta *et al.,* 2004), but other cytokines could be considered such as IL2, IL8, others showed to be less useful for such purpose as INF, IL1, IL6 or TNF (Alluwaimi, 2004).

The application of IL2 has been widely explored and several studies showed that rBo-IL2 induces: higher antibody titres and specific lymphocyte proliferation when inoculated together with bacterial or viral antigens; higher levels of phagocytosis and an overall higher bactericidal activity of leukocytes (Pighetti and Sordillo, 1995;Wedlock *et al.,* 2000). In modulating the immune response induced by vaccination, IL8 could be one of the best candidates. This cytokine is produced by epithelial cell and its gene encoding for this cytokine is up-regulated when cells are stimulated by LPS, *E.coli* and *S. aureus* (Barber and Yang, 1998; Boudjellab *et al.,* 1998). IL8 could also play a major role in the detoxification of LPS through the release of CD14 (Lee *et al.,* 2003, Persson Waller *et al.,* 2003).

A proper stimulation should modulate the immune response driving it toward a T_h1-dominated response instead of a T_h-2 one, as generally obtained by current vaccination protocols (Burton and Erskine, 2003). Indeed, T_h-1 response is more efficient in controlling invading pathogens by inducing a strong IL2 and IFNγ response, stimulating IgG_2 production and improving phagocytosis and intracellular killing.

More research is needed to select the proper immunomodulating molecules to be used for new vaccine development. Moreover, the results of the research in this field would also help the development of new immunomodulating molecules to be used to improve cow immune status, not only at mammary gland level.

Interfering factors

The achievement of an optimal health status in dairy herds is influenced by different factors such as genetics, nutrition, housing, hygiene, and prevention. A balance among different management and physiological factors in needed to reduce the frequency of diseases affecting production such as mastitis (de Kruif and Opsomer, 2002, Zecconi, 1997). These aspects should be also considered in developing vaccine and vaccination protocols. Indeed, even we would have an efficacious vaccine, we cannot forget that cow homeostasis plays a major role in modulating the immune response and therefore affects the efficacy of the vaccine.

There are several studies showing that the periparturient period is one of the most important and critical periods for the immune system, and some impairment of immune

Table 2. Cytokine cell sources and target cells and availability of data from bovine samples (Zecconi and Smith, 2003) (modified).

CYTOKINE	CELL SOURCE	TARGET CELL	DATA
IFN α	Leukocytes	Many	Yes
IFN β	Fibroblast and epithelial cells		
IFN γ			
IL-1 α	Macrophages, endothelial cells, lymphocytes,	Thymocytes, neutrophils, hepatocytes,	Yes
IL-1 β	T & B cell, fibroblasts, epithelial cells,	chondrocytes, muscle cells, endothelial cells,	
	astrocytes, keratinovytes, ostetoblasts	epidermal cells, osteocytes, macrophages,	
		T & B cells, fibroblast	
IL-2	T cells	T & B cells, macrophages	Yes
IL-3	T cells; mast cells	Multipotential stem cells, mast cells	no
IL-4	T cells, mast cells, bone marrow stromal cells	T cells, mast cells, B cells, macrophages,	no
		heamotopoietic progenitor cells	
IL-5	T cells	Eosinophils	no
IL-6	Fibroblasts, T cells, macrophages, endothelial	B & T cells, thymocytes, hepatocytes	Yes
	cells, keratinocytes, mast cells		
IL-7	Thymic stromal cells	Pro-B cells, pre-B cells, thymocytes,	No
		activated mature T cells	
IL-8	Monocytes, endothelial cells, epithelial cells,	Neutrophils, T-cells, basophils	Yes
	fibroblasts, chondrocytes, hepatocytes,		
	keratinocytes, synoviocytes		
IL-9	T cells	CD4 T cells, mast cells	No
IL-10	T cells, macrophages, B cells	Macrophages, mast cells, NK cells	No
IL-11	Stromal cells	Haematopoietic progenitor cells	No
IL-12	T cells, B lymphoblastoid cells	T cells, NK cells, LAK cells	Yes
GM-CSF	T cells, endothelial cells, fibroblasts,	Multipotential stem cells, mature	Yes
	macrophages, mast cells	neutrophils and monocyte, macrophages	
M-CSF	Fibroblasts, monocytes, endothelial cells	Multipotential stem cells, monocyte,	No
		macrophages	
G-CSF	Macrophages, fibroblasts, endothelial cells,	Multipotential stem cells, granulocytes	No
	mesothelial cells, T cells		
TNF	Macrophages, T cells, thymocytes, B cells,	Tumor cells, fibroblasts, macrophages,	Yes
	NK cells	osteoclasts, neutrophils, adopocytes,	
		eosinophils, entothelial cells,	
		chondrocytes, hepatocytes	
LT	T cells	Tumor cells, neutrophils, osteoclasts	No

defences could be observed (Kehrli and Goff, 1989, Mallard *et al.,* 1998). This impairment increased the frequency of reproductive and production diseases as recently reviewed by (Ingvartsen *et al.,* 2003), and of clinical mastitis (Barker *et al.,* 1998; Dosogne *et al.,* 1999; Burvenich *et al.,* 2000).

Physiological, metabolic or psychological stressors induce a T_h2-dominated response, leading to a production of IgM and IgG1, but impairing neutrophil recruitment, phagocytosis

and intracellular killing (Burton and Erskine, 2003). However, a number of studies showed that the impairment of immune defences at parturition or is not an unbreakable rule. Indeed, we can observe differences based on age of the cow (Mehrzad *et al.,* 2002), stage of lactation (Mehrzad *et al.,* 2001), energy balance (Stabel *et al.,* 2003; Dosogne *et al.,* 1999) and herd (Piccinini *et al.,* 2004, Piccinini *et al.,* 2005).

The knowledge on cow immune status and the interfering factors largely increased in the recent years. However, this knowledge have not find many practical applications. One of the possible use of these information would be in the development of vaccination protocols. The studies could help in finding the best timing and frequency of administration to overcome the "physiological" impairment of the mammary gland or to reduce it.

Conclusions

To show the importance of a deeper integration among researches in mammary gland immunity, I select a topic close to my expertise, but other examples can be found. Indeed data on bacteria pathogenesis are available for Staphylococci, Streptococci and E.coli (Calvinho and Oliver, 1998; Almeida and Oliver, 2001; Lammer *et al.,* 2001; Leigh *et al.,* 2004) and therefore a similar approach could be applied.

As a general conclusion, I believe that more integrated efforts will help the development of successful vaccines for mastitis. The research should be directed to collect more information in order to:

- identify antigens highly immunogenic and more related to bacteria pathogenicity;
- identify the proper molecules to enhance immune response;
- identify molecules that could enhance non-specific (cellular) immune response with a long-acting activity and without adverse effects;
- identify effective and easy way of administration;
- develop in vivo model to assess the activity of these substances, taking in account the variability of cow physiological and immunological status;

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Mastitis management in an economic framework

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Abstract

As with many other cattle diseases, the economic damage of mastitis, either clinical or subclinical, can be brought down to a few categories: Milk production losses, drugs, discarded milk, veterinarian, labour, milk quality, culling, clinical mastitis, subclinical mastitis and other diseases. The costs for these factors might differ between countries and regions there fore it is hard to give conclusive answers on the costs of mastitis and the benefits of mastitis management. Management decisions can be taken at various levels: the quarter level (e.g., drying off a single quarter), the quarter/cow level (e.g., treating clinical or subclinical mastitis), the cow level (e.g., culling a cow with clinical or subclinical mastitis), the herd level (e.g., changes in management such as barn and milking hygiene) and the national or regional level (e.g. improving extension services). Using the basic cost elements around mastitis and mastitis management, costs and benefits can be calculated for different circumstances. A good economic analysis starts with the costs associated with different types of mastitis. Using this basis, economic effects of management changes at all decision levels can be made. Many times generic economic calculations are not specific enough for the situation. Each cows, farms and regions differ for production circumstances and price levels. Therefore economic calculations should be as specific as possible.

Keywords: economics, management, clinical mastitis, subclinical mastitis

Introduction

Being an endemic disease on dairy farms all over the world, mastitis is an important cause of a less efficient milk production. Moreover, mastitis affects milk quality directly through a change in technical and hygienic milk quality and indirectly through the intrinsic milk quality (Hogeveen and Lankveld, 2003). Therefore mastitis is a concern for the dairy industry. Mastitis management therefore should have the goal of improving milk quality and the efficiency of milk production and thus make the production of milk more sustainable. Given the multi-factorial nature of mastitis, management consists of a wide range of activities, amongst others the treatment of diseased cows (clinical or subclinical), dry cow therapy, prevention of transmission of infections (either from cow to cow or through the environment) and improvement of the immune system. There is much scientific literature on mastitis management. However, there is less scientific literature on economics of mastitis and many times, this literature is regarding a calculation of economic damage of mastitis or the benefits of one or two management factors. Most studies are normative (using

simulation modelling to estimate economic effects). Less studies are positive (using collected data to estimate economic effects).

Economic calculations for costs and benefits of mastitis and mastitis management depend very much on the specific situation of a country or region. Therefore, clear economic statements are very hard to give. Recently, IDF published an extensive review on economic consequences of mastitis (Østerås *et al.*, 2005). The aim of this paper is to give a comprehensive overview of economic considerations around mastitis management. First the economic damage caused by mastitis is described in general. Secondly, aspects of management at the cow, farm and national level are described in an economic framework.

Economic damage of mastitis

As with many other cattle diseases, the economic damage of mastitis, either clinical or subclinical, can be brought down to a few categories:

- milk production losses;
- drugs;
- discarded milk;
- veterinarian;
- labour;
- milk quality;
- culling;
- clinical mastitis;
- subclinical mastitis;
- other diseases.

Although costs for these factors might differ between countries and regions, the economic principles behind these factors are the same and will be explained below.

Milk production losses

In both clinical and subclinical mastitis, there is a loss in milk production. There is an large amount of published research on these changes in milk production (see e.g., Hortet and Seegers, 1998; Houben *et al.*, 1993; Raubertas and Shook, 1982). Moreover, the loss in milk production does not only occur during the case itself, even after the mastitis case is cured, the milk production level of the cow stays lower. Milk production loss is not obvious to the producer, because this is milk never produced, and therefore never seen. It is a hidden cost or lost income opportunity. The economic damage of a lower milk production per cow depends on the structure of the farming business. First of all, milk payment systems may differ (payment based on kgs of fluid milk or based on milk constituents such as fat and protein). Secondly, the calculation of the economic damage of decreased milk production differs between a quota system (e.g., such as in place in the EU, Norway or Canada) or a non-quota system. In a dairy system where farmers do not face a milk quota, the production potential of the farm is the number of dairy cows present on the farm. The number of dairy cows might be restrained because of seize of the barn, available labour, available foodstuffs or available capital, but the milk that cows produce can be delivered to the factory and will be paid for with the customary milk price. When milk production per cow is decreased by mastitis, less milk will be delivered to the factory and the net return of the farm will decrease. Their might be some savings because, when cows are fed relative to milk

production, the farmer might save on feed (concentrates) which will result in decreased costs. Suppose that the milk price is € 30,-/ 100 kg milk and that the additional feeding costs is € 8,-/100 kg milk, a milk production decrease of 100 kg will result in an economic damage of € 22,-.

In a quota-situation calculation of economic damage for a decrease in milk production becomes much more complicated. The production potential of a farm is in most situations the quota and not the number of animals, therefore, the returns of milk sales are more or less determined and the goal of the farmer is to produce the milk within the quota, not more and not less, as efficient as possible. With a decreased milk production a farmer has several options (depending on the legislation associated with the quota system):

- Milk more cows to fill the quota. In this case, economic damage is calculated as the additional costs to milk more cows. These costs are not easy to estimate and consist amongst others of additional feed costs, additional veterinary costs, additional labour (see description of labour above) and additional costs for use of the barn. Many times additional costs for the barn are 0. However, with a crowded barn, costs might be associated with a lower level of animal welfare. When the farmer used the over capacity of a barn for additional earnings (for instance to raise heifers for sale), the costs associated with higher barn use are the decrease in earnings for these additional activities. The additional costs for milking more cows are therefore very dependent on the specific farm situation.
- Increase the production of the cows (e.g., by more concentrates) to fill the quota. In some farm situations, milk production of the cows can be increased by application of a better (more expensive) feeding regime. Additional costs are associated with the higher amount of (more expensive) feedstuffs which are necessary to do this. In some cases (depending on the management capacities of the farmer), a higher milk production per cow can lead to more health disorders.
- Lease out milk to other farmers when the quota is not filled by own production. In some quota systems, farmers can lease or lease out milk relatively easy. This makes the quota system much more flexible. When farmers do not fill their quota, the additional quota can be leased out to other farmers. When this is done due to mastitis and the associated milk production decrease, the returns from milk sales will be decreased, some savings might occur because of less needed feedstuffs (just as in the non-quota situation), but there are new returns from leasing out milk. So suppose that the milk price is € 30,-/100 kg milk, the savings in feeding costs are € 8,-/100 kg milk and the returns from leasing out milk are € 15,-/100 kg milk the total economic damage of a decrease in milk production is € 7,-/100 kg milk

In general, economic losses due to a lower milk production per cow (and consequently economic gains due to a higher milk production per cow) are lower for a quota-situation than for a non-quota-situation.

Drugs

This is a straightforward economic damage. Drugs, necessary to treat a cow with mastitis, cost money. Depending on the legislation and the infrastructure in a country costs of drugs may vary between countries.

Discarded milk

Economic damage due to discarded milk is comparable with the damage of a decreased milk production. However, there is one difference, the discarded milk is actually produced by the cows, which means that feeding costs for that amount of milk has to be taken into account with the calculations. The economic damage of 100 kg discarded milk is therefore larger than for 100 kg decreased production. Although not advisable from a veterinary point of view (there is an increased risk of developing resistant microbes in the calves), discarded milk is on the farm often fed to calves instead of milk replacer. This will save costs for milk replacer.

Veterinarian

Besides delivering drugs (in many countries), the veterinarian might have to spend time on diagnosis of a (clinical) mastitis case. Depending on legislation, in some countries this is mandatory for each mastitis case or only for severe mastitis cases upon request of the farmer.

Labour

Costs for labour are, from an economical point of view, difficult to interpret. Opportunity costs for labour, e.g., to treat an animal, may differ from farm to farm. In other words, is there an alternative use for the labour involved? When the number of hours of external labour can be decreased by preventing mastitis, the opportunity costs are easy to calculate: hours X hourly wage. When it is the labour of the farmer himself, opportunity costs are much more difficult to estimate. If the labour comes from his own free time, it is the value that the farmer himself gives this free time. If the farmer, because of mastitis, spends less time in other controlling tasks, opportunity costs are the decrease income because less controlling. Finally perception of the value of labour might be important. Treating mastitis cows, while other cows are waiting in the milking parlour is work that a farmer does not like to do. So he is willing to spend money to prevent that. Labour costs are not only made at the farm level. When there are national programs or programs by a dairy company to decrease the level of mastitis, these costs can be associated with mastitis.

Milk quality

Mastitis does influence the quality of milk. Some of these changes cause a less efficient processing of milk and might result in products with less favourable properties. Examples are an unstable and rancid taste of milk, a lower cheese yield and a decreased shelf life (e.g., Ma *et al*., 2000; Santos *et al*., 2003). The associated economic damage for is difficult to calculate and moreover, the direct effect of this economic damage for the individual dairy farmer is even more difficult to estimate. The only changes in milk quality that have a direct effect are the ones influencing factors that are part of the milk payment system, for instance bacterial count and somatic cell count. In most countries there is a regulatory limit for bulk milk bacterial count and bulk milk somatic cell count (BMSCC). In relation to mastitis, especially BMSCC is an important milk quality aspect. The SCC of a cow or quarter might be strongly increased without visible changes in the milk. There are, in experimental studies, examples of quarter milk with 13,000,000 SCC per ml without clinical symptoms (Pyörälä and Mattila, 1987). Therefore, due to a (subclinical) mastitis case BMSCC can strongly increase which might have financial consequences. In some milk payment systems,

penalties are given after exceeding the threshold in one bulk delivery, and in some systems, the geometric average of BMSCC should exceed the threshold level during several (mostly 3) measurements. There are many dairy companies that give a bonus on the milk price, when the BMSCC is lower than a certain threshold.

Besides BMSCC and bacterial count, most milk payment schemes test for antibiotic residues. Although the mastitis in itself does not affect growth inhibition, the use of antibiotics in treatment of mastitis does increase the risk of penalties. Different countries and milk processors use different rules for antibiotic residues, but the financial consequences of antibiotic residues in the milk can be considerable.

Culling

Cows with mastitis have a higher risk of being culled. The cost due to premature replacement of animals due to mastitis is probably one of the largest areas of economic loss. However, it is also a hidden cost. It is very difficult to calculate in a correct way (see for instance Grohn *et al.*, 2003; Houben *et al.*, 1994; Lehenbauer and Oltjen, 1998). Basically, economic damage of premature culling is caused by increased costs for replacement animals and decreased production efficiency. When a cow is culled, direct costs are the costs of rearing or buying a replacement animal (mostly heifers). Indirect costs are a decreased efficiency of milk production by the replacement animal, since the milk yield of multiparous cows is higher than that of primiparous cows. Moreover, the milk production of a heifer might be disappointing (heifers have a relatively high culling rate). On the other hand, there are also returns of culling a cow, mostly the price of meat. The costs of involuntary culling differ over time, depending on milk production, parity, lactation stage and reproductive status. This is partly illustrated in Figure 1, where costs of involuntary culling are given for different parities and lactation stages. Also it can be noticed that the RPO increases when the cow is pregnant (around 5 months in lactation).

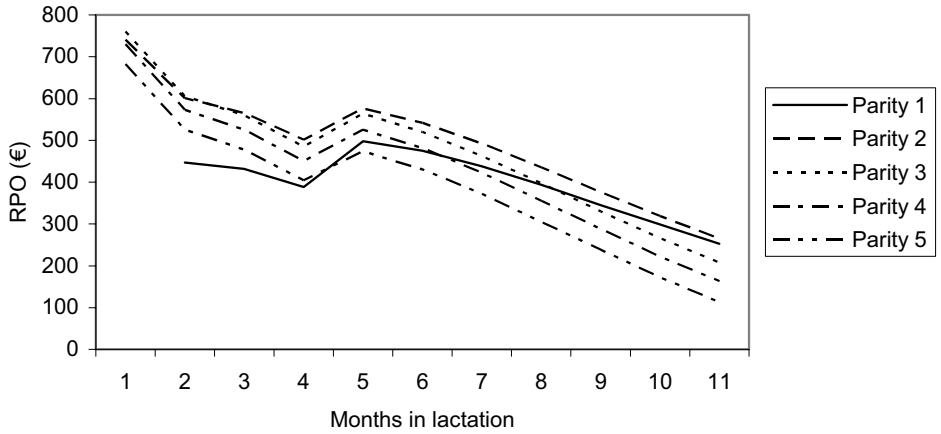

Figure 1. Costs of involuntary culling for a cow under Dutch circumstances with a calving interval of 13 months and an average production level (source: Van der Walle, 2004, based upon the model of Houben et al.*, 1994).*

Clinical mastitis

For some management decisions, prevention of clinical mastitis can be an important benefit. Clinical mastitis in itself is not an economic factor. The factors as described above (milk production, drugs, discarded milk, labour, veterinarian, culling and milk quality) are the economic consequences of clinical mastitis. Mastitis management at the cow level can prevent clinical mastitis in the same cow or can prevent spread of mastitis pathogens. Because of the contagious nature of mastitis, a cow with mastitis increases the risk that other cows get mastitis. There are only few publications on the spread of mastitis pathogens through the herd (Lam *et al.*, 1996; Zadoks *et al.*, 2001). The costs of these new mastitis cases may be attributed to the original mastitis case. Also mastitis management at the herd level does prevent clinical mastitis.

Subclinical mastitis

In the same reasoning as for clinical mastitis, prevention of subclinical mastitis can be an important benefit of mastitis management at various levels.

Other diseases

There is an association between mastitis and other cattle diseases. The causal relation however, is difficult to determine. When the risk of other diseases is increased by mastitis, economic damage of other disease cases attributable to mastitis can be seen as economic damage of mastitis. However, this damage is very hard to establish because the interactions between various diseases are hard to establish and will not be further discussed in this paper.

Mastitis management in an economic framework

In this section, mastitis management is positioned in an economic framework. The basis of economic foundation of mastitis management lies in insight in the costs of clinical and subclinical mastitis. Since the mastitis situation differs from cow to cow and thus from farm to farm, these costs should be differentiated for various pathogens. In the past various calculations have been published on the costs of mastitis (de Vos and Dijkhuizen, 1998; Kaneene and Hurd, 1990; Schakenraad and Dijkhuizen, 1990; Schepers and Dijkhuizen, 1991). However, many of these calculations are general, giving an average economic damage per cow with clinical mastitis, which makes it difficult to calculate the farm specific damage of mastitis. Moreover, not much effort has been carried out in the calculation of damage of subclinical mastitis. Information published so far, is directed at the consequences of production losses due to an increased SCC (e.g., Seegers *et al.*, 2003). However, subclinical mastitis does imply the risk of a cow becoming clinical and the spread of mastitis pathogens due to a cow with subclinical mastitis in a herd. Knowing a current mastitis situation, different management options can be considered.

Mastitis management is carried out at various levels, and at all of these levels economic costs and benefits may be calculated. An overview of some types of decisions (without trying to be complete) is given in Table 1. This is based on the more detailed description of costs and benefits of interventions by Østerås *et al.* (2005).

Table 1. Overview of mastitis management at various levels (based on a more extensive description by Østerås et al.*, 2005).*

Level	Type of decision	Costs	Benefits
Quarter	Drying off quarter	Labour	Milk quality
		Milk production	Clinical mastitis[1]
		Culling	Culling
Quarter/cow	Treating subclinical case	Labour	Milk quality
		Veterinarian	Milk production
		Drugs	Clinical mastitis[1]
		Discarded milk	Culling
		Milk quality	
Quarter/cow	Treating clinical case	Labour	Milk quality
		Veterinarian	Milk production
		Drugs	Clinical mastitis[1]
		Discarded milk	Culling
		Milk quality	
Cow	Culling cow with (sub)clinical mastitis	Labour	Milk quality
		Culling	Clinical mastitis[1]
Farm	Management change	Labour	Milk quality
		Material	Subclinical mastitis[1]
		Investments	Clinical mastitis[1]
Region/country	Extension/service/research	Labour	Milk quality
		Material	Subclinical mastitis[1]
		Investments	Clinical mastitis[1]

[1]Representing subsequent costs

Drying off udder quarters as management tool, is carried in some smaller herds. Benefits of this management are an increase in milk quality and thus a increase in the probability of higher milk payments, reduction in the risk of clinical mastitis, either in the same cow or in other cows, due to a reduction in the spread of pathogens and a reduction in the risk of culling. The costs are additional labour to dry the udder quarter, losses in milk production (although there is compensatory milk production in the other quarters, Hamann and Reichmuth, 1990) and a increased risk of culling because of a lower milk production.

Treatment of a cow, can be regarded as quarter level management (when intramammary antibiotics are used, the antibiotics are applied at the quarter level) but also as cow level management. Consequences of treatment are partly at the cow level (e.g. discarded milk). Recently, results are published on the effectiveness of treatment of subclinical mastitis (DeLuyker *et al.*, 2005; Oliver *et al.*, 2004; St Rose *et al.*, 2004). Benefits of this treatment might be an improved milk quality, higher milk production (although St Rose *et al.* (2004) found no improvement of milk production following cure), a lower risk of clinical mastitis in the treated cow, less spreading of mastitis throughout the herd and a lower risk of culling. Costs of treatment of subclinical mastitis is the treatment itself (consisting of labour, drugs, veterinarian, discarded milk and a risk on antibiotic residues). Recently some studies are published on the economic efficiency of treatment of chronic subclinical mastitis caused by *S. Uberis* or *S. dysgalactiae* (Swinkels *et al.*, 2005), *S. uberis* (Hogeveen *et al.*, 2005;

Steeneveld *et al.*, 2005) and *S. aureus* (Swinkels *et al.*, 2005). These analyses show that the economic efficiency is very dependent on cure rates and specific farm circumstances.

In most farming systems, the treatment of a case of clinical mastitis is hardly a question. It is rather a decision between treatment and culling. Because of animal welfare considerations, almost all cows with a moderate or severe case of clinical mastitis are treated. The costs of treatment are comparable with the treatment of subclinical mastitis cases described above. The benefits are also the same, with the only difference in prevention of clinical mastitis in the same cow. Therefore, publications were scarce and only on a comparison of different treatments (Shim *et al.*, 2004; Van Eenennaam *et al.*, 1995).

For both clinical and subclinical mastitis, an alternative to treatment is the culling of a cow. Because of mastitis, the productivity of a cow decreases and this affects the expected income of a cow. The costs of culling a cow are the costs for culling as described elsewhere in this paper and a little additional labour. The benefits are an improved milk quality and a lower risk of clinical mastitis in the future. Most calculations of the costs of involuntary culling of dairy cattle, do not take the disease status of the cow into account. It can be expected that the expected future income of a cow with mastitis (either clinical or subclinical) is lower than without mastitis (Grohn *et al.*, 2003). Therefore, culling a cow with mastitis might be economical beneficial because of this lower expected income for the cow. This was studied by Houben *et al.*, (1994), who concluded that in most cases the optimal decision was to keep and treat rather than to replace a cow. For subclinical mastitis an economic analysis showed that in many cases extra culling was justified in order to reduce the level of infection in the herd (Stott *et al.*, 2002). Since culling of infectious cows is an important part of many mastitis control systems, the subject of economic efficiency of culling cows with mastitis deservers more attention in the future.

At the farm level, many different management measures are possible. The benefits of these measures lie in a lower incidence of clinical and subclinical mastitis, and depending on the milk payment system a higher milk price due to a better milk quality (Dekkers *et al.*, 1996). Costs of a change in mastitis management can be a higher need for labour, more materials (such as teat dips) and higher costs for investments (Allore and Erb, 1998; Gill *et al.*, 1990; Hoblet *et al.*, 1991; Yalcin *et al.*, 1999). From an economic point of view, investments should be depreciated and interest should be calculated for the capital used for that investment. Some studies have been published on specific management activities such as vaccination (Degraves and Fetrow, 1991) or dry cow therapy (Berry *et al.*, 2004; Hogeveen, 2003; McNab and Meek, 1991), where specific attention was given to selective dry cow therapy (Østerås and Sandvik, 1996).

At the regional or country level, decisions are not taken by the farmer, but by governments, dairy processors or representatives of the farmers. Types of decisions might be to provide free or cheaper extension on mastitis, provide cheaper service (e.g. in bacteriological testing) or carry out research. The costs of these activities are made by governments (paid by the public then), dairy processors (indirectly paid by farmers throughout the milk price) or farmers' collective funds. Benefits are, on average, a lower incidence of mastitis and a better milk quality. In Norway, for many years the dairy sector is focusing on mastitis. To estimate the effects of this focus, costs associated with clinical and subclinical mastitis are estimated at the National level (Figure 2).

Before cost calculations at the herd level are extrapolated to the national or regional level it is important to know whether it is a quota or a non-quota situation. In a non-quota

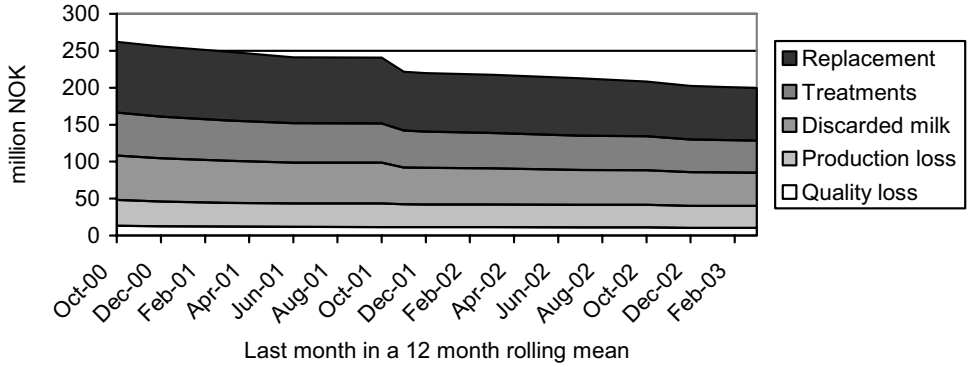

Figure 2. Distribution of the total mastitis loss to quality, production loss, treatment cost and replacement during 2000 trough 2003 for the Norwegian dairy farmers.

situation, costs at the national level cannot be based on individual herds. When all herds succeed in lowering the costs of mastitis, the cost price of milk will decrease, this means that (in an open market) with a given milk price the supply will increase, followed by a decrease in milk price and a stabilization on a lower milk price, taken into account the lower cost price of milk due to mastitis prevention. The benefit at the national level will not be for the farmer but for the consumer. This is referred to as the consumer surplus as is for instance described by Schoenbaum and Disney (2003). In a quota situation, the supply of milk is fixed. A decrease in cost price because of an improved mastitis situation will then completely beneficial for the farmer.

Discussion and conclusions

In this paper the basic elements to calculate the economics of mastitis and decisions around mastitis are given. This paper does not provide a conclusive answer of the costs of mastitis and the benefits of certain mastitis management options. These costs and benefits depend on the specific situations (price levels, production circumstances) of a country region or the farm. Decisions might even differ from cow to cow, given milk production levels, age and reproductive status of that specific cow. Economic calculations should therefore be very specific. Current developments in the use of computers in dairy farming provide opportunities for farm or cow specific economic calculations. The elements described in this paper can be used to calculate costs and benefits of mastitis and mastitis management for different situations.

When economic calculations are used for decision support (which is the primary goal of animal health economics), there are a number of assumptions, such as transparency, perfect information and a clear definition of a utility function. Under these assumptions, the (rational) decision maker follows the most optimal advice. However, in reality, people take other decisions than the most optimal one from an economic point of view. Anecdotal evidence from veterinary practice does support these observations. Neo-classical economists might argue that the problem and choices were not transparent, that there was no complete information, or that the definition of the used utility function was not correct. However, from the field of behavioural economics, where psychological insights are combined with

economic theory, there is an argumentation that behaviour of people might be irrational from an economic point of view, but is rational from a psychological point of view. In this field many experiments are carried out describing the economic behaviour of, mostly, consumers (Kahneman and Tversky, 1979; McFadden, 1999; Rabin, 1998). Since farms are small "companies" in the private household and the business are closely interrelated and in which the decisions are many times taken by one person, economic behaviour of consumers and of farmers might be comparable. Items in this field that deserve more attention are the gain/loss disparity (consumers regard the value of a loss higher than the value of a gain, which shows some resemblance with cure or prevention) reasoning under uncertainty and the time preference of money (discount rates unconsciously used by consumers are much higher than the "economic" discount rates, Thaler, 1981). Insight in this economic behaviour of dairy farmers can explain deviations of economic optimal behaviour. However, to enhance the profit of dairy farms, correct economic calculations for mastitis management remain very important.

Concluding, the economic damage of mastitis, either clinical or subclinical, can be brought down to a few categories: Milk production losses, drugs, discarded milk, veterinarian, labour, milk quality, culling, clinical mastitis, subclinical mastitis and other diseases. The costs for these factors might differ between countries and regions therefore it is hard to give conclusive answers on the costs of mastitis and the benefits of mastitis management. Management decisions can be taken at various levels: the quarter level (e.g., drying off a single quarter), the quarter/cow level (e.g., treating clinical or subclinical mastitis), the cow level (e.g., culling a cow with clinical or subclinical mastitis), the herd level (e.g., changes in management such as barn and milking hygiene) and the national or regional level (e.g. improving extension services). Using the basic cost elements around mastitis and mastitis management, costs and benefits can be calculated for specific circumstances.

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New antimicrobial agents in mastitis therapy

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Summary

Mastitis is a disease caused by Gram-positive and -negative bacteria. For this reason antibiotics have always been essential in treatment of mastitis and almost all have been used. Antibiotics are used in mastitis therapy systemically and/or locally, consisting of intramammary antibiotic administration. The purpose of this presentation is to review new antimicrobial agents introduced to mastitis therapy during the years 1995-2005. Six compounds were considered belonging to the β-lactam, macrolide, lincosamide and fluoroquinolone groups. The β-lactams were amoxicillin combined with clavulanic acid and two advanced cephalosporins; ceftiofur and cefquinome, the macrolide tilmicosin and lincosamide pirlimycin, and the fluoroquinolone marbofloxacin. The β-lactams and marbofloxacin are broad-spectrum antibiotics and suitable for treatment of Gram-positive and -negative pathogens. The macrolide and lincosamide spectrum incorporates Gram-positive bacteria only. The advanced cephalosporins are generally more suitable for treatment of Gram-negative pathogens, but both ceftiofur and cefquinome have excellent activity, low minimum inhibitory concentration (MIC), against Gram-positive pathogens as well. These cephalosporins are also resistant to β-lactamase activity. The two macrolides also have low MIC values against relevant Gram-positive mastitis pathogens. The therapy regimens of some of these new compounds include intramammary only or combined intramammary and systemic treatment. Most studies indicate that combination therapy appears advantageous. It appears justified to conclude that efficacy of the therapy appears more influenced by pharmacokinetics than the MIC.

Keywords: therapy, pharmacokinetics, MIC, antibiotics

Ceftiofur

Ceftiofur is structurally analogous to cefotaxime, one of the first third generation cephalosporins used in human medicine. Contrary to most other cephalosporins, ceftiofur undergoes rapid metabolism and its main metabolite, desfuroylceftiofur, has significant antimicrobial activity. The compound is found in the plasma and tissues mainly as desfuroylceftiofur (Jaglan *et al.*, 1990). Ceftiofur should, therefore, be considered a pro-drug. The analytical methods for determination of ceftiofur concentrations range from non-specific microbiological inhibition assays to specific HPLC methods. The HPLC method of Jaglan *et al.* measures ceftiofur as its desfuroyl metabolite while the bioassays measure total ceftiofur related antimicrobial inhibition against the indicator organism, i.e. no method is able to analyze ceftiofur and its metabolites simultaneously. Because of the analytical problems, no comprehensive pharmacokinetic studies were performed.

Antimicrobial activity of ceftiofur against mastitis pathogens was reported by Erskine *et al.* (2002). Antimicrobial susceptibility of 2778 clinical and subclinical mastitis isolates

were studied. All Staphylococcus aureus and streptococci were susceptible to ceftiofur. Similarly, practically all Escherichia coli, Klebsiella pleuropneumonia and Serratia marcescens were susceptible. None of the tested Pseudomonas aeruginosa strains, however, was susceptible to ceftiofur. The ceftiofur spectrum, therefore, appears to cover the overwhelming majority of mastitis causing organisms. It remains to be seen whether the susceptibility to ceftiofur reflects also susceptibility to desfuroylceftiofur.

Owens *et al.* (1990) studied ceftiofur concentrations in milk and udder tissues. In this study ceftiofur was administered either by intramuscular or both intamuscular and intramammary administration. The observed milk and tissue concentrations at this administration protocol were very low. Ceftiofur pharmacokinetics in experimental coliform mastitis was published by Erskine *et al.* (1995). In this study, using much higher dose than in the above study, ceftiofur concentrations in milk were found to be higher in the mastitic udder quarters. In a subsequent study Erskine *et al.* (2002b) showed that intramuscular ceftiofur treatment at 2.2mg/kg/day during 5 days in clinical mastitis case significantly reduced death or culling related to mastitis caused by coliforms.

Cefquinome

Cefquinome, as ceftiofur, is an advanced cephalosporin compound. Cefquinome pharmacokinetics were described by Limbert *et al.* (1991). The pharmacokinetics in cattle were characterized by short half-life (1.33 h), low volume of distribution (0.23 L/kg) and rapid clearance (0.26 L/min).

The MIC values of cefquinome was determined against a wide variety of pathogens isolated mainly in European hospitals (Limbert *et al.*, 1991). Of the 40 methicillin susceptible Staphylococcus aureus strains, the MIC_{90} was 1.53 µg/mL. However, the respective value for methicillin resistant S. aureus (n=30) was 25 µg/mL. The MIC_{90} in Streptococcus spp. in serogroups A, B and C (n=36) was 0.024 µg/mL. The cefquinome activity against Gram-negative pathogens was also good. The MIC_{90} value ranged from 0.195 to 0.781 µg/mL except for indole positive Proteus spp. and Pseudomonas aeruginosa. The MIC_{50} values of proteus was clearly in the therapeutic range (0.195 µg/mL) while the value for pseudomonas was still high (6.25 µg/mL). Guerin-Faublee *et al.* (2003) showed that all 495 tested mastitis causing organism from clinical cases were susceptible to cefquinome.

In a study by Shpiegel *et al.* (1996) experimentally infected with E. coli recovered significantly better following intramuscular cefquinome treatment (1 mg/kg) with or without intramammary treatment. Cefquinome treatment was significantly more efficacious compared to ampicillin/cloxacillin treatment.

Tilmicosin

Tilmicosin is a macrolide closely related to tylosin. Tilmicosin is a narrow spectrum antibiotic and effective only against Gram-positive pathogens. Ziv *et al.* (1995) reported tilmicosin half-life in cattle of 4.18 h after subcutaneous administration. In the same study tilmicosin volume of distribution was found to be large (>2 L;kg). The tilmicosin milk concentrations exceeded the respective serum concentrations.

The MIC_{90} for S. aureus was 0.78 µg/mL (n=164) whereas the MIC_{90} for other Gram-positive mastitis pathogens were in the same range (Ziv *et al.*, 1995).

Tilmicosin was administered to cattle at dying off (Ziv *et al.,* 1995). Tilmicosin could be detected in dry udder secretions for 8-9 days at concentrations exceeding the S. aureus MIC_{90} values. Tilmicosin was evaluated in treatment of S. aureus mastitis during lactation (Owens *et al.,* 1999). The used treatment protocol, however, was unsuccessful in eliminating the pathogens. In a study concerning dry-cow treatment by Nickerson *et al.* (1999), Tilmicosin was found equally effective with cefapirin after intramammary infusion but inferior after subcutaneous administration. Intramammary administration of 300 mg of tilmicosin to heifers during various stages of pregnancy reduced significantly the prevalence of staphylococcal and streptococcal infections (Owens *et al.,* 2001).

Pirlimycin

Pirlimycin is a lincosamide antibiotic related to clindamycin activite only against Gram-positive bacteria. Pirlimycin pharmacokinetics after intramammary infusion were studied using a population pharmacokinetic model (Whittem, 1999). Elimination of pirlimycin from the milk was strongly and positively correlated to milk production. Pirlimycin was eliminated from the milk during 36 hours. The pirlimycin milk concentrations 12 h after intramammary administration were 7.5-8.7 µg/mL which was subsequently used for MIC breakpoint determination (Thornberry *et al.,* 1993).

Thornberry *et al.* (1993) reported pirlimycin MIC values for mastitis pathogens. The MIC_{90} value for mastitis strains of S. aureus was 1 µg/mL (n=51). The MIC_{90} for Str. agalactiae (n=72) and dysgalactie (n=40) were 0.5 and 1 µg/mL and for Str. uberis (n=52) >32 µg/mL. The same study also observed a problem in determining the breakpoint for susceptibility disk assay. Larger inhibition zone is required for susceptible Str. agalactiae than other strains. Erskine *et al.* (2002) showed that pirlimycin resistance among 2778 mastitis isolates of S. aureus and streptococci (including uberis) was minimal or non-existing during a 7 year period.

Pirlimycin was effective in eliminating prepartum intramammary infections caused by staphylococci and streptococci (Oliver *et al.,* 2004). In an experimental mastitis model using Str. uberis, Oliver *et al.* (2003) showed that treatment results improved as a function of treatment duration among groups treated 2, 5 or 8 days. The cure rate was 58.1%, 68.8% or 80.0%, respectively.

Marbofloxacin

Marbofloxacin is a fluoroquinolone. These drugs are more effective in treatment of Gram-negative pathogens but they are considered broad spectrum antimicrobials. However, they possess considerable activity also against S. aureus. They are less suited for treatment of streptococci. Schneider *et al.* (2004) studied the pharmacokinetics of marbofloxacin in cattle. The half-life was found to be 2.5-6.3 h dependent on the administration protocol. A repeated administration at 2 mg/kg provided C_{max} concentration of 2 µg/mL after the third administration.

The study demonstrated that the MIC_{90} for S. aureus was 0.23µg/mL while the respective value for E. coli was 0.02. No change in susceptibility of veterinary pathogens during a 7-year period was observed, except for enteric E.coli (Meunier *et al.,* 2004).

Amoxicillin and clavulanic acid

Amoxicillin is a broad-spectrum β-lactam antibiotic that has been used in mastitis therapy for decades. However, amoxicillin is susceptible to β-lactamase activity, which inactivates the molecule rapidly. Clavulanic acid is a suicidal β-lactamase inhibitor. Clavulanic acid does not participate in the antibacterial effect, but acts only in elimination of the β-lactamase activity. Amoxicillin is rapidly converted to ampicillin in the body. Ampicillin pharmacokinetics are essentially similar to other β-lactam antibiotics. The half-life is short (about 1 hour) and the volume of distribution low.

According to Erskine *et al.* (2002) about 60% of S. aureus isolates are susceptible to ampicillin. The susceptibility to ampicillin among mastitis causing streptococci is 100% and for E. coli 85%. Therefore, significant improvement by amoxicillin/clavulanic acid treatment compared for amoxicillin or ampicillin alone can be expected only for S. aureus. In a field trial, Taponen *et al.* (2003) compared two mastitis treatments against S. aureus. In this study the pathogens lacking β-lactamase activity were treated with penicillin and the β-lactamase producing strains with amoxicillin/clavulanic acid combination. Comparison of the two groups appears complicated.

Conclusions

Mastitis causing pathogens appear generally susceptible to the new antimicrobial agents. Unsuccessful treatments, therefore, do not seem to result from lack of antimicrobial potency. Hence, significant differences in therapy results appear to be related mainly to pharmacokinetic considerations.

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The contribution of science to progress in milking technology

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Abstract

This review offers a personal perspective on the impact of science and engineering on milking technology over the past decade. Examples of scientific results or engineering inputs that have changed milking systems or practices include:

- research on threshold settings for automatic cluster removers;
- science-based guidelines for monitoring and improving teat condition;
- clarification of the effects of the milking machine on mastitis risk;
- the evolution of ISO standards for milking installations from dimensional specifications to performance-based guidelines;
- the new automation revolution.

Other interesting and potentially important contributions to scientific knowledge (such as blood, lactate or MAA sensing for detection of abnormal milk; reverse pressure gradients; compressive load applied by teatcup liners) have not been included in my list of examples because, to date, they have not had any real impact on commercial practice. Some of these results, and others we don't even know about yet, might turn out to be winners in the next decade - provided that the shrinking pool of milking researchers around the world can be saved from extinction.

Keywords: milking machine, teat condition, vacuum fluctuations, automation

At the IDF Centenary conference in 2003, one review on the development of liners and pulsators over the past 100 years (Mein and Schuring, 2003) concluded that:

> "…. practically all commercially successful liners and pulsators have evolved by trial and error. With few exceptions, the guiding development principle has been to use whatever seemed to work best on the farm. Apart from one major burst of development activity that was stimulated by new research results starting around 1970, scientific knowledge and real engineering input has followed rather than led the evolution of liners and pulsators."

In marked contrast to this "suck it and see" method of development for liners and pulsators, the contributions of science and engineering have had major impacts on many other aspects of milking technology. The following examples are offered as a personal perspective on the contributions of scientists and engineers to the practice of milk harvesting during the last 10 years or so. My list is incomplete and subject to the charge of personal bias. No doubt, others could add more examples of scientific contributions that have had a significant impact on commercial farms. The main point I want to make is that the past decade could be described as a "golden age" for the influence of science on the technology of milk harvesting.

Changing ACR settings

A Danish study (Rasmussen, 1993) provided the springboard for achieving shorter milking times and better teat condition for high-producing cows, especially in North American herds. In that experiment, milking time was reduced by 0.5 min per cow with no loss of milk yield when the end-of-milking setting for automatic cluster removers (ACRs) was raised from 0.2 kg/min to a flow rate threshold of 0.4 kg/min. Teat condition improved markedly and significantly fewer cows developed clinical mastitis in the early detachment group of cows. These research results sparked a 5-year period of cautious field evaluation in the USA. Threshold flowrate settings for ACRs were raised from default settings of about 0.3 kg/min to 0.5 kg/min for herds milked twice per day, and to levels as high as 0.9 kg/min for some herds milked thrice daily. At the same time, the typical settings of 10-20 sec time delay for cup removal were shortened to 0 - 5 sec. The net effect has been to reduce milking times by up to 1 min or more per cow with no reported loss of milk yield, no change in SCC or mastitis levels. In addition to quicker milking, the major benefits have been improved teat condition and calmer cows, especially the fresh cows. Today, only 12 years after the publication of Morten Rasmussen's 1993 paper, almost every manufacturer, installer or user of ACRs around the world has been affected by the practical consequences of his research work.

Teat condition and teat health

Maintenance of healthy teat skin and teat-ends is a key part of any effective mastitis program. In 1994, IDF published an excellent bulletin on "Teat tissue reactions to milking and new infection risk" written by a group of European scientists headed by Prof Dr Joern Hamann. These scientists confirmed that changes to teat tissue, the teat-end and teat canal alter the risk of new mastitis infections (Hamann *et al.*, 1994a,b). Subsequently, Neijenhuis *et al.* (2001) showed a significant association between teat-end callosity and incidence of clinical mastitis. At about the same time, simpler methods for quantifying the short- or medium-term effects of milking on teats were proposed by Hillerton *et al.* (2000) who noted that many effects of machine milking are easily recognisable immediately after cluster removal. These scientific contributions provided a framework for the establishment of an informal discussion group of researchers and udder health advisors, self-styled as the "Teat Club International" (TCI), whose major activities and achievements have been:

- to review non-infectious factors (Mein *et al.*, 2001) and infectious factors (Hillerton *et al.*, 2001) affecting short- or medium-term changes in teats;
- to describe the association between teat-end hyperkeratosis and mastitis (Neijenhuis *et al.*, 2001);
- to propose a simple protocol for systematic evaluation of teats in commercial dairies and guidelines for interpretation of observations (Mein *et al.*, 2001);
- to provide guidelines for data collection and analysis (Reinemann *et al.*, 2001);
- to produce a teat condition portfolio (Hillerton *et al.*, 2002);
- to conduct short courses on evaluating teat condition and interpreting the data.

To date, more than 600 participants have attended short courses and training workshops conducted by TCI members during the past 4 years in the USA, UK, Germany, Spain and Australia. The short courses and CD portfolio on teat condition continue to be in demand,

indicating that TCI activities are highly regarded by udder health advisers who can then apply the information to improve teat health in commercial herds. One important outcome has been a general acceptance of the need to look beyond the limited effects of the machine when trouble-shooting teat health problems and also when making recommendations to improve teat condition.

Effects of the milking machine on mastitis

In 1987, five main milking-related mechanisms of mastitis infection were proposed by an International Dairy Federation (IDF) Group of Experts lead by Jerry O'Shea (IDF 215, 1987). Subsequent research results (eg., Baxter *et al*., 1992; Lacy-Hulbert, 1998; Rasmussen *et al*., 1998; Spencer, 2003) and additional reviews (eg., Dodd, 1987; Hamann *et al*., 1994; Woolford, 1995) have provided some new perspectives on the effects of the milking machine on udder health.

The new perspectives include Dodd's conclusion (Dodd, 1987) that "the main way milking machines will influence the level of exposure [to infection risk] is likely to be their direct effect on the health of the teat duct and the skin of the teat". With the benefit of hindsight, I believe that the Irish research team led by Jerry O'Shea, and later by Eddie O'Callaghan, had figured out the vacuum fluctuations story sooner and more clearly than many of us were prepared to admit in the early 1990s. According to the Irish viewpoint (O'Shea *et al*., 1987), impacts and higher new infection risk resulted only from "acute" irregular vacuum fluctuations with exceptionally fast rates of pressure change. Such acute fluctuations could be measured only in the adjacent teatcups within an individual cluster when one teatcup slipped. During the past 10 years, it seems that most people have come to accept the Irish viewpoint that all other types of vacuum fluctuations are inconsequential in relation to mastitis risk.

Following is a brief summary of the effects of the milking machine on mastitis (adapted from Mein *et al*., 2004).

- Direct and indirect milking machine effects might account for 6-20% of new infections in an "average herd". Further quantification of the overall contribution of the machine contribution is difficult and elusive because of the multi-factorial nature of the disease (Woolford, 1995). Nevertheless, it is clear that most new infections are caused by factors other than the milking machine.
- Because new infection rates are lower during lactation than in the early dry period, regular milking seems to have positive benefits in helping to keep teat canals and teat-ends clean.
- The milking machine can influence new infection rates in four main ways as described below.
  1. By increasing bacterial numbers on the teat skin due to cross-contamination.
     - The New Infection Rate (NIR) is reduced by keeping bacterial numbers low on the cows' teats, especially near the teat orifice.
     - Herd management and milking management practices probably have over-riding effects compared with the potential contribution from milking machines.
  2. By changing the resistance of the teat canal to bacterial invasion.
     - The risk of new infections by contagious as well as environmental pathogens such as *Str. uberis* is increased by machine-induced changes in teat-end condition. Such

changes may include: increased congestion and oedema in the teat wall which results in slower closure of the teat canal and/or hypoxia in teat tissues; slower rate of removal and regrowth of teat canal keratin; greater degree of openness of the teat canal orifice after milking; increased hyperkeratosis of the teat-end.
    - The NIR is reduced by providing pulsator settings and liner characteristics that ensure effective teat massage.
3. By generating forces that increase the risk of bacterial penetration of the teat duct.
    - Air speeds greater than 2 m/s up the short milk tube may assist bacterial penetration into or through the teat canal.
    - Normal liner movement is much too slow to generate air speeds greater than 2 m/s.
    - The real action takes place within an individual cluster due to a sudden, transient air inrush through a teatcup when:
        - a liner slips or squawks loudly;
        - a cluster is kicked off or detached abruptly;
        - a cow is machine-stripped vigorously enough to break the seal between a teat and the liner mouthpiece.
    - Such events can produce "acute irregular vacuum fluctuations". These are large (15-30 kPa), transient drops in claw vacuum (often lasting less than 1-2 seconds) with very fast rates of change (150-300 kPa per sec).
    - The resulting high transient pressure gradients between the claw and adjacent liners can increase the NIR by accelerating milk droplets to speeds > 2 m/s towards the teat-ends in adjacent teatcups but only within the same cluster.
    - Vacuum fluctuations in the milkline or receiver are too slow to increase the NIR unless they increase the frequency of slipping liners or cluster falls.
    - Correlations linking unstable milkline or receiver vacuum with increased mastitis are likely to be associative rather than cause-effect relationships. That is:
        - NIR is increased by sudden air inflow through one or more teatcups.
        - Air admission through teatcups is the primary cause of transient vacuum fluctuations in milklines.
4. By varying the frequency and/or degree of udder evacuation.
    - Compared with new infection rates in the early dry period or when milkings are omitted, the new infection rate is relatively low in cows that are milked regularly two or more times per day. Thus, machine milking has a positive effect in reducing the risk of new mastitis infection.
    - In general, the clinical symptoms of mastitis are decreased as milking frequency is increased provided that teat-end condition is not compromised by milking too many times per day.

Performance-based ISO standards and guidelines

The 1996 revision of the International Standards Organisation (ISO) standards on Construction and Performance of Milking Systems, and on Mechanical Testing Procedures, provided another springboard for the application of science and engineering principles to milking technology. The fundamental shift in emphasis, from the traditional dimensional

specifications to the new performance-based guidelines and standards, was driven by scientists and engineers in four key areas:

- New guidelines for the diameter, slope and configuration of milklines evolved from the hypothesis that stratified milk flow, rather than slug flow, was the preferred flow condition in dual-purpose milklines. These guidelines on effective milk-carrying capacity of milklines resulted from a truly international collaboration between scientists including Paul Thompson (who first proposed the idea of using stratified flow to design milklines at an ISO working group meeting in 1991), Doug Reinemann, Stephen Spencer and Steve Stewart (USA); Pierre Billon (France); and Odd Ronningen (Norway). As a direct result of the new ISO guidelines, milkline sizes have tended to increase in Western Europe. At the other end of the range, milklines greater than 100 mm in diameter are seldom installed in new milking systems in the USA today.
- New guidelines for CIP cleaning of milking systems evolved from the application of simple engineering principles for the production and control of slug flow for cost-effective cleaning. This research and practical application was spear-headed by Doug Reinemann (Reinemann and Grasshoff, 1993). Before 1993, hardly any milking equipment dealers or technicians understood the dynamics of cleaning large diameter milklines or long milklines. Since the mid-1990s, however, the application of these engineering studies has given milking equipment companies and dealers an invaluable set of tools for trouble-shooting cleaning problems, optimising the design and maintenance of CIP systems, and providing significant savings in water and chemical usage on farms all over the world (Reinemann *et al.*, 1997).
- The simple but subtle change to measurement of Effective Reserve in or near the receiver, rather than at a position near the regulator, has had an astonishing impact on the design of milking systems, the preferred placement of vacuum regulators and the recommended size of vacuum pumps, especially in North America. This change in measurement point enabled the milking equipment industry to understand the problems associated with locating the regulator too far from the receiver, and the use of airline fittings that were often too small for the volume of air flowing through them. In the early 1990s, the most common reaction to a real or perceived problem of vacuum instability within American milking systems was to increase the size or number of vacuum pumps. One result was that Regulation Efficiency was often less than 30% in large milking systems in the USA. These common deficiencies became obvious when new guidelines for evaluating vacuum levels and air flows were developed by the Machine Milking Committee of the National Mastitis Council (NMC), a process that was spear-headed by Dr Andrew Johnson (Johnson *et al.*, 1996). The NMC guidelines have been widely adopted by the milking equipment industry. Their widespread application has resulted in marked improvements in vacuum regulation in milking systems across the USA, Canada and Mexico.
- New guidelines for determining Effective Reserve and for sizing vacuum pumps evolved from the performance guideline that vacuum in the receiver should remain stable within 2 kPa of the intended level. In the USA, the Machine Milking Committee of NMC co-ordinated a field project to determine the minimum effective reserve capacity required for maintaining a stable vacuum during milking in commercial milking systems (Mein *et al.*, 1995). The practical application of this research produced significant electrical power savings on dairy farms across the USA, Canada and Mexico. Concurrently, vacuum regulation was improved on the majority of these farms.

Automation of milking

The **mechanization of milking** had an astonishing impact on labour productivity throughout the last half of the 20th century. In The Netherlands, for example, the labour required per dairy cow per annum fell from about 330 man-hours in 1950 to only 50 man-hours per cow by 1980. The widespread adoption of machine milking was accelerated by labour shortages on dairy farms after World War II, especially in the northern hemisphere.

Another industry revolution has begun during the last 15 years. Like the earlier period of mechanisation, the new **automation revolution** will have a huge impact on dairy farming practices. Unlike the mechanisation period, the automation revolution was kick-started by science rather than by trial and error.

The scientific seeds of automation were sown in The Netherlands with much of the early research work driven by Wim Rossing (Rossing and Maatje, 1978). Electronic cow identification (cow ID) was pioneered by scientists at Wageningen in the mid-1970's, for example. From the mid-1970's, Dutch scientists began to explore physiological monitoring of cows in heat, or of sick cows, using physiological variables that could be measured quickly and automatically such as cow activity (as an indicator of oestrus), milk temperature and electrical conductivity of milk (as a screening test for mastitis).

These scientific seedlings began to flower in the 1990s. By that time, it had become clear that continuing developments in automation and the application of computers on farms would change the future of dairying in two major ways.

1. Components such as cow ID tags, milk meters, automatic cluster removers, automatic drafting gates and sensors for monitoring physiological variables such as oestrous activity had the potential to be much more than items of hardware to reduce farm labour. When connected to a central, on-farm data processing system, they could provide the core elements of an information system for more effective and more profitable herd management. The impact on large dairy farms with conventional milking systems has been to enable the milking staff to concentrate on the task of milking while providing the herd manager with daily management information on:
   - Cows - data on milk yields, herd health, reproductive activity.
   - People - quality and repeatability of work routines used by the milking staff.
   - Equipment - the efficient functioning of the milking machine, stall by stall, hour by hour, week after week after week.
2. Automatic milking systems (AMS) were installed on commercial farms starting in 1992. The gradual acceptance of AMS in Western Europe was under-pinned by a large, diverse scientific team in an integrated EU project co-ordinated by a new generation of Dutch scientists (Meijering, van der Vorst and de Koning, 2003). On the opposite side of the world, Murray Woolford had the vision and drive to initiate the scientific evaluation of AMS for extensive pasture-grazing systems in NZ (Woolford *et al.*, 2004). The defining feature of an AMS is that the cows are more or less free to decide when they will come to the milking area. Thus, automatic milking is much more than just another way of milking cows. It is an entirely new way of dairy farming because milking becomes a background operation. Automatic milking has the potential to revolutionize the way of life on dairy farms all around the world. It is only a matter of time.

The last golden age?

It would be foolish and naive to suggest that none of these developments would have occurred without the contributions of science. However, it is quite clear that science and engineering have had major impacts on milking technology during the last 10 years or so. Future generations might look back on this decade as a "golden age" for the science and technology of milk harvesting. There is a dark side to this glowing assessment, however. As the resources available for milking research continue to shrink steadily in all major dairying nations, there is a high likelihood that it could be seen as the last of the golden ages.

Dairy farmers would be the real losers if that were the case. There are still lots of potential problems and opportunities where the milking industry could benefit from continued investment in science and engineering during the next decade. Examples that are high on my 'wish-list' of priorities include:

1. Better tests and guidelines for evaluating the performance of milking units. Although the new ISO standards provide sensible and specific requirements for vacuum pump capacity, regulator function, line sizes, and other system components, they become less specific as they get closer to the "cow end" of the machine.
2. Development of guidelines based on changes in selected physical characteristics of liners to help farmers determine the useful working life of liners and the optimum time to replace their used teatcup liners.
3. More reliable sensors for detecting clots in foremilk or some related physiological indicator of tissue damage. Such sensors would be welcomed by herd managers and milking staff in conventional parlours because fore-stripping is an incredibly inefficient and unrewarding way to find clinical mastitis cases. Such sensors will need to have exceptionally high sensitivity and specificity to be accepted by owners of AMS for automatic diversion of abnormal milk.

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Mastitis in small ruminants

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Abstract

The most recent knowledge about small ruminant mastitis is presented in this text. The authors point out the etiological, epidemiological and control aspects of mastitis by several pathogens, especially those due to *Staphylococcus* spp., which are the most prevalent pathogens responsible for intramammary infection in small ruminants. The public health impact of pathogens causing mastitis is also emphasized and the efficacy of diagnostic tools are discussed, especially bacteriological detection and determination of milk somatic cell counts (MSCC). Several mastitis control strategies are discussed, but milking procedures, teat dip disinfection and selective dry-off therapy are carefully reviewed, including the risk of contaminating milk through use of antibiotics and antibiotic detection.

Keywords: small ruminants, etiology, somatic cell counts, diagnosis, control

Introduction

Current knowledge of mastitis in small ruminants has been recently reviewed by authors such as Bergonier *et al*. (2003). More specifically, the role of intramammary pathogens in mastitis in goats has been reviewed by Contreras *et al*. (2003) and Bergonier and Berthelot (2003) have reviewed the epidemiology and control of mastitis in sheep. Further studies include those of Paape *et al.* (2001), who explored the feasibility of indirectly diagnosing mastitis through milk somatic cell counts (MSCC) in small ruminants, and of Gonzalo *et al*. (2004), who recently discussed the analytical, health, productive and technological aspects of performing MSCC in sheep and goat milk.

Etiology of intramammary infection and epidemiological consequences

The annual incidence of clinical mastitis in small ruminants is generally lower than 5%, but this incidence can increase sporadically. The prevalence of subclinical mastitis has been estimated at 5 to 30% (Bergonier and Berthelot, 2003; Contreras *et al*., 2003), but there is only limited data about incidence of IMI in the literature.

Classic etiology of mastitis in small ruminants

Several pathogens can cause mastitis but *Staphylococcus spp*. are the most frequently diagnosed causal microorganisms of intramammary infections (IMI) in goats and sheep.

Other pathogens such as *Streptococcus spp*., *Enterobacteriaceae, Pseudomonas aeruginosa, Mannheimia haemolytica,* corynebacteria and fungi can produce IMI in small ruminants but occurrence rates are lower. In addition, severe cases of mastitis related to incorrect preventative strategies have been attributed to the pathogens *Aspergillus fumigatus*, *Serratia marcescens, Pseudomonas aeruginosa*, or *Burkholdelia cepacia* (Las Heras *et al*., 1999; Berriatua *et al*., 2001; Bergonier and Berthelot, 2003; Contreras *et al*., 2003; Gonzalo *et al*., 2004b).

Lentiviruses are also known to infect goats and sheep but because they rarely produce clinical symptoms or elevated MSCC (Turin *et al.,* 2005), they are not usually considered as classic small ruminant intramammary pathogens. Nevertheless, caprine lentiviruses should still be included in the general plan for controlling mastitis (Contreras *et al*., 2003).

Contagious agalactia syndrome

Because contagious agalactia syndrome produces symptoms other than mastitis, some authors fail to consider mycoplasmas *as* the etiology of sheep or goat IMI. However, the intense effects of this pathogen in reducing milk production and increasing the MSCC, means that contagious agalactia should be considered as one of the most important causes of mastitis in endemic areas. In herds clinically infected by *Mycoplasma spp*., besides significant losses due to mortality or the need to cull animals, producers cannot comply with the milk quality standards demanded by consumers, industry and public health organizations (Corrales *et al*., 2004).

Mastitis-causing pathogens of zoonotic impact

Rather than risking a human health hazard that could be caused by some mastitis-causing bacteria, milk is generally heat treated to minimize this effect. However, in regions where cheese is made from raw milk, controlling clinical and subclinical mastitis becomes a priority. Even when using pasteurized milk, the ability of some bacteria, such as *Staphylococcus aureus*, to produce thermostable toxins, enhances the zoonotic role of these pathogens. Under European legislation, the control of *S. aureus* is mandatory, such that the marketing of sheep, goat and cow milk containing *S. aureus* is highly restricted (Directive 92/46ECC Council 1992). Because of its zoonotic importance, preventing milk contamination by *Listeria monocytogenes* it a high priority for the industry. Although, most cases of milk-borne listeriosis are related to spoilage of the raw milk through fecal or environmental cross-contamination, a few cases of listerial mastitis have been reported in sheep. One report of clinical mastitis in a ewe caused by *Listeria monocytogenes* described a highly increased MSCC and persistent shedding of bacteria through milk (Winter *et al*., 2004). Similarly, severe human infections attributed to the consumption of nonpasteurized cow milk were associated with mastitis caused by *Streptococcus zooepidemicus* (Balter *et al*., 2000*)*. There have also been descriptions of mastitis due to *S. zooepidemicus* in goats and sheep (Las Heras *et al*., 2002). The identification of *Nocardia* spp., has also been considered important, due to their potential for causing disease in humans, and because *Nocardia farcinica* is known to cause mastitis in goats (Berriatua *et al*., 2001; Maldonado *et al*., 2004). Indeed, *N. farcinica* is a significant public health concern owing to its aggressiveness, its tendency to disseminate, its resistance to antibiotics and its laborious biochemical identification (De La Iglesia *et al*., 2002). These difficulties could have contributed to the increased incidence of disease caused by this microorganism in developed countries (De La Iglesia *et al*., 2002).

Staphylococcus aureus

Intramammary infections caused by *S. aureus* warrant special attention because this bacterium is responsible for both acute clinical mastitis (gangrenous mastitis) and subclinical mastitis. A main priority should therefore be the implementation of programs to eradicate *S. aureus* from dairy herds of sheep and goats.

S. aureus secretes several toxins contributing to the pathogenesis of mastitis, such as leukotoxins. These leukotoxins can selectively kill host polymorphonuclear leukocytes (PMN) and monocytes. In an investigation of the leukotoxic actions of *S. aureus* strains isolated from cows, sheep and goats with mastitis, (Rainard *et al.*, 2003) found that most isolates were leukotoxic and that strains isolated from small ruminants were more leukotoxic towards bovine PMN than *S. aureus* strains of bovine origin. However, these authors also noted that the PMN of small ruminants were more resistant to these leukotoxic effects than bovine PMN. Besides producing toxins, *S. aureus* also secrete exopolysaccharides ("slime"), which form a protective barrier that restricts the efficiency of both the host immune response and chemotherapy (Baselga *et al.,* 1994). The best strategy for controlling this pathogen is to remove infected animals from the herd, along with conventional precautions such as milking hygiene and dry therapy.

Coagulase-negative staphylococci

Coagulase-negative staphylococci (CNS) are the most prevalent pathogens causing subclinical mastitis. Although less pathogenic than *S. aureus*, CNS can also produce persistent subclinical mastitis, significantly increase MSCC, cause clinical mastitis (Deinhofer and Pernthaner, 1995; Contreras *et al.*, 1997b; Ariznabarreta *et al.*, 2002), as well as producing thermostable enterotoxins (Meyrand *et al.*, 1999; Udo *et al.*, 1999). Nevertheless, despite the accepted role of these bacteria as major IMI-causing pathogens in small ruminants, the pathogenicity of the different CNS species varies widely.

The most commonly isolated CNS species in persistent subclinical IMI in goats and sheep are *S. epidermidis, S. xylosus, S. simulans* and *S. chromogenes; S. caprae* is among the most prevalent causal microorganisms in goats (Gonzalo *et al.*, 2002; Contreras *et al.*, 2003; Bergonier *et al.*, 2003). The presence of different CNS species could be attributable to certain practices for controlling mastitis, such as the protocol and type of disinfectant used for teat dipping or dry-off treatments (Contreras *et al.*, 2003). Because novobiocin-sensitive CNS seem to be the most pathogenic, we should consider including this antibiotic in the dry-off treatment procedure (Deinhofer and Pernthaner, 1995; Gonzalo *et al.*, 2002), although maximum residue limits for sheep and goat milk have not yet been defined for this antibiotic.

Milk yield losses and increased MSCC in infected goat and sheep udders have been widely documented (Gonzalo *et al.*, 1994; Gonzalo *et al.*, 2002; Leitner *et al.*, 2004a; Leitner *et al.*, 2004b). However, despite the high incidence of CNS linked to IMI in sheep and goats, the pathogenic mechanisms that underlie these subclinical infections remain largely unknown. Using *S. epidermidis* to induce IMI in ewes, Winter *et al.*, (2002) reported that lactating udders are capable of a prominent local inflammatory response. Cytokine levels were significantly elevated soon after infection, peaking between 8 and 24 h, and increased IL-1β levels persisted for 144 h. In parallel, the MSCC peaked at 8 h but counts returned to normal values between 48 to 144 h, despite the presence of bacteria in milk. These authors suggested a complex relationship between cytokines and the course of infection,

because cytokines and PMN decreased as infection progressed. In addition, when comparing goat and sheep IMI, it seems that the sheep mammary gland is more affected by CNS (Leitner *et al.*, 2004a; Leitner *et al.*, 2004b).

Diagnostic tools

Bacteriological detection

The gold standard for the diagnosis of IMI in dairy species is bacterial culture. Selective bacteriological testing serves to cut the cost of extensive sample collection and could help poorer areas adopt mastitis control programs. In this sense, the viability of frozen intramammary pathogens in milk is longer than the lactation period, such that frozen samples can be used in the design of goat mastitis control programs (Sanchez *et al.*, 2003). For economic and practical reasons, usually only one milk sample is used for the diagnosis of IMI. Indeed, taking as a true positive diagnosis, the isolation of the same pathogen in consecutive samples from the same udder half, premilking and single sampling shows high sensitivity (96.2%) and specificity (96.1%) (Contreras *et al.*, 1997a). Nevertheless, because the specificity and positive predictive values of this test were found to be higher for postmilking, compared to premilking samples, collecting a postmilking sample is recommended when only one milk sample is used for the diagnosis of goat IMI (Sanchez *et al.*, 2004).

Somatic cell counts

The most important differences between goats and sheep affecting their diagnosis are related to the MSCC. These differences are mainly due to the higher MSCC in uninfected goat halves, the higher apocrine component of goat milk secretion and the larger number of non-infectious factors increasing the MSCC of goats compared to sheep (Paape *et al.*, 2001). Today, most dairy laboratories use MSCC methods (fluor-opto-electronic counters) that are adequate for the apocrine composition of milk from small ruminants, especially goats. However, given that the MSCC is an indicator of milk quality and that bonus/penalty schemes for the farmer are based on the bulk tank MSCC, it is important that the MSCC is as accurate as possible. Unfortunately, the standardization of methods according to the type of preservative used or the storage time and temperature etc. of the milk samples has not yet been sufficiently documented (Gonzalo *et al.*, 2004a).

Control and prevention strategies

Vaccination

Vaccines against clinical grangrenous mastitis, that are available on the market for small ruminants, are widely used when there is a high incidence of clinical gangrenous mastitis. However, owing to the reported different efficiency of these vaccines for dairy cows and sheep, and their inability to prevent new infections, it has been suggested that vaccines should be used in dairy herds with a high prevalence of *S. aureus* IMI to reduce clinical symptoms. The effectiveness of vaccination programs against mastitis caused by *S. aureus* has been reported for sheep but not for goats (Amorena *et al.*, 1994; Tollersrud *et al.*, 2002). The efficiency of a vaccine including *S. aureus* and *S. simulans* was assessed in field conditions (Marco, 1994), with results indicating the reduced prevalence of clinical mastitis

but not of subclinical infection. At present, vaccination studies have failed to find this tool decisive for controlling mastitis in small ruminants, and more immunization studies are needed to improve this strategy.

Milking procedures and teat dip disinfection

To improve the health status of the herd, the whole farm has to be subjected to conditions of strict hygiene. In effect, through optimizing milking machine standards and parlor systems, the udder health of dairy sheep herds was found to improve (Gonzalo *et al.*, 2005). Most of the routines implemented for dairy cows, including milking order, are also applicable to small ruminants, especially when the herd shows a high incidence of IMI. Because of the opportunistic nature of CNS, their prevalence increases with deficiencies in mechanical milking systems or in milking hygiene. To control CNS-induced IMI, all milking routines should be revised and milking equipment must be periodically checked to ensure correct milking variables such as vacuum level, pulsation rate and ratio, vacuum reserve per unit, etc. Similarly, adequate quality control of the water used to clean the milking equipment is needed to avoid infection outbreaks, as has been reported for *Pseudomonas aeruginosa* (Las Heras *et al.*, 1999).

Teat dipping has been demonstrated to be highly effective at preventing new infections in cows' udders due to different pathogens, especially CNS (Hogan *et al.*, 1987). In small ruminants, postmilking teat dipping has been used, mainly in highly infected herds (Paape *et al.*, 2001; Bergonier and Berthelot, 2003; Contreras *et al.*, 2003). The quality control of the teat dip disinfectant is very important, because some outbreaks have been related to an inadequate disinfectant acting as an infection source, as reported for *Serratia marcescens* causing mastitis in sheep when using a quaternary ammonium based teat dip (Tzora and Fthenakis 1999).

Conventional teat dipping solutions are iodine or chlorine based, which are not suitable for organic farming, and studies are underway on the efficiency of new disinfectants. In one such study, a dodecyl benzene sulfonic acid spray failed to maintain and/or restore the udder health of a sheep herd subclinically infected by CNS (Klinglmair *et al.*, in press).

Other management measures

Controlling the epidemiological situation of *S. aureus*-provoked IMI in herds of nursed lactating animals is difficult, because the lambs or kids of infected mothers will transmit the infection to the rest of the lactating females, when trying to supplement their own mothers' milk. Also, in sheep with an IMI due to *Mannheima haemolytica*, suckling lambs are the main source of infection as they spread the infection to their mothers. Weaning leads to a drop in the incidence of *M. haemolytica* mastitis. Fortunately, artificial lactation is on the increase in modern farms for production and health reasons, such as combating lentivirus infection (Contreras *et al.*, 2004). This practice is improving the health status of both the lactating kids or lambs and the animal's udder.

Antibiotic dry-off treatment

Based on the following findings, selective rather than generalized dry-off antibiotic treatment seems to be preferable:

- The spontaneous cure rate at parturition, which can be especially high for small ruminants, is 20 to 60% (Paape *et al.*, 2001; Contreras *et al.*, 2003; Bergonier and

Berthelot, 2003). In a study in which the incidence of IMI during the postpartum was compared in goats and sheep, spontaneous cure rates were significantly higher in sheep (McDougall *et al.*, 2002).

- Antibiotic dry-off treatment was unable to reduce the incidence of new IMI in goats and sheep (Paape *et al.*, 2001).
- The good improvement of udder health, especially in goats, when adequate programs to control mastitis are correctly implemented (Contreras *et al.*, 2003; Gonzalo *et al.*, 2005).
- Selective antibiotic dry-off therapy was found to significantly reduce the incidence of IMI, minimizing antibiotic residues in milk (Gonzalo *et al.*, 2004b).
- This treatment requires veterinary surveillance to ensure its adequate and hygienic administration. Massive outbreaks of mastitis have been attributed to an iatrogenic origin through syringe contamination by *Pseudomonas aeruginosa* or *Aspergillus fumigatus* (Las Heras *et al.*, 1999; Bergonier *et al.*, 2003; Contreras *et al.*, 2003; Gonzalo *et al.*, 2004b). A sporadic outbreak caused by *Burkolderkia cepacia* was also associated with contamination during antibiotic dry-off treatment (Berriatua *et al.*, 2001).
- Overuse of antibiotics increases the risk of antibiotic resistance and multiply antibiotic resistance has become a public health problem. The detection of *S. aureus* strains causing sheep mastitis resistant to aminoglycoside antibiotics should be considered a public health concern, given the similar resistance mechanism to strains isolated in humans (Goni *et al.*, 2004).
- Few drugs are specifically licensed for use in small ruminants, particularly goats. The use in small ruminants of antibiotics or other drugs registered for cows, or even the use in goats of products registered for sheep carries a high risk because the safety and efficacy of these products in each species are largely unknown (Mavrogianni *et al.*, 2004).
- The absence of antibiotic residues is mandatory in the European Union for milk from cows and other species, although it seems that positive results are higher in milk from small ruminants than in cow's milk (Yamaki *et al.*, 2004). Despite the availability of registered non-specific methods of residue detection for the milk of small ruminants, (Contreras *et al.*, 1997b) demonstrated the high selectivity of several antibiotic residue kits registered for cow's milk towards goat's milk. However, because the techniques routinely used for identifying antibiotic residues are unable to detect all positive cases, antibiotic detection methods need to be standardized for sheep and goat milk (Yamaki *et al.*, 2004; Montero *et al.*, 2005).

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Animal welfare: Of increasing importance in modern dairy production

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Abstract

It is well documented that consumers are interested in and often express concern about the welfare and well being of animals in modern agriculture. Swedish consumers have, in repeated studies, rated the way cows are managed and fed among their 5 main concerns about dairying. Internationally the Farm Animal Welfare Council (FAWC) in the UK has defined "the five freedoms" of animals:

1. Freedom from hunger and thirst - by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort - by providing an appropriate environment including shelter and a comfortable rest area.
3. Freedom from pain injury or disease - by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour - by providing sufficient space, proper facilities and company of the animal´s own kind.
5. Freedom from fear and distress - by ensuring conditions and treatment which avoid mental suffering.

Modern dairying would be wise to incorporate these basic cow rights into its quality control programs as soon as possible.

Swedish and Danish dairy companies have decided to develop a common system called "Program for cow welfare" that eventually is to be used by all Swedish and Danish dairy farmers. The intention is to increase cow welfare and well being in order to keep consumer confidence and strengthen the economic return on dairy farms and dairy companies. Current systems in research that evaluate animal welfare are time consuming and costly, however. The hypothesis is that information in the Nordic animal disease and treatment recording systems and information from the cow control data bases on fertility, milk production and culling can be combined to an index that will indicate animal welfare. The production parameters should make it possible to also calculate the economic benefits of increased cow welfare.

Keywords: animal welfare, data base, consumer concern, profitability, five freedoms

Animal welfare and dairy production

The consumer is interested in and often expresses concern about the welfare and well being of animals in modern agriculture, dairy cows included (Donelly, 2004). Swedish

consumers have, in repeated studies, rated the way cows are managed and fed among their 5 main concerns about dairying (Swedish Dairy Assoc (SDA), 1999; SDA, 2000; SDA, 2003).

These consumer concerns go far beyond the demands of the animal protection acts that generally only address acts of cruelty against animals. The Swedish Animal Protection Act (SAPA) of 1988 (L1, SFS 1988:534; www:djurskyddsmyndigheten.se), however, goes further than that and also states that animals have the right to be protected against disease and the right to express their natural behaviour. This latter right has been the focus of much debate in Sweden during the last decade and the conflicts have ranged from how cages for egg laying hens should be constructed and which functions, such as sand-bathing, nests for egg-laying, etc should be included, to, among other things, the width and length of cubicles for dairy cows. The SAPA states the right for Swedish dairy cows to be let out to graze in the summertime. The current interpretation is that cows should be allowed access to pasture at least during the time between two milkings, either in day- or nighttime.

Internationally animal rights people as well as philosophers and researchers have contributed to the debate. In UK the Government already in 1979 established an independent advisory body called the Farm Animal Welfare Council (FAWC; www.fawc.co.uk). The FAWC has the rights to investigate any topic falling within its remit, to communicate freely with outside bodies, the European Commission and the public and to publish its advice independently. The FAWC in 1993 defined the five freedoms:

1. Freedom from hunger and thirst - by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort - by providing an appropriate environment including shelter and a comfortable rest area.
3. Freedom from pain injury or disease - by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour - by providing sufficient space, proper facilities and company of the animal´s own kind.
5. Freedom from fear and distress - by ensuring conditions and treatment which avoid mental suffering.

The consequence of these five freedoms for the dairy industry is that we must look not only at physical resources and abuse of animals but also address how animals are reared and managed. Does modern dairy production abuse or exploit animals? Are cows “rights” or freedoms violated? The answer is perhaps that it depends - on whom your asking and whose views you take. It is clear, however, that culling rates are too high, in the Nordic countries about 30-40% annually, disease levels are also too high - treatments against mastitis are sometimes above 50% on a national level (NMSM, 2004), too many cows have sore feet or legs - a study in UK found 55% of dairy cows to be lame (Clarkson *et al.*, 1996) and a death rate in calves of 15-20 % is not uncommon (Mikel Jensen *et al.*, 2001). Issues to address and take seriously if the dairy industry wants to keep up its image (Caswell, 2004).

The focus of the discussion in the present paper will be on animal welfare and animal well being in the sense expressed in the 5 freedom statements above rather than in the traditional sense of protection of animals against cruelty.

Research in animal welfare

In the last five years research into animal welfare and how it may be measured on herd basis has increased dramatically. Two international conferences on the topic have been held (Sorensen and Sandoe 2001; Webster *et al.*, 2003).

It is our opinion that the research community has reached a consensus on which indicators of animal welfare to use when evaluating animal health on herd level. Winkler *et al.*, in an article (2003), list the herd level variables for which there exist enough documentation to warrant inclusion in a system intended to measure animal welfare on herd level. The majority of variables are animal based, such as lameness and cleanness rather than the more traditional ones defined in laws and regulations, such as available area, width and length of cubicles, type of floor or bedding, etc (www/djurskyddsmyndigheten.se, ANI and Freedom Food Scheme). The large variation demonstrated between animal based welfare indicators (Manske, 2002; Rousing, 2003) shows that these are influenced both by management/stockmanship and type of production system.

During the year 2000 a network of researchers within the EU was formed, focusing on the topic of assessing animal welfare indicators on herd level. The network is called COST 846 (www.cost846.unina.it). As a result of the efforts of this network an integrated EU project was started in May 2004, with the aim to develop a European system for the assessment of animal welfare (www.welfarequality.net). The hypothesis is that it will be possible to create a "welfare-index" that can be used to improve conditions for animals on farms and communicate with consumers when marketing foods derived from these animals. The aim is that a preliminary system will be presented in 2008.

Stockmanship important

FAWC, who defined the 5 freedoms, also states that stockmanship is the key to animal welfare and that "training and supervision, necessary to achieve required standards, are key factors in the handling and care of livestock". Researchers have shown this to be true for decades. Seabrook in early work showed that the stockmans handling and attitude toward his cows influenced milk production (Seabrook, 1984). Studies in the Nordic countries and the Netherlands illustrate the same. Early research by Ekesbo (1966), Grommers *et al.* (1972), Schmidt-Madsen (1978), Bakken (1978) and Saloniemi (1980) clearly demonstrated the adverse effects of, for instance, open manure gutters with grids and electrical cow trainers but also the effects on animal health from other physical factors such as stall length and width, presence or absence of partitions, amount of litter in the stall and type and amount of play in the neck tie, etc. This led to that technical solutions that did not take basic or behavioral needs of animals into consideration were prohibited in new stalls and hence the physical environment surrounding the cow gradually improved. Østerås (1990) and Ekman (1998), when investigating risk factors for udder health on Norwegian and Swedish dairy farms with tie stall systems a decade later, could find no or only few traditional stall construction risk factors for udder health. Management-related variables, such as clipping of haircoat, trimming of claws, milking your cows in a milking order with the healthy cows first and the infected ones with higher cell counts later, etc., dominated as explanatory variables. Recent studies show great variation in selected health variables between herds with the same production systems (Fregonesi and Leaver, 2001; Winckler and Willen, 2001;

Arvidsson and Hallén Sandgren, 2004). The proportions of lame cows, for instance, in loose housing systems in Sweden and Denmark have been shown to vary from 0 to 27% (Manske, 2002; Rousing, 2003). This leads to the conclusion that studying the physical resources around the cow is not enough. We also have to look at how the cows are fed and managed - the degree of stockmanship present on the farm. The combined effects of these "hard" and "soft" factors are best expressed and possibly also measured in the animal or in animal related variables.

Measuring animal welfare

Examples of existing systems for assessing animal welfare are the "Animal Needs Index" (ANI) in Switzerland and "The R.S.P.C.A. Freedom Food Scheme" in Great Britain. The ANI system grades important factors in animal rearing, such as available space, possibilities for social contacts, quality and construction of the floor and cubicles, indoor climate, management routines etc into an index (Bartussek, 2001). Both the ANI and the "Freedom Food Scheme" (www.rspca.org.uk) are based on the "Five Freedoms" (www.fawc.co.uk/freedoms.htm) and aim at describing available resources and documentation on evaluated farms. No or only very few animal based measurements or management variables are used in these systems, however. An evaluation of the Freedom Food Scheme was not been able to show increased animal welfare on participating herds (Main *et al.,* 2003). Experiences from users of the ANI indicate good repeatability in measurements at different visits and between different evaluators, but this does not mean that farms with the best or worst welfare are classified in the right category, or in other words, the validity of the index is not satisfactory (Bartussek, 1998).

FRISKKO: The swedish preventive herd health program

The Swedish Dairy Association and the local livestock associations have, in co-operation with scientific institutes and veterinary practitioners, developed a system for animal health care. Testing different models has resulted in a current concept called FRISKKO- HealthyCow (Sandgren and Carlsson, 2000).

The aims of FRISKKO are:

- Improved animal health.
- Better production economy in the herd by reducing costs due to poor animal health.
- Providing the food industry with safe and ethically produced products.

The program aims at improving animal health by improving management and feeding rather than using antibiotics and other medicines. FRISKKO is voluntary and has, in controlled studies in Sweden, been shown to increase the earnings on average by 600 SEK (approximately € 70) per dairy cow and year mainly by reducing costs (Hallén Sandgren, 1999; Hallén Sandgren and Carlsson, 2000; Hult and Hallén Sandgren 2004; Lindberg 2004). The major parts of costs associated with disease in dairy herds are indirect, such as involuntary culling, wastage of milk and increased labor hours. Direct costs, such as veterinary bills including medicine, account for less than 15% of total costs (Emanuelson and Hallén-Sandgren, 1996). FRISKKO comprises computerized tools for working with udder health, fertility, claw disorders and various other herd health problems. It also features a computer program for comparing the economic result on a particular farm, based on its

production records, with other farms in Sweden (for more information on FRISKKO see other paper by Ekman *et al.* in these proceedings).

An important part of FRISKKO is the use of key figures for animal health and production, which give a good indication of the disease situation in the herd over a period of time. It is is also possible to compare the economic performance of the herd with other herds of similar size and breeds, within the region and the entire country.

Key figures that mirror not only animal health and disease but also other aspects of cow welfare, such as how well the current management and production system is able to meet the physiological and behavioural needs of the animals, are presently missing, however. The dairy farmer, and his advisor, therefore is not able to compare the welfare of his animals to that in other herds.

"System for cow welfare"

Swedish and Danish dairy companies have decided to develop a common "System for cow welfare" that eventually is to be used by all Swedish and Danish dairy farmers. The aim is to increase cow welfare and well being in order to keep consumer confidence and strengthen the economic return on dairy farms and dairy companies. Current systems in research that evaluate animal welfare are time consuming and costly, however (Tind Sorenson, 2003). The hypothesis is that information in the Nordic animal disease and treatment recording systems and information from the cow control data bases on fertility, milk production and culling (Emanuelson, 1988; Philipsson *et al.*, 2003) can be combined in an index that will indicate animal welfare (Fraser, 2004). The production parameters should make it possible to also calculate the economic benefits of increased cow welfare and demonstrate that to the farmer. The " System for cow welfare" is intended to be used as a tool that, in a simple, clear, reproducible and consistent manner will identify, solve and prevent animal welfare problems in dairy herds.

Marketing preventive herd health programs like FRISKKO is not easy, mainly due to that the main costs associated with clinically or subclinically diseased animals are indirect. Costs are often hidden in lowered production or not seen because they are accepted as part of production. Therefore voluntary advisory systems for herd health, like FRISKKO, are unlikely to be popularly accepted/adopted by producers unless they are supported by a driving force, such as marketing interests from, for instance, a dairy company that is interested in promoting its public image by engaging in a project like "System for Cow Welfare". Support from such a program, backed by the marketing interests of a dairy company, also could solve the problem of quality assurance of preventive herd health programs like FRISKKO. These prerequisites for success are currently in place in Sweden and Denmark, which hopefully, in the not so distant future, means good news for our dairy cows, dairy producers and their co-ops and in the end - all consumers of dairy products.

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Diagnosis of mastitis and indicators of milk quality

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Abstract

Worldwide structural changes in dairying reflected in larger herds and high yielding cows now require re-evaluation of standard mastitis diagnostics. Economic considerations are the driving motivation for the need to develop and apply new diagnostic systems which make possible continuous udder health monitoring at a reasonable cost-benefit relationship. Automated on-line systems to measure inflammatory parameters could be an appropriate alternative to traditional mastitis diagnostics.

Milk quality indicators must include:

- aspects of hygiene in the sense of food safety (consumer protection);
- compositional criteria in terms of physiological concentration of milk constituents (technological properties), and
- consumer acceptance (milk image). A variety of parameters can be applied to all these criteria. However, the most important overall indicator for milk quality seems to be the somatic cell count, because it includes hygienic, compositional and technological aspects in addition to consumer demand for healthy animals.

Keywords: diagnostic strategies, inflammatory parameters, hygienic, compositional and technological milk quality, physiological cell count

Introduction

Especially during the last decade, there have been major changes in dairying reflecting a marked evolution worldwide. In nearly all industrialized countries, current trends in dairy farming can be characterized by reduced numbers of cows and herds on the one hand, and by increased herd size and lactational yield per cow on the other. A comparison of pertinent data from the United States and Germany over the last ten years shows a reduction of approximately 50% in the number of herds and of 10% in cows in both countries, while the average yield per cow and lactation has steadily increased by about 2% annually on well-managed farms to 10,000 kg per cow. The proportion of larger farms and mean herd size also increased markedly. It has to be stressed that, despite these structural changes, the total milk volume produced increased only slightly or remained stable.

Overall, it can be seen that larger farms necessitate a higher degree of specialization of farm labour and cow group management in order to attain sufficient productivity and to secure employees' salaries.

These new conditions of dairying require new methods, procedures, strategies and concepts for the routine examination of udder health status in addition to diagnostic procedures applied in connection with clinical cases.

Without a doubt, the health status of the cow and particularly that of the mammary gland are fundamental to sufficient profitability of dairying. As it can be seen from Figure 1, the most important factors for efficacy in dairying in addition to health are secretory activity of the mammary gland and the milk yield level per lactation, perhaps better expressed as lifetime production.

In the following the most common mastitis diagnostic procedures are briefly described and evaluated with regard to their potential for integration into a modern system of health surveillance in a dairy herd. Moreover, as dairying is a business that must produce milk that is acceptable to consumers, it will also be considered to what extent parameters used as mastitis diagnostic criteria could also serve as indicators for milk quality.

Diagnosis of mastitis

Definition of mastitis

The term mastitis describes an inflammation of the mammary gland characterized by several physical and chemical alterations of the milk and corresponding pathological changes in the mammary tissue depending on the type of the disease.

Although any foreign matter in or injury to the internal tissues of the mammary gland can lead to mastitis, infectious micro-organisms are the predominant cause of mastitis. The combination of the parameters causative agent and the inflammatory response resulted in the categorization of udder health status shown in Table 1 (DVG, 1994). Originally, this scheme was published by the IDF in 1967 with a cell count threshold of 500,000 cells/ml. In the meantime, it is internationally accepted that the physiological cell count threshold is in a range of 100,000 cells/ml.

In addition to this scheme for udder health categorization based on cyto-bacteriological parameters, criteria such as development of the disease, symptoms, causative agents, etc. can also be used to distinguish several types of mastitis. Examples for such differentiation of different mastitis types are given in Table 2.

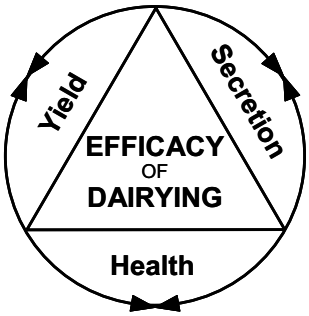

Figure 1. Factors determining efficacy of dairying.

Table 1. Categorization of udder health based on quarter foremilk examination.

Somatic cells (SCC) [/ml]	Pathogens absent	Pathogens present
< 100,000	normal secretion	latent infection
> 100,000	non-specific mastitis	mastitis

Table 2. Classification of mastitis types related to different criteria.

Criterion	Type of mastitis (examples)
Development, severity	Peracute, acute, chronic
Morphology	Catarrhal-purulent, abscedative
Symptoms	Subclinical, clinical
Location	Thelitis, galactophoretis, mastitis
Pathogen	Mastitis by coliforms, by *S. aureus*, etc.

Therefore, the German Veterinary Society (DVG) changed the cell count threshold to 100,000 cells/ml, as shown in Table 1 (DVG, 1994).

Diagnostic pathways

As the term mastitis is applied to both the udder health category "unspecific mastitis" (with an increase in SCC, but no identification of pathogens) and to the category "mastitis" (increase in SCC and detection of pathogens), a diagnosis of mastitis can be made without the detection of mastitis pathogens. Inflammatory reactions can thus be used as the criteria for the characterization of mastitis cases.

Particularly in cases of clinical mastitis, there are a variety of mastitis diagnostic tests for the examination of the milk sample as to its appearance, contamination with pathogens, concentration of milk constituents and indicators of inflammation, etc., in addition to clinical examination of the cow, the mammary gland, and the secretion. Some determining factors for the most commonly applied mastitis tests are summarized in Table 3.

The methods to be chosen will depend on the objectives of the work and on the information and accuracy required. Examples of such procedures are given in Table 4.

The main interest of the farmer and the herd veterinarian is to reduce the losses associated with mastitis, for most net income losses in dairy farms are doubtless the result of subclinical mastitis. The first aim must be to improve the efficacy of mastitis prevention; for while there is a need for efficacious mastitis therapy, it must be realized that farmers seldom earn money when the cows have to be treated.

A variety of milk constituents can be used as inflammatory parameters, either in combination with or as alternatives to somatic cells. This is particularly the case in continuously applied udder health monitoring systems. Furthermore, cyto-bacteriological examination of the quarter foremilk is the best parameter for the diagnosis of mastitis, especially if performed twice at an interval of at least one week. However, this reference procedure is not feasible as a routine test, as it is very time-consuming and expensive.

Table 3. Determining factors of mastitis diagnosis tests in milk (selection).

Determining factor	Characteristic
Level of milk sample	Bulk tank - cow composite - quarter
Severity of mastitis	Clinical; or subclinical
Test performance	Manual; or automated
Bacteriological examination	Yes; or no? Positive or negative?
Inflammation determination	Yes; or no? Positive or negative?

Table 4. Comparison of current priorities in mastitis diagnostic procedures between scientific studies and field application (herd veterinarian).

Parameter	Sci. study	Field appl.
Clinical assessment	+	+++
Herd bulk milk	+	+++
Cow composite milk	++	++
Udder quarter milk	+++	+
Sampling frequency	+++	+
Culturing	+++	+ (+)
SCC - direct measurement	+++	++
SCC - indirect measurement	(+)	+++
Inflammatory parameters other than SCC	++	+ (+)
Milk yield	+++	+
Automation	(+)	(+)

The herd veterinarian should determine the udder health status by cyto-bacteriological examination of foremilk samples of all cows at ca. one-year intervals. These results will indicate the most important pathogens in the herd in question and may help determine the most appropriate measures for therapy and prevention. If routine udder health monitoring is performed, it is generally not necessary to culture quarter foremilk samples. (Exceptions are for example unsuccessful treatment of clinical cases or abrupt increase of mastitis cases, etc.).

Table 5 summarizes the procedure proposed for future mastitis diagnosis in well-managed dairy herds.

In conclusion, the measures for mastitis diagnosis should be focussed on a balanced cost-benefit relationship. Further developments of automated on-line udder health monitoring systems to measure different inflammatory parameters such as electrical conductivity, NAGase, lactose, lactate, etc., are expected to result in a very efficient and inexpensive diagnostic tool which ideally will include an inter-quarter evaluation score. Such a system is needed for economic reasons not only in larger herds but also in average-sized

Table 5. Proposal for future mastitis diagnostic procedures.

1.	At every milking (on-line-measurements)
1.1	Determination of cow individual milk yield
1.2	Determination of at least 2 inflammatory parameters (quarter/cow level)
2.	At weekly intervals
2.1	Herd bulk milk SCC
2.2	Herd bulk milk culturing
3.	At monthly intervals
3.1	Evaluation of cow composite and herd bulk milk data
3.2	Evaluation of clinical cases and efficacy of control measures
4.	At yearly intervals
4.1	Cytobacteriological examination of all quarter foremilk samples
4.2	Decision on future control measures in the particular herd

herds. Overall, the application of such an automated method would provide sufficient diagnostic information to control mastitis effectively and to ensure good milk quality.

Indicators of milk quality

Definition of quality

Milk quality can be defined in very different ways. Here, milk quality is considered only in regard to hygienic, compositional and consumer-relevant aspects.

Milk quality is a term that includes all favourable properties of milk. The quality grading of raw milk is based on predetermined thresholds for various hygiene and composition parameters. Since food safety is the bedrock of quality, all elements determining food safety are non-negotiable. However, other aspects like taste, appearance, availability, price (affordability) and so on are largely matters for the market place to decide.

It is nevertheless clear that general production conditions in the current global market network will have a manifold influence on the potential to affect food safety both positively and negatively. There is increasing awareness that it is not enough to establish correct legislative thresholds. A holistic view is necessary to solve food safety problems that will also solve existing problems of sustainable animal production.

Milk hygiene criteria are decisive for food safety, but may also influence milk composition. In nearly all countries, established legal or voluntary standards for the

hygienic quality of milk are principally focussed on the prevention of any potential impairment to consumer health by milk consumption (e.g. consumer protection). For this reason, certain aspects of milk composition such as somatic cell count thresholds are also used to grade milk hygiene. The somatic cells per se have no hygienic importance, but it is well documented that the risk of chemotherapeutic residue from mastitis therapy will increase with increasing cell counts (Ruegg and Tabone 2000). Milk of good hygienic quality can be characterized by the following criteria:

- low or very low numbers of saprophytes (spoilage agents);
- absence or low numbers of pathogenic microbes;
- absence of chemotherapeutic residues;
- reduction or minimization of contaminants.

Milk which fulfils all these parameters can be assumed to be a clean, safe and wholesome foodstuff (WHO 1992).

For the farmer, *the compositional quality* of raw milk is of prime interest as long as the milk price is related to milk composition. Responses to an IDF questionnaire (IDF 1998) indicate that this is very often the case for butterfat and crude (total) protein, but that other criteria such as lactose, milk solids, etc. are applied only in a few countries. However, public health standards for bacteria and somatic cell counts of raw milk are designed to protect public health and not to maximize dairy product quality and shelf-life (Barbano 2004). Therefore, healthy mammary glands (cell counts below 100,000 cells/ml) are needed to minimize the enzymatic deterioration of proteins and fat by plasmin and lipases. Low bacteria count and low cell count guarantee that milk maintains its flavour stability and that the shelf-life of milk and milk products is increased. In other words, the technological quality of milk, which is one aspect of its compositional quality, depends on healthy glands with low cell counts.

Milk quality has also been considered in regard to *consumer demands* and acceptance. Increasingly, consumers evaluate milk quality according to both objective and subjective parameters; for example, they demand that the foodstuff milk is not only clean, safe and wholesome, but also produced under ethical and environmentally responsible conditions. Thus, the health status of the cow and the mammary gland are crucial to meeting consumer expectations (Hamann 2002).

As shown in Figure 2, the milk composition in terms of variation of major and minor milk constituents is mainly influenced by lactation physiology, herd management and the health status of the cow and the mammary gland.

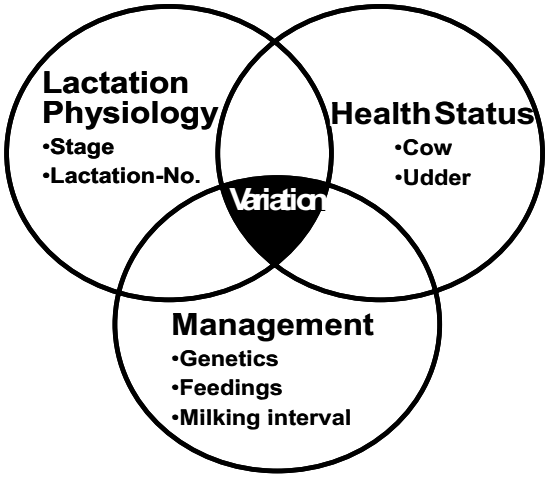

Figure 2. Variation of compositional milk quality.

Milk quality indicators

Hygienic quality

As indicated in the section "definition of milk quality", indicators for hygienic quality include the total bacteria account (TBC), absence of pathogenic bacteria and chemical residues, and contaminant levels below the maximum residue limits (MRL). Worldwide, these criteria are taken into consideration in special legal rules and/or related payment schemes. The dairy farmer thus has a direct economic interest in maintaining hygienic quality in a range that will assure the maximum price (premium class). Modern cleaning and disinfection techniques, proper application of hygienic measures during milking, and appropriate storage of the milk (e.g. cooling) make it possible, at least in modern dairy countries, that an average of more than 90% of the milk delivered to the dairy processors corresponds to premium milk class thresholds.

Compositional quality

There is very limited large-scale information on other milk quality parameters such as lactose, lactate, enzymes, fatty acids etc. This is due to the fact that, butterfat and milk protein, which are mainly influenced by lactation stage, feeding and breed; are included in payment schemes for milk in addition to cell count However, at least those milk

components can be regarded as indicators of milk quality in terms of milk composition if they indicate significant variations depending on udder health.

Only clinically healthy cows with four healthy (normally secreting) udder quarters will produce milk both in the quantities pre-determined by the animal's genetic potential and in a quality fit for all processing purposes - while satisfying consumer demand for a natural, healthy and beneficial/nutritious foodstuff. Keeping the bovine udder healthy is therefore in the interest of producers, processors and consumers.

A review of the literature makes it clear that only milk from a healthy udder has a physiologically normal composition (Tolle *et al.*, 1971, Barbano 1999, Reichmuth 1975). An udder quarter can be considered healthy if it has an SCC < 100,000/ml and is free of mastitis pathogens (Dohoo and Meek, 1982, Hamann 1996, Harmon 1994). The official standards for milk of highest quality were set using criteria that aim to exclude any possible health risks to humans. However, these criteria bear no relationship to the criteria used to define udder health, generating a discrepancy between the financial incentives for quality milk and the maintenance of udder health and consumer expectations. While today's international definition of a healthy udder may be a cell count limit of 100,000/ml, the cytological limits that define milk as acceptable for processing and manufacture vary internationally from 150,000 - 750,000/ml (IDF 1998). Udder health is commonly assessed on the basis of a cyto-bacteriological investigation of the quarter foremilk, whilst milk quality is determined on the basis of cytological assessment of a milk sample taken from the herd bulk milk. The somatic cell count of quarter foremilk samples (QFM) of healthy udder quarters is significantly lower than those of the corresponding composite quarter milk samples (QCM), yet the measurements are more comparable at a somatic cell count below 100,000/ml (Hamann 2001). This means that, the cell count for the composite cow milk should not exceed 100,000/ml for an udder with four healthy quarters (Laevens *et al.*, 1997, Ma *et al.*, 2000).

Significant differences ($P < 0.01$) were found for six different milk components (NAGase, lactate, lactose, chloride, potassium and electrical conductivity) determined for 9,326 QCM samples at five different cell count ranges (< 50,000, 50,000 - 100,000, > 100,000 - 200,000, > 200,000 - 400,000 and > 400,000 cells/ml). All means were transformed to log at base 10 and compared with the overall mean of all samples (= 0.0). Thus, the reference for all components across the somatic cell count ranges is zero. Figure 3 shows the deviation of the milk components in the various somatic cell count ranges.

There was a distinct change in the proportion of every milk component as the cell count exceeded 100,000/ml. This suggests that the physiological norm is in the region of 100,000 cell/ml. Regardless of the level of measurement (udder quarter, mammary gland or herd bulk), concentrations of milk components will differ significantly ($P < 0.01$) from the physiological norms in milk with a cell count above 100,000 cells/ml. In light of the detrimental changes in milk processing properties (technological quality) and the milk production losses associated with non-physiological cell count values, more attention should be paid to maintaining bovine udder health and meeting consumer demands.

The most important single indicator of milk quality is the somatic cell count. SCC is a criterion of hygienic quality and is a key parameter for the health status of the mammary gland on quarter, cow and herd bulk milk levels, and thus is indicative of the risk of non-physiological composition of the milk (compositional quality). Furthermore, the consumers can at least be sure that the overall health situation of the cows was at an acceptable level if the cell count is low (<100,000 cells/ml).

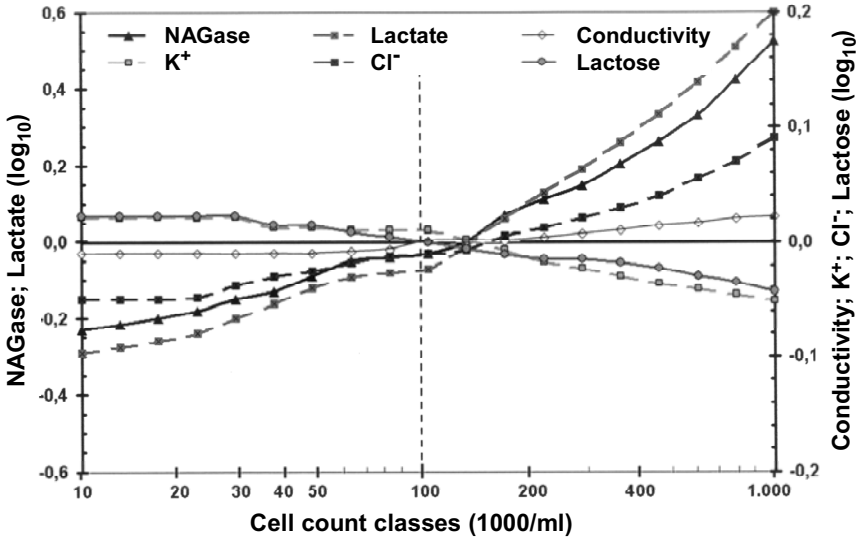

Figure 3. Mean deviation (in log 10) of selected milk constituents from the overall means in relation to cell count classes (n= 9326 QCM samples) (Hamann, 2001).

Conclusions

We should try to continue to improve the udder health of the dairy cow in order to improve the hygienic and compositional quality of milk.

The most promising way to reach this goal would be the gradual inclusion of cell count classes far below today's (e.g. 400,000 cells or higher) in payment schemes. This would mean that better udder health in a herd would be immediately rewarded with higher milk prices for the farmer. Moreover, since more and more milk and milk products are required to maintain their stability and natural properties, both milk producers and milk processors will profit from a closer partnership to ensure milk with those features, which can be expected only from low cell count.

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Mastitis control systems: The Norwegian experience

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Abstract

The aim of this paper is to describe the methods, results and to suggest possible improvements in the Norwegian Udder Health Control Program implemented from 1982. The program is an integrated part of The Norwegian Cattle Health Services from 1995. The goal of the program is to improve udder health by keeping the BMSCC low, to reduce the use of antibiotics, to keep the cost of mastitis low at herd level and improve the consumers' attitude to milk products. In 1996 a decision was made to reduce the use of antibiotics in all animal productions in Norway by 25 % within 5 years. Relevant data has been sampled through the Norwegian Cattle Herd Recording System (NCHRS); Health records from 1975, SCC data from 1980, all integrated within NCHRS. From 2000 the mastitis laboratory data was included in the NCHRS. Data on clinical disease, SCC and mastitis bacteriology has been presented farmers and advisors in monthly health periodicals since 1996 and on the Internet from 2005. In 1996 Norwegian recommendations on the treatment of mastitis was put in practise. Correct milking and correction of milking machines has been emphasized and less effort has been put on dry cow therapy and teat dipping. A selective dry cow therapy program is about to be implemented these days. The results so fare show a 50% reduction in clinical treatments of mastitis from 1994 to 2004, a reduction in BMSCC from 250,000 to 114,000 and a total reduction in the mastitis cost from 0.23 NOK to 0.13 NOK per litre milk corresponding to 9.2% and 3.7% of the milk prize, respectively. The reduction is attributed to changed attitudes, breeding, eradication of BVDV and preventive work. The improvements seem to continue.

Keywords: environment, breeding, dry cow therapy, teat dipping, BVDV

Introduction

Mastitis is defined as any inflammatory process in the mammary gland (IDF, 1987). Mastitis has a clinical or a subclinical appearance. Clinical mastitis is defined as mastitis with clinical signs from the udder or visible changes in the milk and is divided in; severe, moderate or mild according to the recommendation of IDF (IDF, 1999). Subclinical mastitis is only detected by laboratory methods such as analysis of somatic cell count (SCC) or other parameters related to the inflammatory process.

Clinical mastitis is measured as appearances of events and presented as events per defined time - e.g. cases per year (or month) at risk as an incidence rate (population measure) or risk of having one case in one animal during a specified time at risk - e.g. risk rate or incidence risk (individual measure). A case is defined as clinical appearance as

detected by the herdsman or more often as treated cases. There is a defined number of days, usually eight days from the case appears until a new case can be recorded.

Subclinical mastitis is measured using, inflammatory responses in a number of units, usually at the same time. The presentation of results will therefore be; Number of positives per analysed unit (quarter, animal or herd) or prevalence. The importance of this is described by IDF (IDF, 1997).

The main motivation for mastitis control is economical as stated by Morris (1975). Economical benefits from mastitis control should therefore be linked to different mastitis parameters. Not much scientific work has been done on this subject, however the IDF has tried to sum up its relevance (IDF, 2005). The subject will also be discussed in another lecture on this conference (Hogeveen, 2005). As economical gain is a main goal, the economic parameters must be included when mastitis control programs are evaluated.

Other important parameters are animal- and farmer welfare. Most clinical cases are painful for the animal and work demanding for the farmer. Subclinical mastitis might also be related to some pain (Eshraghi *et al.*, 1999). Mastitis caused changes in the milk content and -characteristics such as shelf-life and properties for cheese processing. Therefore the dairy processors demand quality payments for a low content of inflammatory parameters in the milk (SCC). The absence of careful mastitis control as example when pointing only at reducing the SCC might lead to huge costs of extra treatment and culling at farm level as the method. The economic analyses should therefore absorb all the important parameters of mastitis control. Other goals are to minimize the risk of having the milk contaminated with pathogens or toxins that are a hazard to human health (*Str.agalcatiae*, *Staph.aureus*-toxines, *Listeria spp* etc) and to eliminate as far as possible specific highly pathogenic strains of bacteria or bacteria that are carriers of resistant genes. These bacteria cause a continuous need for new and sophisticated antibiotics and they might cause transfer of pathogens or resistant genes into the human food chain which again would influence consumer attitude towards milk.

As most of the pathogens involved in mastitis are very common in the dairy cow environment, it is not economically feasible to try to eradicate mastitis. Pathogens like *Mycoplasma bovis* and*Str.agalactiae* are not usually found in the environment of the cow and would be easier to eradicate. Mastitis can, however, be controlled to a much lower level than we see today.

A full-scale mastitis control program have to include information on the level and status of the parameter to be controlled in an integrated analyses in an information system, The pathogen involved must be recognized to know how to deal with these to prevent and interfere with the pathogeneses of mastitis. Exact knowledge should be based on unbiased research. Finally there is a need for personnel and resources to do the information and advisory work and tools to motivate and the farmer or herds(wo)man to make the correct decision at the correct time.

The aim of this paper is to present results achieved in Norway from mastitis control and as far as possible to compare with other countries. The progress of mastitis control will be evaluated and future improvements will be suggested.

Material and methods

Records of mastitis at quarter level

Traditional mastitis diagnostics were done by cow side tests of clinical cases by farmers and /or veterinarians. Our experience is that this is costly, and gives little information without knowledge of the total health status. Our aim is to accumulate such information over time and to use it at herd level - to reveal which pathogen is involved in a particular herd - in clinical as well as subclinical cases. The information is used to develop a control program and a therapy program adapted to specific pathogens. From 1996 this information is therefore included in the NCHRS and presented in the health periodical together with information on cow milk somatic cell count (CMSCC) and clinical disease. From 2005 this information is available on the Internet for farmers, advisors and veterinarians.

Records of clinical mastitis

Norway introduced records of treatments already in 1975, probably as the first country worldwide. Computerized records were kept to control for certain reproductive disorders and also assess whether an association between breeding for higher milk production and mastitis existed. From 1978 focus has been on clinical mastitis resistance in the Norwegian Cattle Breeding Program. Since 1992 there has been more focus on mastitis than on milk yield. Another paper in this conference will go into details on the effect of the breeding program.

All historical events on clinical mastitis, CMSCC, milk yield as well as bacteriology is now available for both herd and cow analyses. This will give feed-back to the farmers and advisors on how different environmental changes and therapy protocols work at that specific farm.

Records of subclinical mastitis

Subclinical mastitis is recorded as somatic cell counts usually either at cow (composite) level (as CMSCC) or at bulk tank level (as BMSCC). These records started in the late 1970's

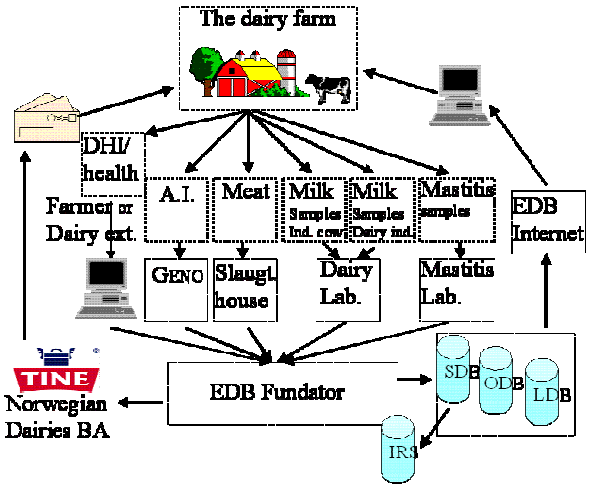

Figure 1. Integration of all relevant information in the NDHRS.

or early 1980's. From 1975 to 1979/80 it was performed by the mastitis laboratories twice a month using Coulter Counter. However from 1980, CMSCC was measured every second month or every month and BMSCC four times a month. All information was incorporated into the NDHRS (Figure 1). The most important result is that the information on consecutive analyses is presented and mathematically transferred to more useful data. This presents data so they can be used directly in herd and cow analyses in a problem solving process as well as for prognostics and diagnostics at cow level.

The usage of information

At cow level information on diagnostics can be used to predict the prognosis for different cows within a herd according to expected cure after therapy or no therapy and hence select cows for bacteriological culturing of milk samples to add more information to the system. Cows can then be selected for dry cow therapy, culling at the optimal lactation stage breeding etc. Finally, the information can be used to separate milk from delivery to the dairy processor. CMSCC together with available bacteriology is thus the most important decision-making tool in the daily dairy herd management of mastitis.

At herd level the information can be used to analyse the economics of mastitis, the herd characteristics, as the new infection rate, incidence of clinical cases, duration, prevalence etc. Interventions can be made as early as possible to avoid further destruction of the herd's economics.

The Norwegian Action Plan in mastitis control

The Norwegian mastitis control has followed the main principles stated by Dodd (1980) and Morris (1975). An effective mastitis control should aim to reduce the new infection rate. Morris (1975) questioned the usefulness of introducing the duration of infections stated by Dodd (1980). Morris (1975) stated that the term duration is easily comprehended, but that it is very difficult and time-consuming to measure in a significant population of animals. With new computer technology the Norwegian recording system is calculating both the new infection rate based on CMSCC and clinical mastitis records as well as the duration each month in each herd. The variables are described in more detail by Valde *et al.* (2005)The duration is simplified according to Dodd's equation; the prevalence equals the new infection rate times duration.

The Norwegian action plan in mastitis control is as follows:

1. Avoid high new infection rate
    a. Proper and exact environmental action according to the pathogens and problems at present
        i. Good milking routines
            1. Cleaning (hygiene)
            2. Good interaction with the cows (let down and welfare)
            3. Proper handling and milking equipment (air inlet)
            4. Good and proper preparation (let down and welfare)
            5. Careful removal of clusters (air inlet and over milking)
        ii. Good functioning milking machine
            1. Proper vacuum condition (teat handling)
            2. Proper liners (teat handling, impacts)
            3. Proper pulsation (teat handling)

4. Vacuum capacity (teat handling, impacts)
5. Proper capacity and dimension of pipelines (impacts)

iii. Good environment
1. Clean (hygiene and management)
2. Dry (hygiene, management and building)
3. Good stall function (animal welfare, hygiene)
4. Proper bedding area (animal welfare, hygiene)

iv. Diminish the contact between the pathogens reservoir and the teat canal
1. Culling chronic infected cows (management)
2. Clean and dry environment (management, building)
3. Avoid bedding material that act as reservoir for actual pathogens (hygiene, management)

2. Shorting down the duration of existing infections
   a. Removal of udder pathogens reservoir
      i. Culling chronically infected cows (*Staph.aureus* and others)
      ii. Clean and dry environment (CNS and environmental)
      iii. Therapy at an appropriate time and of the correct cows (dry cow period, *Staph.aureus* and streptococcae)
   b. Establish a therapy protocol adapted to the pathogens and environment at present.
      i. Selective dry cow therapy (for expected responders)
      ii. Appropriate therapy of clinical cases (for expected responders as well as the need for animal welfare)
      iii. Appropriate detection and therapy of subclinical cases during lactation (for those with economic benefits - very few cases)
      iv. Segregation (for unpromising cases until slaughtered)

All points should refer to the actual pathogen at present. This means you should first of all know the pathogen involved in the mastitis problem both at herd level as well as at regional and national level. The pathogens involved are different from herd to herd and from country to country, and the type of pathogens is changing over time (Pitkala *et al.* 2004). This is probably caused by the environmental changes and the therapy pressure.

At regional and national level

It is important to assess which pathogens are involved. In Norway a survey was done during year 2000 (Sølverød and Østerås, 2001). The results gave highly relevant information for the implementation of new strategies. *Staph.aureus* is the most prevalent bacteria, however half of them were associated with fairly low CMSCC and the incidence of clinical mastitis was not increased significantly in cows with this bacteria. The milk yield is higher just after calving but reduced later in lactation. The prevalence was highest at the start of the first parity and lowest in the second parity. The prevalence of *Staph.aureus* decreased during lactation while *Str.dysgalactiae* increased. There was also a strong seasonal effect, with higher prevalence during late indoor season and the summer compared to the autumn. The survey illustrated the importance of good surveys before implementing a control program. Surveys from Finland also illustrate that the pathogen panorama is shifting during time (Pitkala *et al.,* 2004). The control program has to be changed over time and also has to be different from country to country because we are not battling the same organisms.

Breeding program for resistance

Research have documented that it is possible to breed for a higher resistance to mastitis (Heringstad *et al.*, 2005). The heritability is found to be 3-5% for clinical mastitis and approximately 15 % for SCC. Many countries have included SCC in the breeding index. Finland and Sweden have also included clinical mastitis in the index and Norway has only included clinical mastitis. To achieve effective breeding development on traits with low heritability, it is important with large daughter groups. As there is a negative genetic correlation between clinical mastitis and milk yield sufficient weight has to be put on mastitis to get a positive effect. The Norwegian breeding program is probably the only program that has put enough weight on clinical mastitis to get a net positive effect on mastitis in the population. The Norwegian breeding program towards a higher individual resistance against clinical mastitis is an integrated part of the Norwegian mastitis control program. Some pathogens are more likely to cause clinical signs (*E. coli),* while other pathogens usually cause subclinical infections (*Staph.aureus* and CNS). Therefore it is a question on the effect if it is as good as possible both in clinical and subclinical cases.

Other diseases

There is a significant connection between a few other diseases and mastitis. This means that the control of mastitis can be even more effective if relevant diseases are included into the program (eg. BVDV, milk fever, reproduction, ketosis). It is documented that a herd newly infected with BVD virus got a 7% increase in the risk of clinical mastitis due to effect on the immune system during the infectious stage of the disease (Waage, 2000). When starting the BVD eradication program in Norway in 1992, the prevalence of BVD was 26% serological positive on bulk milk tank samples. In 2004 there was only 3 herds left with restrictions due to possible infected animals. The rest of the Norwegian cattle population is tested and found to be free from BVD virus. This has also had a positive effect on the results of the mastitis control program.

Dry cow therapy and teat dipping

The Norwegian mastitis control program is different from the five point plan as there has been no or very little dry cow therapy and only approximately 12 % of the herds have practised regular teat dipping. Reasons for this are that dry cow therapy was almost "banned" from the Norwegian School of Veterinary Science in the 1960's and 1970's. We are now trying to implement selective dry cow therapy and using our information system in the selection process. We recommend all cows with more than 100.000 in CMSCC the last three samples before drying off to be selected for bacteriological testing. Those who are positive for *Staph.aureus* and *Str.dysgalactiae* or other major pathogens should be dry cow treated. No treatment is recommended if CNS is identified. Cows with high CMSCC (above 600.-700.000) and with major pathogens should be culled at the economic optimal time in lactation. The whole program is scientifically based on the estimated formulas of prediction of success or failure according to Østerås *et al.* (1999a and 1999b) and Østerås and Edge (2000). At present about 0.05% of our cows are dry cow treated. According to our data, approximately 35 % should be sampled and of those, 35 - 40% should be assessed for dry cow therapy.

The restrictive use of teat dipping is due to the traditional way of thinking in Norway, that teat dipping would be detrimental to minor pathogens and normal skin germs and hence

ease the colonization by pathogens like *Staph.aureus* etc. At present we run a large project in more than 200 herds to evaluate the effect of teat dipping and dry cow therapy under Norwegian conditions. So far we see that our selection process for the dry cow therapy is effective in the way that we have achieved a cure rate on *Staph.aureus* in approximately 80 %. The results from this study will be presented soon.

Results

The results of the Norwegian mastitis control program are illustrated in figure 2 and figure 3. There has been a progress in BMSCC from 1980 till 2004 and for clinical mastitis from 1994 till 2004. From 1985 the BMSCC was reduced, but the clinical cases increased until 1994. Figure 4 illustrates that the economic losses due to mastitis in Norway has decreased from 1991 until 2004. The main reason for this is reduced losses due to clinical mastitis and production losses. The rate of clinical mastitis can be reduced further as long as the production and replacement loss does not increase. This will be followed closely and is an important part of the management of mastitis control in the country and regions.

The mastitis pathogens isolated from milk during routine sampling in Norway shows this prevalence during 2003 at quarter level: *Staph.aureus* 8.0 to 11.2 %, penicillin G resistant *Staph.aureus* 0.9 to 1.1 %, *Str.dysgalactiae* 1.7 to 2.9 %, *Str.agalactiae* 0.01 to 0.02 %, CNS 1.0 to 1.7 % and dry quarters 2.0 to 2.2 %. At cow level the prevalence is: 27.0 %, 2.8 %, 7.1 %, 0.05 %, 4.4 % and 7.0 %, respectively. The survey in 2000 identified the same prevalence to be 22.2 %, 2.8 %, 3.8 %, 0.0 %, 5.7 % and 8.8 %. Thus the proportional rate of *Staph.aureus* showing resistance to penicillin G is 10.5 to 12.6 %.

For samples taken from clinical mastitis cases we find typically 49.8 % of cows with *Staph.aureus*, 4.1 % with penicillin G resistant *Staph.aureus*, 17.3 % *Str.dysgalactiae*, 6.4 % coliforms and 7.7 % CNS. The same prevalence on quarter level is 16.6 to 22.8 %, 1.2 to 1.6 %, 4.2 to 6.3 %, 1.6 to 2.0 % and 1.8 to 2.8 % respectively, depending on quarter

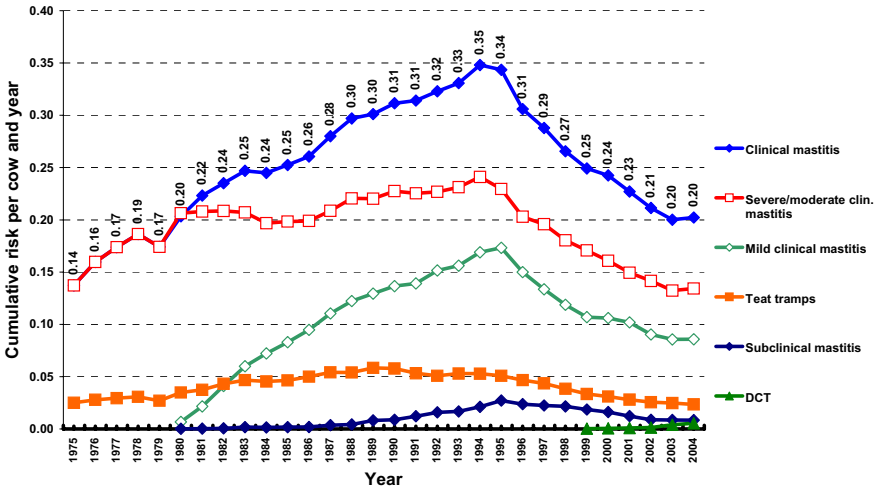

Figure 2. The cumulative risk of a cow being treated for different diagnoses of mastitis and teat tramps per year from 1975 till 2004.

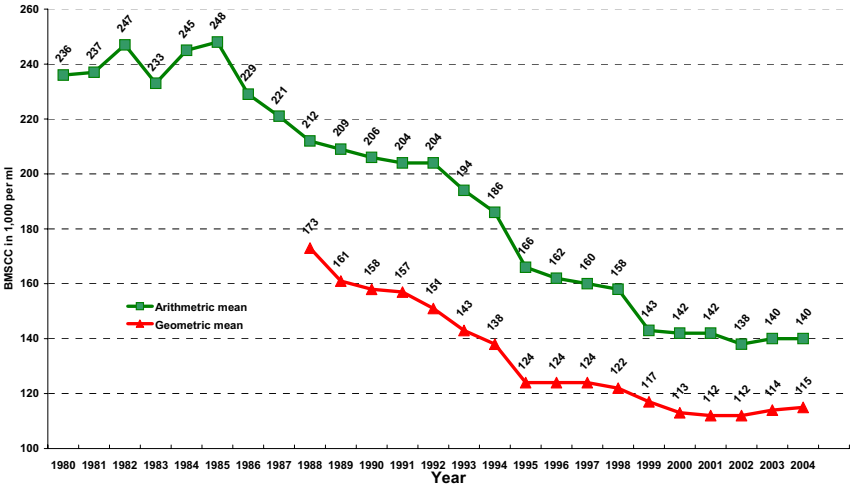

Figure 3. The bulk milk somatic cell count (BMSCC) in Norway from 1980 till 2004 expressed as both arithmetic and geometric mean.

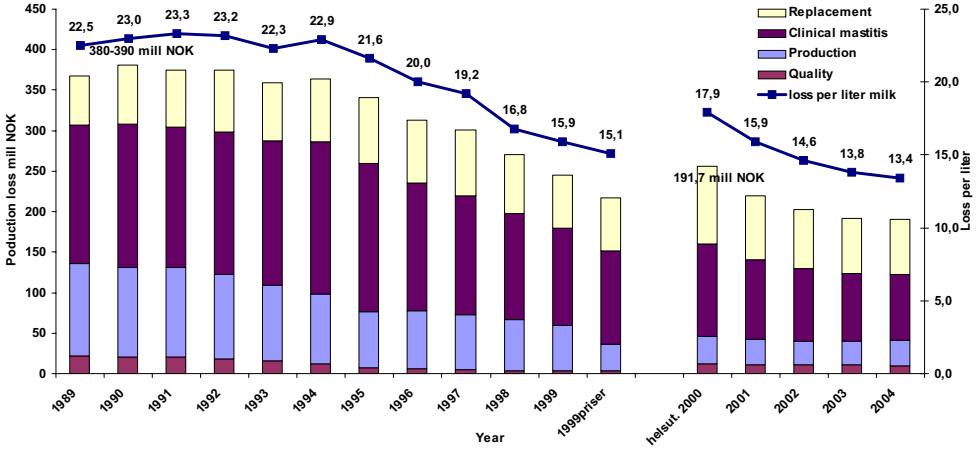

Figure 4. Estimated total mastitis loss in Norway from 1989 to 2004 divided in different types of losses and in total øre per liter milk delivered to the dairy processor.

site. The proportional rate of resistant *Staph.aureus* is thus 8.2 % on cow level and approximately 7 % at quarter level.

Figure 4 illustrates that the economics of this work has a value of 0.10 NOK per liter milk delivered to the dairy. In Norway this comprises a total amount of 150 million NOK or approximately 40 % of the level in early 1990's. More detailed analyses on the economical benefit of the control program illustrates that of the total benefit from 1990 to 1994 was 17 million NOK, of these 8 mill NOK was generated from better quality payment, 24 mill NOK from reduced production loss related to lower SCC, however an extra loss of 11 mill NOK from increased (means more) clinical treatments (both veterinary fees and discharged milk) and finally another extra loss of 4 mill NOK from increased (means more) replacement

due to mastitis. From 1994 to 2004, after the change in treatment strategy, the total gain of the program was 173 mill NOK, of these was 2 mill NOK generated from better quality payment, 55 mill NOK from reduced production losses related to lower SCC, 106 mill NOK from fewer clinical treatments (both veterinary fees and discharged milk) and finally 10 mill NOK from reduced replacement due to mastitis. It is obvious that the treatment strategy to lower the BMSCC during the 1980's was wrong as the extra treatment and culling costs was hardly significant in improving the economic performance in udder health, despite a large improvement in BMSCC. The new strategy from 1994 on reduced the treatment cost of more than 100 mill NOK without any unfavorable effect on quality or BMSCC.

Discussion and conclusion

The Norwegian mastitis control program has shown huge progress and the extra money earned is approximately 200 million NOK since 1994. The main reason for the progress is probably the well organized cooperatives which have made it possible to collect all relevant information in one database. The data is easily accessed by the farmer, the breeding organization and by the university. The last is important to be able to use the data in relevant research. The data is has now become more easily available for local advisors and veterinarians through the internet.

From 1984 there were large improvements in the level of BMSCC, however, there was also a large increase in the number of treatments of clinical cases of mastitis. This was probably due to more treatments to lower the BMSCC and get quality payments. This leads to over-treatments and excessive costs.

To avoid this, a treatment protocol on mastitis therapy during 1995/96 was introduced. This involved a more restrictive use of antibiotics, especially during lactation. Instead of declaring *Staph.aureus* to be treated as soon as possible, these bacteria should not be treated as subclinical infections unless at drying off.

The health periodical presents for farmers and advisors the key parameters at herd level to formulate a correct control program. The new infection rate, duration, prevalence as well as economic estimates of total mastitis losses in NOK per liter milk delivered was presented every second month To avoid over-treating cows and also to avoid too intensive culling strategies.

The tools of the strategy of reducing antibiotics by 25 % in 5 years had three main targets: 1) Through attitude changes of farmers, advisors and veterinarians to avoid uneconomic therapy of subclinical and mild cases of mastitis (short term effect), 2) Through making progress with good mastitis control protocols (medium fast effect), and 3) Through breeding for resistance to mastitis and to improve the cows ability to cope with mastitis (long term effect). This goal was obtained within 3 years and after 10 years the reduction is more than 50 percent.

We see from the paper of Herigstad *et al.* (2005) that the genetic improvement of the Norwegian cows has been 3 % unit per 10 years. This means that of 0.15 (from 0.35 to 0.20), 0.03 can be attributed to the effect of the breeding program. This is 20 % of the total reduction of 173 mill NOK, or 35 mill NOK. A BVDV herd will show a 7 % increase in the mastitis loss due to the introduction of BVDV according to Waage (2000) and 25 % of the herds were infected in 1994 compared to zero in 2004. The BVDV contributed with 173 mill NOK times 0.25 times 0.07, which equals 3 mill NOK. The rest of 173 minus 38 equals

135 mill NOK will partly be due to attitude changes, changed treatment strategies and better mastitis control due to a better information system etc. It should be noted that vaccination has not been an issue in mastitis control in Norway, although there was some research going on in the 1990's. It seems that Morris (1975) was correct when he stated that: "immunization has been promoted for many years as a long-term answer to mastitis. But, on economic grounds this is most unlikely to be true for most pathogens, even had vaccine development been reasonably successful, which it has not."

This sentence I will suppose is still true after a new 30 years of experience, hard work and lots of money input on that particular area.

Finally I would like to put up a question after reading Morris paper from the same conference in 1975 - which I as you understand, still found extraordinarily correct and informative. Why is the progress in mastitis control so slow? Are we working with the right areas and are the research in the area constructed and being conducted in the correct way.

The information technology with lots of coordinated data and modern epidemiological research is an important tool when trying to fight mastitis. The constraint now in Norway is to teach farmers, veterinarians and advisors to use all available information in the correct way. Experiments from the Norwegian breeding program have proven that breeding is one of the ways to go. Mastitis has to be included as a part in the total herd health management. There are relations between different diseases. however, the same risk factors might have different effect on the different diseases. Finally it is up to the farmer to implement a control program and this demands motivation.

It is all a question of good management practices at farm level.

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Antimicrobial resistance in mastitis organisms as a public health threat

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Introduction

Development of antimicrobial resistance is considered to be one of the major public health threats. It is a consequence of selective pressure on bacteria by exposure to antimicrobial agents. By genetic exchange mechanisms resistance can spread between bacteria. This spread is not inhibited by phylogenetic, ecological or geographical boundaries. Therefore development of resistance in the animal reservoir may have an impact on the resistance development in bacteria regularly infecting humans and may ultimately interfere with the treatment of infectious diseases in humans. This can happen directly as zoonotic agents become resistant, or indirectly when commensal or animal pathogenic bacteria transfer their resistance to human bacteria. For this reason a continuous monitoring of the development of resistance in bacteria of the veterinary and agricultural sector that may have an impact on public health is necessary. The exposure of animals to antibiotics is one of the main factors contributing to resistance. For policy making it is important to identify this hazard, therefore a system to obtain detailed data on the exposition of animals to antibiotics is also necessary.

Monitoring of resistance in bacteria circulating in animals should focus on bacteria of public health importance from food animals, considering that the food chain is a major route for transfer of bacteria from animals to humans. Bacterial species included in the surveys are food-borne pathogens and food-borne commensal organisms, preferably isolated from healthy animals at slaughter. To represent food-borne commensal organisms, sentinel or indicator organisms are used that may be a source for transmission of resistance determinants to pathogens. *E. coli* is included as indicator for the Gram-negative bacteria; enterococci are included as indicators for the Gram-positive bacteria. The inclusion of animal pathogens is of indirect importance for public health, but still very relevant. These pathogens usually represent a worst-case scenario because they are isolated from diseased animals that were treated with antibiotics. Therefore the surveillance of these strains in animal husbandry can be used for early warning purposes regarding the detection of new emerging resistances.

Although contamination of carcasses at slaughter and meat products at retail are considered the major routes of transmission, other important routes may exist. E.g. contamination of the environment, direct contact and contaminated products like milk or eggs may contribute to the dissemination of bacteria and their genes. Bacteria that are associated with milk and milk products are a potential source for transmission of genes.

In this paper the importance of milk related organisms for the potential transmission of resistance determinants to humans is discussed for two examples: macrolide and methicillin resistance.

Macrolide resistance

Resistance to macrolides in streptococci is most frequently caused by modification of the ribosome by a methylase encoded by an *erm*-gene and drug efflux by a membrane bound protein encoded by *mef* or *mre*A-genes(Clancy, Dib-Hajj *et al.*, 1997, de Azavedo, McGavin *et al.*, 2001, Duarte, Bellei *et al.*, 2005). Presence of Erm-methylases confers resistance to erythromycin and inducible resistance to lincosamides and streptogramin B (MLS phenotype). Subtypes *erm*(B), *erm*(F) and *erm*(Q) genes have been found in macrolide resistant streptococci from mastitis milk samples (Roberts and Brown, 1994).The Mef-pump confers resistance to 14- and 15-membered macrolides only (de Azavedo, McGavin *et al.*, 2001). Mre-pumps are described as second efflux mechanism, but are also detected in susceptible strains. Therefore *mre* may also be a housekeeping gene (de Azavedo, McGavin *et al.*, 2001). Recently a new Lincomycin-Streptogramin A (Lsa) resistant phenotype was detected in *S. agalactiae* in New Zealand. The resistance mechanism and genetic basis remains unknown (Malbruny, Werno *et al.*, 2004).Resistance to tetracycline and macrolide is often found in the same mobile unit, Tn*1545*, which is commonly found in streptococci and enterococci (De Leener, Martel *et al.*, 2004). This and similar transposons have a wide host range and are probably responsible for a large part of the tetracycline and macrolide resistance observed in streptococci.

In staphylococci different genes encoding macrolide resistance have been described. Four of these have been found in staphylococci from animals. The most common genes encoding for methylases in animals are *erm*(C) and *erm*(A) (Aarestrup and Jensen, 2002).

Methylases encoded by Erm-genes are plasmid mediated and can therefore rapidly spread in bacteria populations during selection pressure by antibiotic treatment. Optimum circumstances for selection exits during dry cow therapy, because long term low concentrations are present in the udder. This was observed during and after i.m.m. treatment with tilmicosin, a member of the macrolide family (Tikofsky, 2003). During and after treatment a significant increase of resistance to macrolides and lincosamides occurred. Moreover, resistance to tetracyclines increased, which may be explained by linkage on Tn*1545*.

In The Netherlands the majority of the udder pathogen *Staphylococcus aureus* is susceptible to macrolides (Table 1). But macrolide resistance is considerably higher in the penicillin- resistant strains if *S. aureus* compared to the penicillin-sensitive strains. Since

Table 1. Percentage of penicillin-sensitive and penicillin-resistant Staphylococcus aureus strains resistant to other antimicrobial (Sol, 2002).

	Penicillin-sensitive			Penicillin-resistant					
	1996	1997	1998	1996	1997	1998	1999	2000	2001
Number	322	898	844	221	494	486	216	283	347
Amoxy/Clav.	0	0	0	0.0	0.6	0.2	0.0	0.0	0.0
Cephalotin	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0
Neomycin	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0
Erythromycin	1.6	0.8	1.0	8.6	11.7	9.5	10.2	4.9	3.4
Lincomycin	3.8	3.7	5.0	16.8	15.0	17.7	17.6	14.9	10.1
Trim-sulpha	0	0.1	0	0.4	0.6	0.2	0.0	0.4	0.3

1999, erythromycin is no longer used in intramammary products in The Netherlands and this may explain the decrease in resistance to erythromycin (Sol, 2002). On the other hand macrolide resistance is high in the Dutch streptococcal udder pathogens *Streptococcus uberis* and *Streptococcus dysgalactiae* (Table 2) (Anonymous, 2004).

Usage of macrolides in treatment of mastitis and especially in dry cow treatment will select for transferable *erm*-genes in streptococci and staphylococci in the udder. Whether these transferable genes are a potential public health threat depend on several factors as milk hygiene and effect of pasteurisation of milk and host specificity. It is not unlikely that in raw milk and its products bacteria carrying these genes are present and consumed by humans. The subsequent transfer to human pathogens may take place. However, these genes are already present in human bacteria. Therefore the contribution of genes being present in bacteria in milk to the gene pool in human bacteria will only constitute an indirect and if any, a very small effect.

The white areas indicate the dilution range tested for each antimicrobial agent. Values above this range indicate MIC values > the highest concentration in the range. Values at the lowest concentration tested indicate MIC-values ≤ the lowest concentration in the range. The vertical bars indicate the breakpoints.

Table 2. MIC-distributions (in %) and resistance percentages (R%) of Streptococcus uberis and Streptococcus dysgalactiae isolated from mastitis milk samples from Dutch cattle by the Animal Health Service in 2003 (Anonymous, 2004).

	MIC-distribution (µg/ml)															
	0,015	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128	256	R%
S. uberis (N = 83)																
Penicillin	66.3	3.6	12	10.8	6	1.2	.	.|	.	.	.	.	.	.	.	0
Amox/clav. acid	43.4	24.1	4.8	20.5	6	.	.	.	.	.|	.	.	.	.	.	0
Cephalothin	6.0	8.4	16.9	24.1	9.6	12	22.9	.	.	.	.|	.	.	.	.	0
Erythromycin	6.0	20.5	47	7.2	.	.|	3.6	2.4	2.4	.	.	1.2	9.6	.	.	19.3
Lincomycin	1.2	3.6	10.8	14.5	3.6	2.4	4.8	19.3	6|	4.8	2.4	.	26.5	.	.	38.7
Pirlimycin	10.8	9.6	34.9	9.6	3.6	3.6	2.4	12|	7.2	2.4	.	.	3.6	.	.	13.3
Trim/sulpha	.	1.2	13.3	39.8	34.9	9.6	.	.|	1.2	.	.	.	.	.	.	1.2
Tetracycline	.	.	1.2	12	26.5	19.3	.	.	.|	1.2	3.6	20.5	12	3.6	.	41
S. dysg. (N = 94)																
Penicillin	97.9	.	.	1.1	1.1	.	.	.|	.	.	.	.	.	.	.	0
Amox/clav. acid	96.8	1.1	.	1.1	1.1	.	.	.	.	.|	.	.	.	.	.	0
Cephalothin	1.1	1.1	7.5	86	3.2	.	2.2	.	.	.	.|	.	.	.	.	0
Erythromycin	.	21.3	57.4	7.4	.	1.1|	.	.	.	.	.	.	12.9	.	.	12.8
Lincomycin	.	.	1.1	24.5	33	1.1	1.1	1.1	11.7|	4.3	1.1	.	21.3	.	.	26.6
Pirlimycin	.	4.3	50	20.2	4.3	.	.	.|	2.1	5.3	1.1	.	12.8	.	.	21.3
Trim/sulpha	.	1.1	1.1	38.3	47.9	11.7	.	.|	.	.	.	.	.	.	.	0
Tetracycline	.	.	.	.	.	1.1	2.1	7.4	12.8|	3.2	1.1	16	56.4	.	.	76.6

Methicillin resistance in staphylococci

Semisynthetic penicillins such as oxacillin and nafcillin are often used for treatment and prevention of *S. aureus* infections in bovine mastitis. These antibiotics are not degraded by the common staphylococcal penicillinase enzyme. Some staphylococci, including *S. aureus* and coagulase-negative staphylococci are resistant by a mechanism that renders them resistant to all current β-lactam antibiotics. This resistance mechanism is encoded by the chromosomally located *MecA*-gene, which encodes a transpeptidase, PBP2a, with low affinity to β-lactams. Expression is regulated by two regulatory genes *MecI* and *MecR*.

Methicillin resistant *Staphylococcus aureus* (MRSA)

In the past 20 years MRSA strains have become a major cause of hospital acquired infections. The incidence of MRSA in hospitals varies by country. In general these hospital acquired MRSAs (HA-MRSA) are multiple resistant to many different classes of antibiotics (Munckhof, Kleinschmidt *et al.*, 2004; Tiemersma, Bronzwaer *et al.*, 2004). Their prevalence varies in Europe from <1% in northern Europe to > 40% in southern and western Europe (Tiemersma, Bronzwaer *et al.*, 2004). In the USA the proportion of nosocomial infections due to MRSA increased from 2.5% in 1975 to 54.5% in 1999 (Stemper, Shukla *et al.*, 2004). Community acquired MRSA (CA-MRSA) is an emerging infection in individuals not specifically at risk for infection, although a propensity for infection exists in ethnic groups like Native Americans in the Mid West and aboriginals in Australia and Canada (Stemper, Shukla *et al.*, 2004). Most CA-MRSA strains are susceptible to antibiotics and more frequently associated with superficial skin and soft tissue infections than HA-MRSA, but in children deaths have occurred with necrotizing CA-MRSA pneumonia (Dufour, Gillet *et al.*, 2002; Stemper, Shukla *et al.*, 2004). Most *S. aureus* strains causing skin infection and necrotizing pneumonia harbour the Panton-Valentine leukocidin (PVL) genes (Lina, Piemont *et al.*, 1999; Dufour, Gillet *et al.*, 2002).

Clonally distributed CA-MRSA carrying PVL genes has been described in Europe and the USA(Dufour, Gillet *et al.*, 2002; Wannet, Heck *et al.*, 2004; Witte, Braulke *et al.*, 2004; Francis, Doherty *et al.*, 2005).

MRSA is rarely isolated from animals and most of the strains found are associated with exposure to infections in humans (Devriese and Hommez 1975; Gortel, Campbell *et al.*,1999; Seguin, Walker *et al.*,1999; Manian 2003; van Duijkeren, Box *et al.*,2004). MRSA is described to occur in milk samples from cattle (Devriese and Hommez 1975; Tomlin, Pead *et al.*, 1999; Lee, 2003), chickens (Lee, 2003), horses (Hartmann, Trostle *et al.*, 1997; Seguin, Walker *et al.*, 1999) and dogs (Gortel, Campbell *et al.*, 1999; Tomlin, Pead *et al.*, 1999; Manian, 2003; Duquette and Nuttall, 2004; van Duijkeren, Box *et al.*, 2004). Although human to animal transmission will play an important role for the cases in animals, clonal distribution in of MRSA in animals may contribute to the dissemination of MRSA. The latter was described in horses in Michigan State Veterinary Teaching Hospital (Seguin, Walker *et al.*, 1999) and in cattle and chickens in Korea (Lee, 2003). Both in Michigan and in Korea the MRSAs were multiple resistant indicative of being related to HA-MRSA.

In coagulase negative staphylococci the *MecA*-gene is more commonly present.

This includes *S. intermedius*, *S. schleiferi* subsp. *schleiferi*, *S. schleiferi* subsp. *coagulans*, *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. xylosus* from dogs (Gortel, Campbell *et*

al., 1999; van Duijkeren, Box *et al.*, 2004), *S. haemolyticus* from cow, *S. lentus* from cat, *S. haemolyticus*, *S. lentus, S. sciuri, S saprophyticus, S xylosus* and *S. epidermidis* from horses (Yasuda, Kawano *et al.*, 2000; Yasuda, Kawano *et al.*, 2002; van Duijkeren, Box *et al.*, 2004) and *S. sciuri*, *S. saprophyticus* and *S. epidermidis* from chickens (Kawano, Shimizu *et al.*, 1996). In The Dutch National Antimicrobial resistance Monitoring Programme of 2003, 25% of coagulase negative staphylococci isolated from bovine milk samples were classified resistant to oxacillin(Anonymous, 2004). Part of these strains were *MecA*-positive, this included *S. epidermidis, S. sciuri* and *S. xylosus*.

In the evolution of methicillin resistance *S. sciuri* is considered the source for *MecA* in MSRA(Wu, Piscitelli *et al.*, 1996). *MecA*-genes present in bovine staphylococci may act as reservoir for evolution of resistance to *S. aureus*. Because the *MecA*-gene is located chromosomally and therefore not easily transmitted to other bacteria the change of this event to occur and contribute of the spread to human pathogenic strains is not large. If *MecA* would transfer to *S. aureus* during treatment of mastitis or prevention during dry cow treatment, this would not necessarily pose an extra public health risk because the bovine *S. aureus* strains are not multi resistant and lack known virulence associated genes. The public health risk would be increased if the gene would be transferred to staphylococci able to survive and spread in human populations.

The real public health risk of methicillin resistance is not the selection and potential spread of genes in bovine staphylococci, but the survival and spread of human MRSAs in the animal population. The fact that this phenomenon has been described in cattle in Belgium in 1975(Devriese and Hommez, 1975) and recently been described to occur in cattle in Korea, where the genomes of the six bovine MRSA isolates were very closely related to those of human MRSA isolates and were a possible source of human infections caused by consuming food products made from these animals (Lee 2003), demonstrates that this risk is not science fiction. Continuous monitoring of methicillin resistance in bovine strains of *S. aureus* and subsequent typing of resistant strains found is of great importance.

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Environmental control for mastitis prevention, milk quality and food safety

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Abstract

Environmental mastitis affects all herds and is the major mastitis problem on well managed dairy farms with low bulk tank somatic cell counts. Significant sources of environmental pathogens are bedding materials, manure, exercise lots, and pastures. Molecular methods have facilitated detection and characterization of environmental pathogens and revealed a shifting epidemiology for some pathogens. Improvement of cows' ability to withstand infections and use of best management practices aimed at reduced exposure to environmental contamination are advised to prevent intramammary infections with environmental pathogens. These same practices also have an impact on milk quality parameters such as coliform counts and preliminary incubation counts. Finally, reduction of the exposure to environmental contamination will reduce food borne hazards for the consumer.

Keywords: environment, *Streptococcus uberis*, *Escherichia coli*, food safety, milk quality

Introduction

The environment of the cow is an important source of bacteria that may lead to intramammary infections (IMI) and clinical mastitis. Therefore, "the environment provided for dairy cows should be designed and maintained so that cow comfort and sanitation are maximized and disease exposure is minimized. Too often, housing facilities are designed for the dairyman rather than for the cow. This factor can result in reduced cow comfort, which, in turn, leads to reduced performance. The environment should be designed to keep them clean, dry, and comfortable" (Jarret 1984). While this statement, made two decades ago, is as true now as it was then, there have been several new developments in the past ten years. The use of molecular methods has provided new insights into the epidemiology of environmental mastitis. In addition, the epidemiology of mastitis itself appears to be changing. Finally, our focus has shifted from mastitis control to total milk quality management. In this contribution, we highlight some of the changing insights and attitudes developed over the past decade with respect to environmental control of mastitis, milk quality and food safety.

Environmental mastitis

The economic loss due to environmental mastitis has been estimated at $107 per clinical case with 88% of the cost resulting from decreased milk yield and discarded milk (about 1,000 lb from reduced production and 570 lb of discarded milk per case). The remaining

costs are attributed to increased labor, treatment and veterinary services, premature culling, and reduced genetic improvement. Losses in older cows were twice as high as in first lactation animals (Gröhn *et al.,* 2004).

Environmental mastitis is defined as mastitis caused by bacteria from environmental origin. It is contrasted with contagious mastitis, which is caused by bacteria that come from other cows. Typically, environmental mastitis is considered to be caused by opportunistic infections that make use of a lapse in the immunity of the cow to establish themselves in the mammary gland. In general these infections are of short duration. Gram-negative bacteria and non-agalactiae streptococci are commonly considered to be of environmental origin (NMC fact sheet "a practical look at environmental mastitis"). In recent years, it has become clear that these generalities cannot be applied to all farms or to all strains within a bacterial species. Exceptions to the classical dichotomy of contagious versus environmental have become evident since the introduction of strain typing techniques in mastitis research.

Gram negative mastitis

Escherichia coli

E. coli used to be considered the ultimate environmental pathogen because it is wide-spread in the dairy environment, and more opportunistic in nature than any of the other mastitis pathogens, taking advantage of the reduced chemotaxis that is associated with negative energy balance in cows during early lactation (Suriyasathaporn *et al.,* 2000). The incidence and severity of acute *E. coli* mastitis can be limited by a reduction in pathogen exposure or an increase in cow resistance. Environmental hygiene, adequate nutrition (energy, vitamin E, selenium) and vaccination may all contribute to *E. coli* mastitis control. Since 1995, however, there has been a steady stream of publications reporting the occurrence of chronic *E. coli* IMI (reviewed in Schukken *et al.,* 2004). Using DNA fingerprinting, the presence of indistinguishable isolates from repeated cases of clinical mastitis in the same quarter of the same cow was shown. Because of the high number of *E. coli* strains in the dairy environment, it is unlikely that recurrent isolation of one strain from the same quarter was the result of repeated new infections. It appears that *E. coli* is adapting to the mammary gland as host environment. As a result, it is expected that recurrent clinical mastitis due to underlying chronic *E. coli* IMI will be observed more frequently in years to come.

Klebsiella species

Few detailed studies of the epidemiology and impact of *Klebsiella* mastitis have been published. IMI with *Klebsiella* respond very poorly to treatment, and most *Klebsiella* infected animals are culled prematurely because of high SCC and continued clinical flare-ups. *Klebsiella* usually originates from the environment, particularly from wet or green sawdust. In recent years, *Klebsiella* mastitis has become an increasingly common problem in well managed herds in the USA, including herds that do not use wood-based products as bedding material. In humans, gastro-intestinal *Klebsiella* carriage occurs. *Klebsiella* is also ubiquitous in nature and can be found in surface water (Struve and Krogfelt, 2004). Re-use of sand contaminated with *Klebsiella* of fecal or environmental origin may play a role in *Klebsiella* mastitis in herds that use inorganic bedding materials. In addition, contagious transmission of *Klebsiella* may take in place in some herds (Kikuchi *et al.,* 1995). Studies into strain typing, fecal shedding and environmental sources of *Klebsiella* mastitis have been initiated

and should lead to improved recommendations for environmental control of mastitis caused by this genus.

Gram positive mastitis

Streptococcus species

Streptococcus uberis is among the most common causes of clinical and subclinical mastitis in many dairy countries. It is also the main cause of mastitis in dry cows. During the dry period, infections can't results from contagious transmission, implying that dry period infections are of environmental origin. Straw and other organic matter supports high *S. uberis* counts. Other environmental sources include soil, water and pasture plants. A wide variety of strains is present in environmental samples and per sample, as many as five different *S. uberis* strains have been detected (unpublished data). For *S. uberis,* as for *E. coli*, the large variety of strains occurring in the environment makes it unlikely that a large proportion of cows in a herd would get infected with the same strain of *S. uberis* by chance. Predominance of one *S. uberis* strain in a herd has been described in the USA (VanWorth *et al.,* 2005) and in The Netherlands (Zadoks *et al.,* 2003). In the latter case, survival analysis showed that infections with the dominant strain lasted significantly longer than infections with other strains. Thus, infections with the dominant strain would have a long window of opportunity for spread, which most likely occurred during the milking process.

S. dysgalactiae is more contagious in nature than *S. uberis*, but is also found in the environment. While *S. uberis* incidence is highest during the pasture season, *S. dysgalactiae* incidence is highest during the winter housing season (Barkema, 1998). Until recently, *S. agalactiae* was considered to be the ultimate contagious mastitis pathogen. It was not found in the environment, and transmission was strictly from cow-to-cow. Comparison of temporally and geographically matched human and bovine strains of *S. agalactiae* showed that cows and people have different strains of *S. agalactiae* or group B streptococcus (Sukhnanand *et al.,* 2005). On rare occasions, a cow may become infected with a human strain of the pathogen, giving rise to what could be called "environmental *S. agalactiae* mastitis". Such infections might explain the observation of incidental clinical *S. agalactiae* mastitis in herds with annual BMSCC < 150,000 cells/ml (Barkema, 1998).

Staphylococcus species

Like streptococci, *Staphylococcus aureus* and coagulase negative staphylococcus species (CNS) are common causes of mastitis. *S. aureus* is a contagious pathogen that spreads easily from cow to cow and usually manifests as chronic subclinical mastitis with elevated SCC and occasional clinical flare-ups. In low SCC herds where contagious transmission is largely controlled, *S. aureus* is still an important mastitis pathogen (Elbers *et al.,* 1998). In these herds, infections are often clinical and of short duration, a manifestation that is considered typical of environmental pathogens. In addition, failure to disinfect stalls regularly or to replace bedding frequently contributed to a high incidence of clinical *S. aureus* mastitis, pointing at an environmental epidemiology of infections (Elbers *et al.,* 1998). Strain typing studies confirm that different clinical manifestations are associated with different strains of *S. aureus* and that some strains appear to be host adapted while others are more likely to be of environmental origin (Zadoks *et al.,* 2000). The group of CNS comprises multiple staphylococcal species that differ in sources, transmission, and consequences of infection. For CNS, as for *Klebsiella* species, much work remains to be done to elucidate epidemiology

and control options. With improved control of other udder pathogens and increased emphasis on low SCC as quality parameter of milk, control of CNS will become more and more important.

Milk quality

Environmental contamination of milk will lead to a reduction in milk quality, suitability for processing and fluid milk shelf life. When suboptimal hygiene is present during the milk harvesting process, environmental bacteria will be present in the raw product. Typically, high coliform counts are the result of poor milking time hygiene (Figure 1) and poor cleanliness of animals and stalls. When environmental contamination is present in milk, high pre-incubation counts (PI-Counts) may also be observed (Jayarao *et al.,* 2004). Although faulty animal, process or equipment hygiene or poor milk cooling are likely causes of high counts of environmental organisms, a recent study in New York State showed that cows with mastitis are also a common source of high bacteria counts in bulk tank milk. This is particularly true in the case of streptococci. Comparison of riboprint patterns of *S. uberis* isolates from bulk tank milk and individual showed that a farm-specific dominant ribotype was present in each bulk tank sample. The dominant ribotype was also isolated from at least one cow within each herd of origin (Zadoks *et al.,* 2004). Similar results have been obtained for herds with high coliform counts. Hence, bacteriological and strain typing data indicate that control of both intramammary and extramammary "environmental" pathogens is important for improvement of raw milk quality.

Food safety

To reduce the risk of food-borne infections in humans, complete process control is essential. Processes on the dairy farm, in the processing plant, at the retailer and in the home affect the likelihood of bacterial contamination of dairy products (Schukken *et al.,* 2003). A schematic representation of processes and infection routes is presented in Figure 2. The process of milk harvesting is probably one of the most important steps in reducing the infection risk in dairy products. In a European study of 249 herds that were followed

Figure 1. A milking machine liner in use.

for 12 months, the prevalence of *Listeria monocytogenes* in milk reflected the prevalence in fecal material, but not the prevalence in roughage. Fecal material was considered the most important source of contamination of raw milk (Husu *et al.*, 1990). Attention to good milking and barn hygiene is important to diminish the risks of contamination of raw milk by *L. monocytogenes, Campylobacter spp., Salmonella spp*. and shiga-toxin producing *E. coli* during milk harvesting (i.e. Murinda *et al.*, 2002).

Discussion

Environmental control of mastitis pathogens is a very important issue in the dairy industry. The classical argument for environmental control is to reduce environmental mastitis. Cows are exposed to environmental bacteria throughout their lives. Most of these bacteria are opportunistic bacteria that may cause short-term ('acute') infections in the mammary gland. However, in a number of situations strains may emerge that are more adapted to survival in the mammary gland, resulting in chronic infections and a prolonged window of opportunity for transmission from cow-to-cow. Hence, environmental control is important for the general reduction of short term environmental infections, but also in the prevention of emergence of cow-adapted strains that move from the environment to the mammary gland of the cow and maintain infection over longer periods of time.

There are other reasons for maintaining a clean and low-exposure environment, too. Many studies have highlighted the importance of environmental control for improvement of milk quality and food safety. The milieu of the dairy cow harbors multiple zoonotic pathogens. It is often difficult or even or impossible to eradicate these pathogens from the dairy farm.

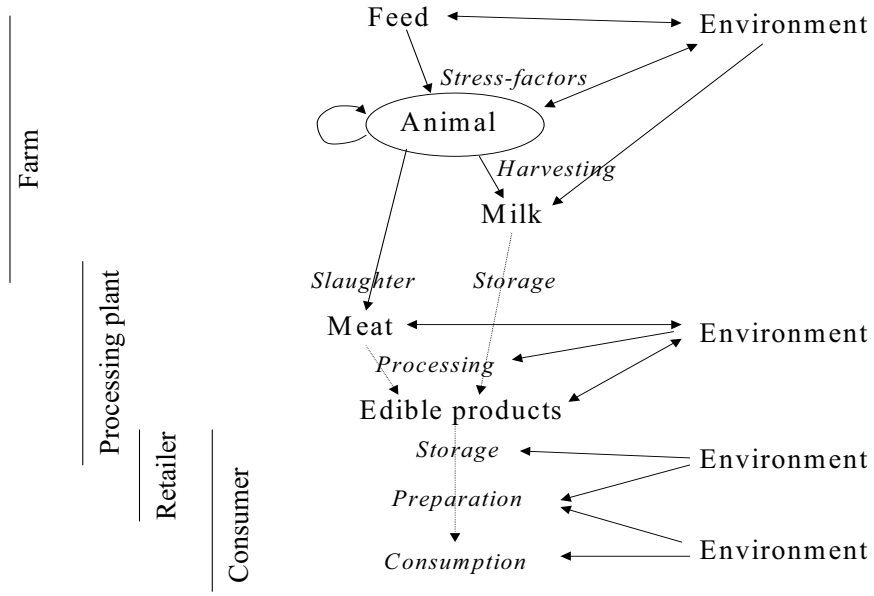

Figure 2. Schematic representation of the flow of products from farm to consumer (indicated by dotted arrows) and the possibilities for infection with environmental zoonotic pathogens (line arrows). Processes associated with risk of infection are indicated in italic (Adapted from Schukken et al.*, 2003).*

The only mechanism that is available to prevent contamination of raw milk with zoonotic pathogens is optimizing environmental control and exposure of the cow, the mammary gland and the milking equipment to environmental contamination. Not only is there a direct benefit to the dairy producer in the reduction of environmental mastitis and improved milk quality premiums, but there is also a long term issue at stake: consumer confidence in the harvesting process on the dairy farm.

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Genetic improvement in mastitis control programmes

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Abstract

Generally, mastitis control programs are advocated as the way forward to reduce on farm mastitis incidence. These programs provide guide-lines for udder health management, treatment protocols, hygienic measures and other preventative instruments. However, generally genetic improvement is still ignored in these control programs, despite that there is much evidence of the large genetic differences between animals in mastitis incidence. One of the major reasons why genetic selection is generally ignored is that the low heritability for mastitis incidence has been misinterpreted in that genetic selection has little role to play in mastitis control programmes, and that, compared to management improvements, genetic selection can simply be ignored. Over the past ten years this view has changed considerably. Effective selection tools have become available in most countries and new developments in the areas of genomics and proteomics promise to make genetic selection even more effective. This, together with the increasing concerns with the use of antibiotics, the need to reduce costs of production at farm level, and the constraints of labour on growing family farms, poses the question where the major successes in mastitis control are going to be made in the next decade: particularising some of the management aspects, diagnostics or treatments of mastitis, or a concerted effort to recuperate and further improve the genetic resistance to mastitis in dairy cows.

Keywords: genetic selection, heritability, genomics, proteomics

Mastitis control programs

The goal of the standard mastitis program is to reduce the number of new infections, and to limit the duration of the existing infections. The foundation for the basic "five-point mastitis control program" is laid in the 1960s (Neave *et al.,* 1969). The program generally consisted of (1) optimisation of the milking procedures and optimal functioning of the milking machine, (2) application of teat disinfectant after removal of the milking unit, (3) dry cow treatment with antibiotics for all cows, (4) adequate treatment and documentation of all cases of clinical mastitis (CM), and (5) culling of chronically infected cows (Neave *et al.,* 1969). The implementation of the five-point mastitis control program has led to control of *Strep. agalactiae* mastitis and, to a lesser extent, *Staph. aureus* and *Strep. dysgalactiae* mastitis (Neave *et al.,* 1969; Hillerton *et al.,* 1995). However, the program is less successful in preventing new infections with environmental pathogens (Schukken *et al.,* 1990; Lam *et al.,* 1997a). Recommendations to control both contagious and environmental pathogens have been combined in a ten-point mastitis control program, issued by the National Mastitis Council (2001).

Although undoubtfully the programs are effective, it may be naïve to assume that this one recipe will automatically lead to a general uptake by farmers. Apart from the fact that each enterprise has its own constraints and economic optimal situation, there is also a general pressure on labour requirements and cost reduction on dairy farms in The Netherlands. Therefore, there will be reluctance to accept any measures that increase cost of production, even though there are economic benefits for the average farmers in return in the form of a reduction in mastitis. Furthermore, it can be questioned if the growing concern in the western society over excessive use of medications like antibiotics and the wish to extent longevity of dairy cows were taken on board when these plans were devised.

Genetic selection is a complementary to the management tools to combat (sub)clinical mastitis. Genetic selection is a slow process that results in a steady change in the genetic composition of the dairy herd that accumulates and is permanent (Shook, 1989). Costs to the dairy farmer of selecting and using a bull that reduces mastitis are relative small compared to most management measures. At the same time labour requirements for bull selection are insignificant for a dairy farmer.

Genetic differences in mastitis resistance

It is well known that heritability estimates of mastitis are generally below 0.05 (e.g. review by Mrode and Swanson, 1996). This is often misinterpreted in that genetics has little role to play and that improvement of management is the only option to reduce mastitis. However, the low heritability is better explained by the random nature of which cows get mastitis and which cow not, within a herd. The remaining 95% (if 5% can be explained by genetics) of the phenotypic variation is very hard to get under control by any means. One way to improve the effectiveness of selection for traits with low heritability is to test more daughters per progeny-test sire, to reduce the influence of the random nature of mastitis incidence. But testing more daughters per sire is costly for breeding organisations. Another option is to use more heritable information from indicator traits like SCC, milking speed, linear type traits and electrical conductivity.

Leaving the low heritability a side, it is more important to note that considerable variation between bulls exists (Pryce *et al.*, 1998). In a small Dutch study the range in breeding values found in a subset of heavily used sires was: -7% to +7%. This large range indicated that daughters of the worst and best bull are expected to have 23.5% and 16.5% mastitis per lactation, respectively. On farms with a higher than average incidence of mastitis, this effect might even be larger, and when more mastitis records would have been available the full extent of the genetic merit might even have been larger. With two of the extreme bulls having had more than 75,000 daughters in the Netherlands alone, the potential impact of genetic selection might become clearer. If in each generation the poorer mastitis bulls are selected, e.g. by chance, than these effects will accumulate over generations. Hence, there are clear opportunities for genetic selection, but also risks of ignoring the genetic merit of animals.

Genetic selection

Selecting on increased genetic resistance of mastitis can be done directly or indirectly. Direct selection means that the actual trait is measured on the animal or its relatives.

Indirect selection means that an indicator trait for CM is measured on the animal itself or its relatives.

The strongest emphasis to direct selection for udder health is given in Scandinavian countries with the joint use of direct mastitis incidence and SCC in Denmark, Finland and Sweden (as reviewed by Heringstad *et al.,* 2000), while Norway only uses information on CM. Other countries in the world select for udder health using indirect measures, such as: SCC, udder conformation and others.

Lactation-average SCC is the most commonly used indirect measure for CM, because it is readily available through most milk recording systems. Moderately high to high genetic correlations have been estimated between CM and SCC (0.3 - 0.9), indicating that selecting purely on lower lactation-average SCC would increase the CM resistance (reviewed by Mrode and Swanson, 1996), which makes lactation-average SCC a suitable trait to include in selection indices. More recently, it was suggested that selection on diminishing peaks in SCC during lactation, would decrease the incidence of CM even more effectively (De Haas *et al.,* 2004).

Next to SCC, conformation traits are widely used as indirect indicators of CM. Linear type traits represent descriptions of udder characteristics of a cow. They are scored nation-wide and therefore cheaply available for genetic selection. Genetic correlations between CM, SCC and type traits are generally low, but indicate a higher risk for CM with a weak udder suspensory, deep udders, bad front teat placement, loose rear udder attachment and long teats (Lund *et al.,* 1994; Rogers *et al.,* 1998; Van Dorp *et al.,* 1998; Rupp and Boichard, 1999; Sorensen *et al.,* 2000). In The Netherlands, milking speed is also included in the udder health index.

Selection strategies

Selection for improved udder health is of primary importance in dairy cattle populations, and it is now included in selection indices in most countries (VanRaden, 2002). Genetic correlations between milk yield and both CM and SCC are unfavourable (Syvajarvi *et al.,* 1986; Groen *et al.,* 1994; Uribe *et al.,* 1995), suggesting that selection solely for yield will increase the CM incidence. However, they do not present a fatal flaw in breeding schemes as breeders can create selection indices that contain traits with unfavourable associations (Cameron, 1997). Combined selection on yield and breeding values for CM (either from direct or indirect information) might counteract the unfavourable correlated response in CM (Pryce *et al.,* 1998) or pathogen specific mastitis (De Haas *et al.,* 2002).

Can somatic cell count be too low?

One concern of using lactation-average SCC in genetic selection has been that it would not only select for a reduced susceptibility to intramammary infections (IMI), but also against the possibilities for a cow to respond to an IMI. The estimated genetic relationships suggest that daughters of sires that transmit the lowest SCC had the lowest number of cases of overall CM (Philipsson *et al.,* 1995; Mrode and Swanson, 1996; Nash *et al.,* 2000; Rupp and Boichard, 2001). Therefore, SCC should be decreased to the lowest possible value, at least within the range covered by the population mean and the genetic variance in their

populations, and the theory that selection for the lowest SCC will result in dairy cattle that are unable to respond to IMI is not supported.

Still, these studies might not fully address the question if there is a higher risk for clinical *E. coli* mastitis when using bulls that transmit low SCC. Relationships among pathogen-specific CM and sire transmitting abilities for SCC have only recently been determined (Nash *et al.,* 2000; Nash *et al.,* 2002). These authors concluded that selection for lower SCC may also improve genetic resistance to pathogen-specific CM, as daughters of sires that transmit higher SCC showed higher incidence rates of all pathogen-specific CM (including *E. coli*). This is in line with the genetic correlations between SCC and pathogen-specific CM estimated by De Haas *et al.* (2002).

Environmental mastitis (ENV_CM) is generally of shorter duration than contagious mastitis (Fox and Gay, 1993; Smith and Hogan, 1993). Therefore, Shook (1993) hypothesised that selection for lower SCC may not improve resistance to ENV_CM, because with monthly test-day recordings the elevated SCC due to ENV_CM may not be detected. However, this seems contradictory to the fairly strong positive genetic correlation estimated between SCC and ENV_CM (De Haas, 2003). One explanation could be that exposure to environmental pathogens occurs daily, and the less resistant cows may become infected more often, or have a longer lasting increased SCC. As a result, the less resistant cow may be more likely to have elevated SCC on the test-day. Another explanation might be that a case of clinical *E. coli* mastitis causes such a strong increase in SCC, that the predominant source of variation in SCC is due to cows having CM or not, rather than having a high or low SCC prior to infection. This suggests that genetic selection on diminishing presence of peaks in SCC would decrease the incidence of pathogen-specific CM without risking an increased susceptibility of the cows to IMI because of a too low SCC.

Molecular genetics

Traditional methods of selection have difficulties improving traits of low heritability, such as disease resistance. A great majority of disease resistance traits are likely to be influenced by the aggregate of many genes, each with a relatively small effect (Kelm *et al.,* 2001). Recently, efforts have been undertaken to locate genes affecting economically important traits in dairy cattle. Genetic markers associated with these genes can be used in marker-assisted selection to increase genetic progress (Kashi *et al.,* 1990). Application of marker-assisted selection for milk production and other economically important traits would increase the rate of improvement for these traits. The use of genetic markers allows selection on the basis of a DNA profile. However, to find genetic markers for mastitis resistance, large datasets are required both in terms of mastitis records as in terms of population structure, which is a problem for most countries, except maybe the Nordic countries (Klungland *et al.,* 2001).

Chromosome 23 contains the BoLA locus (Weigel *et al.,* 1990; Dietz *et al.,* 1997; Starkenburg *et al.,* 1997; Ashwell *et al.,* 1998). Dietz *et al.* (1997) identified an allele at the BoLA locus as a potential risk factor for acute IMI (= allele DRB3.2*16). This allele was also significantly associated with lower SCC in Holsteins and higher estimated breeding values for SCC (Kelm *et al.,* 1997; Sharif *et al.,* 1998). Several other alleles at the BoLA locus were reported to be associated with a decreased number of cases of CM, a smaller amount of discarded milk, and lower udder health costs (Weigel *et al.,* 1990; Kelm *et al.,*

1997; Sharif *et al.,* 1998). These relationships suggest that alleles at the BoLA locus may serve as markers for health and production traits (Weigel *et al.,* 1990). Sharif *et al.* (1999) concluded that increasing or decreasing the frequency of BoLA alleles to increase resistance to CM did not have adverse effects on production in the population they studied.

Indications for markers affecting milking speed and fore udder attachment were identified on chromosome 23 (Schrooten *et al.,* 2000). These traits are genetically correlated to resistance of CM (Lund *et al.,* 1994; Mrode *et al.,* 1998; Sorensen *et al.,* 2000).

It could be argued that an increased incidence of CM is caused by high milk yield, rather than a change in the immune response. Because of the unfavourable genetic correlation between milk production and incidence of CM, it can be expected that some markers affecting mastitis are positioned close to markers of milk production traits (Klungland *et al.,* 2001). A number of markers affecting milk production traits have been published in different breeds, of which several map to chromosome 6 (Ashwell *et al.,* 1996; Spelman *et al.,* 1996; Schrooten *et al.,* 2000). Chromosome 7 may contain a marker which affects SCC (Heyen *et al.,* 1999; Ashwell *et al.,* 2001), but markers affecting SCC were also identified on chromosome 5, 21, 22, 23 and 26 by Heyen *et al.* (1999).

Functional genomics

The basic physiologic factors, molecular mechanisms, and gene systems responsible for mastitis susceptibility in dairy cows are still almost completely unknown, and this has seriously impeded our ability to substantially improve overall resistance to mastitis through therapies, vaccination programs and genetic selection schemes (Burton and Erskine, 2003). The combined power of gene mapping technologies, gene expression studies in immune cells and udder tissues, and modern bioinformatics tools with available and expanded genome sequence will help to solve the puzzles of disease susceptibility and resistance. This could, for example, be accomplished through a new ability to precisely regulate molecules of the normal defense systems that control neutrophil responsiveness to IMI (Burton and Erskine, 2003). Inverse relationships have been found between the neutrophil recruitment capacity of the udder and the outcome of an IMI. Cows with spontaneous cures have rapid and massive milk neutrophil responses, whereas cows with no or slow neutrophil responses get severe forms of mastitis (Kremer, 1993). Mutations in key genes regulating the dynamics of the neutrophil defense system could be searched, and could then form the basis for mastitis susceptibility screening and genetic selection programs geared towards improving mastitis resistance at the herd or population level. Also the potential impact of functional genomics is expected to be large in the areas of mastitis diagnostics tool.

Conclusions

In most countries effective selection tools have become available to utilise the large genetic variation in mastitis resistance. New developments in the areas of genomics and proteomics promise to make genetic selection even more effective. This, together with the increasing concerns with the use of antibiotics, the need to reduce costs of production at farm level, and the constraints of labour on growing family farms, will make it more important to improve the genetic resistance to mastitis in dairy cows.

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Milk production and mastitis control in emerging dairy countries: The experience in Chile

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Milk production in Chile

From a geopolitical point of view, Chile is divided from north to south in 13 Regions. The Regions have very different climates, ranging from a dry and desert climate in the north to a Mediterranean climate in the central zone and a cooler and wetter climate in the south. There also are substantial differences in management systems and herd sizes within the Regions. Management differs according to herd size, soil type and climate and contains everything from zero grazing to year-round grazing.

Milk production is an important economic activity and is for many smallholder dairy farmers an important mean of subsistence. Herd sizes vary from a few cows on smallholder farms to very large commercial herds with several thousand cows. Smallholder herds represent 81.9% of total dairy herds and 36.6% of total dairy cows but only 13.6% of total milk supply delivered to processing plants (Anrique, 1999) (Table 1).

Although dairy farms are established all over the country, most of the dairy cows (73.2%) and milk supply for industry (83.3%) are concentrated in southern Regions (IXth and Xth Regions) (Table 2, Fig. 1).

Milk production in Chile has consistently increased during the last two decades at an average of 7% yearly. In 2004, milk supplies to dairy industries reached 1676,5 million liters, 184.9% more than twenty years ago (Table 3). Furthermore, about 500 million liters do not

Table 1. Average dairy cows according to herd size (Chile, 1997).

Milk production (Lt/yr)	Dairy cows	Dairy herds	Average cows/herd
< 100,000	225,898 (36.6%)	11,040 (81.9%)	20,5
100,000 - 1,000,000	268,485 (43.5%)	2,128 (15.8%)	162,2
> 1,000,000	122,207 (19.8%)	310 (2.3%)	394,2

Table 2. Milk received by dairy industries and dairy cow population in Chile (2004).

Region	Total milk supply[1] (thousand liters)	Dairy cows[2]
Metropolitan	155,558.6 (9.3%)	31,857 (5.1%)
VIIIth	124,256.5 (7.4%)	73,112 (11.9%)
IXth	229,009.5 (13.7%)	71,844 (11.7%)
Xth	1,167,655.8 (69.6%)	378,853 (61.5%)
Total country	1,676,480,5	615,924

[1]Adapted from ODEPA, 2005

[2]Adapted from Anrique, 1997

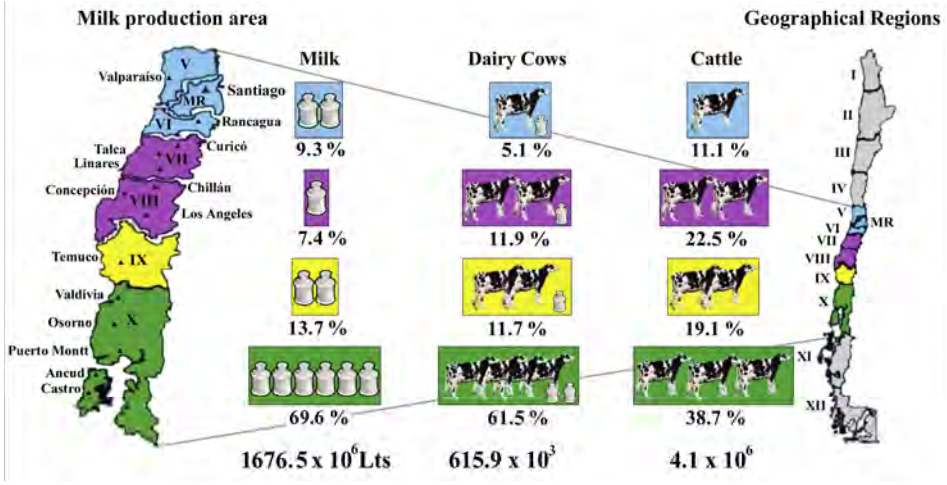

Figure 1. Milk production and cattle population by geographical Regions in Chile (2004).

Table 3. Milk received by dairy industries in Chile (1985-2004) (Lt x 1000)[1].

Year	Milk	% Variation		Year	Milk	% Variation	
		(a)	(b)			(a)	(b)
1985	491,517.0			1995	1357,869.5	+ 9.9	+ 139.8
1986	588,453.2	+ 13.2	+ 13.2	1996	1406,426.3	+ 3.6	+ 139.0
1987	666,324,5	0.0	+ 13.2	1997	1496,833.4	+ 6.4	+ 154.4
1988	666,572.5	+ 2.2	+ 15.7	1998	1530,024.5	+ 2.2	+ 160.0
1989	770,583.0	+ 13.1	+ 30.9	1999	1469,716.3	- 3.9	+ 149.8
1990	890,301.5	+ 15.5	+ 51.3	2000	1447,213.0	- 1.5	+ 145.9
1991	947,707.1	+ 6.4	+ 61.1	2001	1636,461.3	+ 13.1	+ 178.1
1992	1021,060.8	+ 7.7	+ 73.5	2002	1605,391.8	- 1.9	+ 172.8
1993	1121,114.7	+ 9.8	+ 90.5	2003	1563,169.3	- 2.6	+ 165.6
1994	1235,640.0	+ 10.2	+ 110.0	2004	1676,480.5	+ 7.2	+ 184.9

[1]Adapted from ODEPA, 2005

(a) Respect to previous year

(b) Respect to 1985

reach the formal market and is instead used for calf feeding, small cheese makers, and direct sale for human consumption.

It is important to note that though in 2004 there were 24 milk processing plants operating throughout the country, 74.9% of total milk submitted for sale was bought by only four large dairy companies: Soprole S.A. (22.5%), Nestlé Chile S.A. (19.8%), Colún (18.5%), and Loncoleche S.A. (14.1%) (Table 4).

Mastitis control and milk quality in Chile

In contrast to raw milk received and milk products produced by dairy industries, which is officially recorded and published every month by the Ministry of Agriculture (ODEPA),

Table 4. Raw milk received by Chilean to dairy industries (2004)[1].

Dairy Company	Plants	Milk Reception (Lt)	%	
SOPROLE S.A.	5	377,795,544	22.5	
NESTLÉ CHILE S.A.	3	332,893,972	19.8	42.3
COLÚN	1	310,450,258	18.5	60.8
LONCOLECHE S.A.	3	235,632,478	14.1	**74.9**
CUMELEN-MULPULMO	1	91,827,451	5.5	80.4
SURLAT S.A.	1	88,316,182	5.3	85.7
CALAN LTDA.	3	69,981,616	4.2	89.9
CHILOLAC	1	38,168,433	2.3	92.2
QUILLAYES PETEROA LTDA.	2	35,282,847	2.1	94.3
CAFRA LTDA.	1	32,022,411	1.9	96.2
LÁCTEOS PUERTO. VARAS	1	30,541,196	1.8	98.0
AGROLÁCTEOS CUINCO LTDA.	1	23,126,308	1.4	99.4
VITALAC S.A.	1	10,441,797	0.6	100.0
Total	24	1,676,480,493		

[1]Adapted from ODEPA, 2005

there are no official records of mastitis or milk quality in Chile. Therefore, it is rather difficult to assess the real progress in mastitis control and milk quality throughout the years. However, it is evident that little progress was made in the past, as this was not on the focus of the national dairy industry. As milk production increased, the milk industry soon realized that mastitis control and milk quality needed to be improved. The first important step to achieve this was legally taken in 1978 when a law (Ordinance Nº 271, Ministry of Agriculture) come into force establishing a national classification system of milk submitted for sale based on SCC (*Viscometer* test) and bacterial content (*Reductase* test) (Table 5).

It is difficult to assess the impact of this law on mastitis prevalence and milk quality because there are no previous records on SCC or mastitis prevalence. However, a number of research reports show that in the 70's over 70% of cows suffered some degree of mastitis, probably with an average SCC >1 million, and >85% of producers delivered milk with >1 million cfu/ml (von Baer *et al.*, 1976, Zurita, 1988). Before the 80's mastitis control measures were not applied, there was no control for hygienic quality, antibiotic residues were not tested, and over 80% of producers delivered non-refrigerated milk (Kruze, 1988).

Despite there was no obligation for the milk industry to pay farmers based on milk quality, most companies started to pay a bonus for milk Class A. This encouraged milk producers to implement mastitis control measures (dry cow therapy, post milking teat

Table 5. First classification system of raw milk based on hygienic quality in Chile (Ordinance Nº 271, Ministry of Agriculture, Chile, 1978).

Class	Reductase (h)	SCC (cell/ml)[1]	Density (gr/ml)
A	> 3	< 500.000	> 1.029
B	3 -1	500,000 - 1,000,000	> 1.029
C	< 3	> 1,000,000	< 1.029

[1]Viscometer test

disinfection, udder washing and drying before milking with disposable paper towels, testing milking equipment) and to gradually changing from non-refrigerated churns to refrigerated bulk milk tanks. At present, over 90% of dairy farmers have bulk milk tanks and many dairy industries are no longer processing non-refrigerated milk. Some research carried during the 80's showed that mastitis prevalence went down by 15%, about 15-30% of producers had less than 500,000 cell/ml, and over 70% had a MB reduction time >3 h. (Rossi, 1982; Gómez, 1981; Haverbeck, 1982). However, when bulk tank samples that had passed the *Reductase* test were simultaneously tested with the *Standard Plate Count* method, a high number of bacteria were shown to be present in such samples (Pedraza *et al.*, 1987). It also was shown that somatic cell counts were up to 30% lower when tested by the *Viscometer* test compared to electronic cell counting on duplicate samples. Because milk production was steadily increasing and milk quality showed no real improvement, in 1993 the milk industry decided to replace the *Reductase* test with the *Standard Plate Count* method for determination of microbiological quality and the *Viscometer* test with the *Fossomatic* for SCC determinations. Also, in 1995 the *Bactoscan* was introduced in Chile by COOPRINSEM (Osorno, Xth Region), the largest private milk quality testing laboratory in the country, and this method has been adopted by most dairy industries to assess the bacteriological quality of their raw milk supplies. The effect of these changes on mastitis prevalence was soon evident among farmers enrolled in the Official Milk Record Control Program of COOPRINSEM; mean SCC on composite milk samples went down to 458.00 cell/ml in 1993 and to 389.000 cell/ml in 1994 (Agüero, 1995). Later on, two new milk quality laboratories were set up in southern Chile and equipped with *Fossomatic* instruments to measure SCC on individual composite milk samples (CAFRA in Frutillar, Xth Region, and INIA-Carillanca in Temuco, IXth Region). At present, all dairy industries use the *Fossomatic* instead of the original *Viscometer* test to measure SCC in milk.

One of the most important facts contributing to a significant reduction in mastitis prevalence (reduction in SCC) was the introduction in 1995 of quality payment schemes for raw milk. At the beginning, these payment schemes were voluntarily introduced by a few milk plants with a bonus paid for top class milk based on SCC (*Fossomatic*) and TBC (SPC or *Bactoscan*). Payment schemes were then progressively introduced by all dairy industries. Further reduction of bacteria and cell counts was observed when penalties were introduced for lower quality milk and the ranges of both SCC and TBC were adjusted for the highest quality milk. At present, all dairy plants in Chile have quality payment schemes with a small bonus for high quality and large penalties for low quality milk. In addition, antibiotic residues in milk are heavily penalized and milk that tests positive is rejected; also an extra bonus has been now introduced in most payment schemes for Tuberculosis and Brucellosis certified-free-herds. The improvement in reduction of TBC and SCC after the introduction of quality payment schemes according to data compiled from the milk industry is presented in Table 6.

The annual mean SCC on composite milk samples of dairy farmers enrolled in the *Milk Record Control Program*s of COOPRINSEM and CAFRA are presented in Table 7 and Table 8, respectively. Among COOPRINSEM's cooperates, the percentage of producers with <200.000 cell/ml increased from 56.3% to 67.8 % between 1994-2002, and the mean SCC consistently decreased from 389,3 cell/ml to 272,7 cell/ml in the same period; however, there are has not been an apparent improvement among producers controlled by CAFRA between 1997 and 2002.

Table 6. Bulk milk TBC and SCC reported by main dairy industries in Chile (1996-2002).

	1996	1997	1998	1999	2000	2001	2002
TBC[1]	321	482	324	86	81	51	80
SCC[1]	420	460	401	374	342	318	311

[1]Weighted means (cfu/ml x 1000)

[2]Weighted means (cell/ml x 1000)

Table 7. Weighted SCC on composite milk samples of all dairy farmers enrolled in COOPRINSEM's Official Milk Record Control Program (1994-2002).

			Ranges of SCC (cell/ml x 1000)			
Year	Cows[1]	Mean SCC	< 200	200-500	500-1000	> 1000
1994	22,213	389,3	56.3 %	24.4 %	10.7 %	8.6 %
1995	28,267	494,6	53.1 %	23.3 %	11.7 %	11.9 %
1996	33,796	389,7	60.8 %	20.3 %	9.9 %	9.0 %
1997	40,938	393,4	60.5 %	20.3 %	10.0 %	9.2 %
1998	49,428	348,5	65.5 %	17.6 %	8.8 %	7.9 %
1999	53,323	329,2	68.5 %	16.4 %	7.8 %	7.3 %
2000	63,754	292,7	67.0 %	13.9 %	11.6 %	7.5 %
2001	69,664	281,6	68.1 %	13.6 %	11.2 %	7.1 %
2002[2]	34,692	272,7	67.8 %	13.9 %	11.4 %	7.0 %

[1]Average cows under control; [2]Jan-Jul 2002

From: COOPRINSEM, Osorno, 2002 (pers.com.)

Table 8. Weighted SCC on composite milk samples of all dairy farmers enrolled in CAFRA's Official Milk Record Control Program (1997-2002).

			Ranges of SCC (cell/ml x 1000)			
Year	Cows[1]	Mean SCC	< 200	200-500	500-1000	> 1000
1997	9,358	304,8	61.8 %	19.5 %	9.8 %	8.9 %
1998	14,148	287,5	64.5 %	19.7 %	9.0 %	6.8 %
1999	13,580	297,2	63.9 %	23.8 %	9.3 %	3.0 %
2000	14,574	341,8	61.4 %	20.5 %	9.7 %	8.5 %
2001	16,628	338,1	61.7 %	20.0 %	9.7 %	8.6 %
2002	10,014	345,8	60.8 %	20.3 %	10.0 %	8.9 %

[1]Average cows under control; [2]Jan-Jun 2002

From: CAFRA LTDA., Frutillar, 2002, (pers.com.)

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Pathogenesis and immunology

Pathogenesis of chronic intramammary *Escherichia coli* infections

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Abstract

Chronic intramammary *Escherichia coli* infections are an increasingly important problem in dairy herds. In this study we investigate the pathogenesis of chronic coliform mastitis. Three well-defined *E. coli* strains associated with chronic infections and three well-defined *E. coli* strains associated with acute infections were used as experimental pathogenic strains. Our results showed that the *E. coli* strains internalized by bovine mammary epithelial cells (MAC-T). This could provide *E. coli* with a survival advantage, allowing the microbes to better resist detection and clearance by both innate and adaptive immune defense mechanisms. By using gentamycin protection assay and immunofluorescence staining we showed that *E. coli* strains associated with chronic infections internalized mammary epithelial cells more efficiently than strains associated with transient infections. Antibacterial resistance was much higher among *E. coli* strains associated with acute infections. We demonstrated that internalization of mammary epithelial cells by *E. coli* was an active process involving host cell cytoskeletal rearrangement and intracellular signaling cascades. Two cytoskeletal disrupting agents, cytochalasin D and colchicine inhibited the internalization of *E. coli* into mammary epithelial cells, which indicates both actin filaments and microtubules play a role in the uptake process. Genistein and wortmannin both inhibit internalization. Polymerase chain reaction analysis showed the presence of type-1 pilus adhesin, FimH in strains used. Blocking FimH with mannose decreased bacterial adherence and internalization.

Keywords: chronic coliform, FimH, adhesion, invasion, antibiotic resistance

Introduction

E. coli is an important pathogen which causes mastitis in dairy cows. Typically, *E. coli* infections in the mammary gland were of short duration, resulting either in bacterial clearance or the death of the host (Hogan *et al.*, 1989). As early as 1979, however, the presence of recurrent infections of the mammary gland with *E. coli* microorganisms was described (Hill and Shears, 1979). Genotypic analysis of *E. coli* strains involved in these repeated infections has shown that these are indeed chronic intramammary coliform infections (Dopfer *et al.*, 1999). The pathogenesis of chronic intramammary *E. coli* infections is still unclear. Chronic coliform infections are also known to occur in urinary tract infections in humans. In these urinary tract infections the same *E. coli* strain caused

recurrent clinical symptoms. These bacteria are not strictly extracellular pathogens, but a sub population of these bacteria are internalized by bladder epithelial cells and these bacteria appear to have a distinct survival advantage over their extra cellular counterparts (Mulvey *et al.*, 2000). Although multiple hypotheses derived from the mechanisms involved in urinary tract infections in humans, none of these have actually been evaluated in the bovine. To be able to develop effective strategies for therapy and prevention, a more thorough understanding of intramammary *E. coli* infections is required.

In this study, we demonstrate the potential of *E. coli* strains isolated from acute and chronic mastitis cases to be internalized by Mac-T cells. In addition, we present evidence for the involment of cytoskeletal and cellular transducing elements in the process of invasion.

Bacterial strains

Three well-defined *E. coli* strains associated with chronic infections and three well-defined *E. coli* strains associated with single infections were used as pathogenic strains. The strains from acute infections, EC-O157, EC-727 and EC-G, are well documented (Lohuis *et al.*, 1990;Hogan *et al.*, 1999;Dopfer *et al.*, 2000a) and two of them repeatedly used in challenge infections and shown to cause only brief transient infections. The other three strains have been isolated from chronically infected cows. Presence of chronic infections was confirmed by PFGE typing of the isolates collected from the infected cows (data not shown). A strain of *Salmonella typhimurium* 14028 (ATCC) was used as a positive control for adhesion to and invasion of mammary epithelial cells. Data on somatic cell counts and coliform counts of one of the chronically infected cows is given in Figure 1.

Bacterial adherence to and invasion of mammary epithelial cells

The initial step in the establishment of a bacterial infection is the interaction of bacterial adhesive proteins with epithelial cells, which is often followed by invasion of the epithelial cell. Various researches shows that invasion of nonphagocytic cells can provide bacterial pathogens with a survival advantage, allowing the microbes to better resist detection and clearance by both innate and adaptive immune defense mechanisms (Finlay and Cossart, 1997a). In order to characterize adhesive and invasive potentials of *E. coli* isolates associated with acute and chronic infections, bovine mammary epithelial cell line, MAC-T

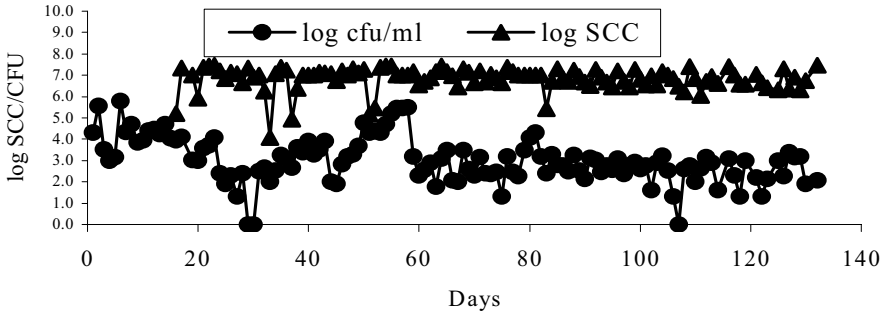

Figure 1. Time series of a chronic intramammary coliform infection. Daily observations on bacteria counts and somatic cell counts in a chronically infected cow with isolate EC-C2.

was infected with each isolate. Adhesion and invasion assays were conducted as described previously (Dopfer *et al.*, 2000b). The results of invasion assays were confirmed by immunofluorescence microscopy (data not shown). Although all *E. coli* strains adhere to mammary epithelial cells strongly, the strains from chronic infections invade mammary epithelial cells more effectively than the strains from acute infections (Figure 2).

E. coli strains, that manage to avoid rapid clearance from mammary gland with milk flow can establish a bacterial reservoir. This reservoir can persist for several months since bacteria can avoid host defenses and protected from antibiotics. Supporting this hypothesis resistance to antibiotics was much higher among *E. coli* isolates associated with acute infections (Table 1).

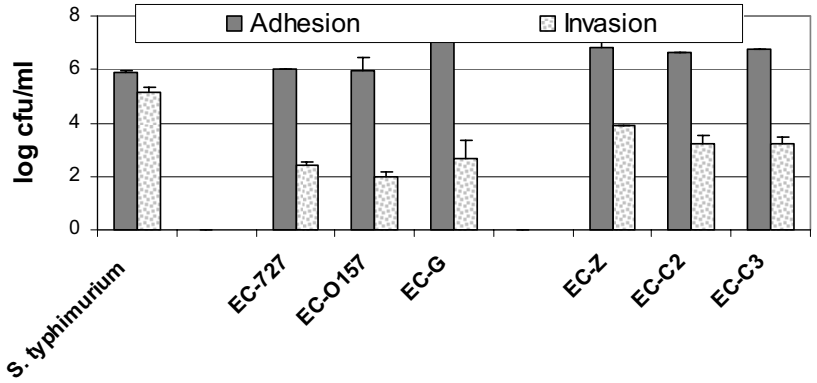

Figure 2. Adherence to and invasion of mammary epithelial cells by E. coli.

Role of type 1 pili on bacterial adhesion and invasion

Type 1 pili are the most common filamentous bacterial appendages of *E. coli* and promote bacterial adhesion to various types of eukaryotic cells. Type 1 pili composed of several subunits including FimH, a mannose-binding lectin that is responsible for promoting bacterial adherence and colonization of mucosal surfaces. FimH has been shown as an important virulence factor for uropathogenic *E. coli* (UPEC) and adherent invasive *E. coli* (AIEC) (Martinez *et al.*, 2000;Boudeau *et al.*, 2001). To determine the role of FimH in mammary cell invasion, FimH with was blocked with 2.5% mannose in adhesion and invasion assays.

Blocking of FimH with D-mannose decreased adhesion of all chronic and one acute strain significantly (Figure 3). This suggests that FimH mediated adhesion plays an important role during chronic infections since it will allow these bacteria to avoid rapid clearance from mammary gland with milk flow. Although blocking FimH with D-mannose decreased invasion of mammary cells, the level of inhibition was not as high as that of adhesion. Also significant amount of bacteria could invade mammary epeithelial cells, suggesting the presence of other mechanisms for invasion.

Uptake pathways of *E. coli* isolates into Mac-T cells

Most invasive pathogenic bacteria including *Salmonella, Listeria*, and *Shigella* have been shown to trigger microfilament dependent entry pathways, while both microfilaments and microtubules are required for invasion of some bacteria such as

Table 1. Antibacterial resistance of E. coli strains used in the study

	Isolate	Antibiotic resistance for
Acute		
	EC-727	Clindamycin, Kanamycin Rifampin, Streptomycin Sulfizoxazole, Sulphadimethoxine Tetracycline
	EC-O157	Clindamycin, Rifampin Spectinomycin, Tetracycline
	EC-G	Amoxicillin[I], Amphicillin, Cephalothin, Chloramphenicol Clindamycin, Kanamycin, Rifampin, Streptomycin, Spectinomycin, Sulfizoxazole, Sulphadimethoxine, Tetracycline, Ticarcillin,Trimethoprim
Chronic		
	EC-Z	Clindamycin, Rifampin
	EC-C2	Clindamycin, Rifampin, Spectinomycin
	EC-C3	Clindamycin, Rifampin

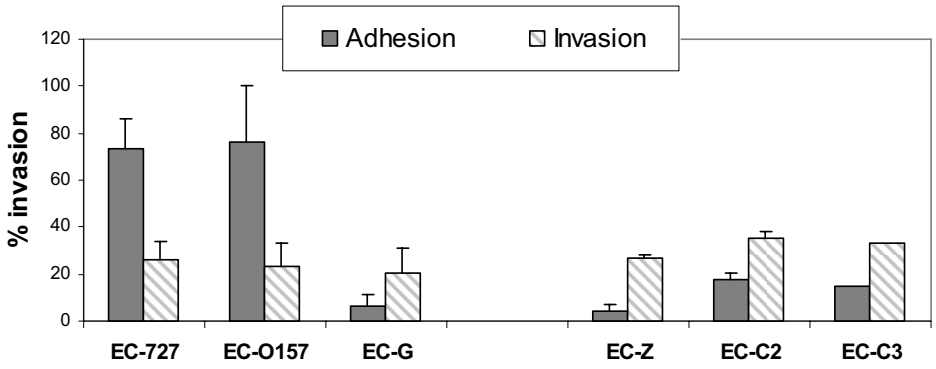

Figure 3. Role of FimH on adhesion and invasion. (FimH is blocked with 2.5% mannose).

Campylobacter jejuni and *Klebsiella pneumoniae*. Furthermore many bacterial pathogens activate host cell signal transduction pathways which involved in mediating invasion (Finlay and Cossart, 1997b). To determine the mechanism of *E. coli* invasion, we used different inhibitors as described previously (Dopfer *et al.*, 2001). Most invasive chronic strain EC-Z and least invasive acute strain EC-O157 were used as representative strains for inhibition assays. Two cytoskeletal disrupting agents, cytochalasin D, which causes microfilament depolymerization and colchicines, which causes microtubule depolymerization, inhibited

invasion by both *E. coli* strains (Figure 4). Effect of cytochalasin D was more profound than the effect of colchicine. Cytochalasin D inhibits the internalization more than %95 while colchicine inhibited around %80. Cytochalasin D inhibited invasion of control *S. typhimurium* strain while colchicine has no effect on it. The involvement of different protein kinases in bacterial internalization was tested using genistein and wortmannin. Genistein inhibited internalization of both acute and chronic strains by around %50. Wortmannin had no effect on the invasion of the control *S. typhimurium* strain while the invasion of both *E. coli* strains were inhibited more than 70%.

The inhibitors had no effect on bacterial adhesion (data not shown). The epithelial cells remained viable by trypan blue exclusion throughout the concentration ranges of the drugs used.

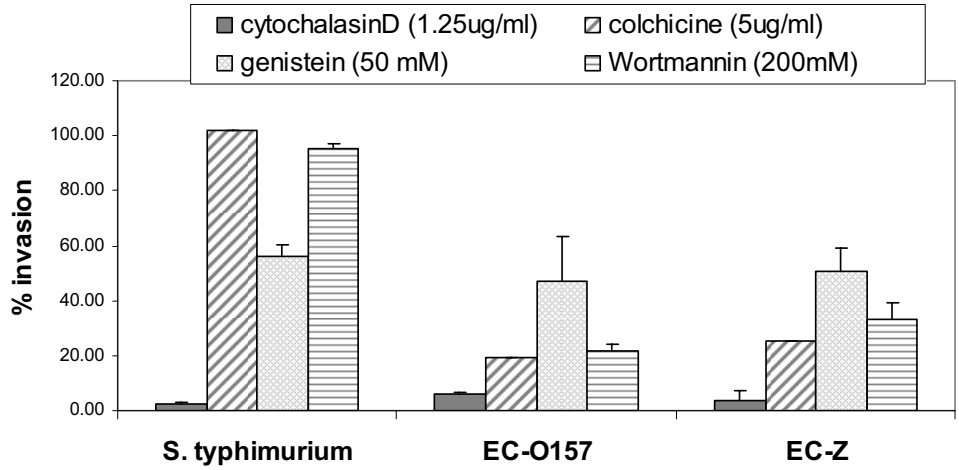

Figure 4. Effect of cytochalasinD, colchicine, genistein and wortmannin on bacterial invasion.

Conclusion

This work demonstrated that *E. coli* can adhere to and invade mammary epithelial cells. In our study, the *E. coli* isolates associated with chronic coliform mastitis appeared more invasive than the *E. coli* isolates associated with acute coliform mastitis infections. This could provide *E. coli* with a survival advantage, allowing *E. coli* to better resist detection and clearance by both innate and adaptive immune defense mechanisms. Our results also suggest that invasion of Mac-T cells by *E. coli* is an active process involving host cell cytoskeletal rearrangement and intracellular signaling cascades.

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Persistence of *Streptococcus uberis* in bovine mammary epithelial cells

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Abstract

Streptococcus uberis is an important mastitis pathogen that has been associated with chronic persistent intramammary infections (IMI) in dairy cows. To define intracellular persistence, studies on time dependent internalization into and intracellular survival of *S. uberis* in bovine mammary epithelial cells were conducted. Two strains of *S. uberis* (UT366 and UT888) and a strain of *Staphylococcus aureus* (UT955) isolated from cows with clinical mastitis were cocultured with bovine mammary epithelial cells. Survival of bacteria in host cells for extended periods was studied. *Streptococcus uberis* UT366 showed highest internalization after 60 min of incubation. However, after 8 h of incubation, internalization values for UT888 were the highest. These results suggest that during the early stages of the internalization process, *S. uberis* UT366 appeared to internalize into bovine mammary cells more efficiently than UT888, but after 8 h following internalization, UT888 survived better than UT366. Nevertheless, both *S. uberis* strains survived intracellularly for up to 120 h without affecting host cell viability. *Staphylococcus aureus* internalized more efficiently than both strains of *S. uberis* evaluated causing death of mammary epithelial cells after 72 h of incubation. These results indicate that *S. uberis* can survive within mammary epithelial cells for extended periods of time without affecting host cell viability. Intracellular persistence of *S. uberis* may be associated with spread of the infection to deeper tissues and subsequent development of persistent IMI.

Introduction

Mastitis remains economically the most important disease of dairy cattle throughout the world, accounting for 38% of the direct costs of the common production diseases (Kossaibati and Esslemont, 1997). *Streptococcus uberis* is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is the predominant organism isolated from mammary glands of multiparous cows during the nonlactating period (Oliver, 1988; Todhunter *et al.*, 1995). This organism also causes a significant proportion of major pathogen IMI in heifers during the prepartum period and during early lactation (Oliver *et al.*, 2004). Bovine IMI caused by *S. uberis* could progress from clinical or subclinical mastitis and possibly acute IMI to chronic infections that can persist in the infected mammary gland for more than one lactation (Oliver *et al.*, 1998). Internalization of bovine mastitis pathogens including *S. uberis* into mammary epithelial cell was described previously (Matthews *et al.*, 1994a; Almeida and Oliver, 1995; Almeida *et al.*, 1996; Dopfer *et al.*, 2000). An intracellular microenvironment provides these pathogens with protection from the immune system and non-specific antibacterial factors

present in bovine milk. Research from our laboratory showed that exploitation of host cell cytoskeleton, host signal transduction, and expression of "de novo" proteins by pathogens (Matthews *et al.,* 1994b; Gilbert *et al.,* 1994; Almeida *et al.,* 2000) were required for internalization of *S. uberis* into bovine mammary epithelial cells. However, little is known about events that occur following internalization of bacteria into mammary epithelial cells. Therefore, the objective of this study was to characterize post-internalization events and elucidate the fate of intracellular *S.uberis* using time dependent internalization and fluorescent dyes.

Materials and methods

Bacterial strains

Two strains of *S. uberis* (UT888 and UT366) were used. *Streptococcus uberis* UT888 was isolated originally from a cow with chronic mastitis and has been used extensively in our laboratory for experimental challenge exposure studies to induce mild clinical mastitis. *Streptococcus uberis* UT366 is a highly virulent strain isolated from a cow with acute clinical mastitis and used previously for experimental induction of bovine IMI (Doane *et al.,* 1987). *Staphylococcus aureus* UT955 was isolated from a dairy cow with clinical mastitis and used as a positive control. All strains were characterized extensively by PCR-based DNA fingerprinting and biochemical analysis (Jayarao and Oliver, 1994). The non-pathogenic strain of *Escherichia coli* DH5α (Gibco, BRL, Bethesda, MD) was used as a negative control strain.

Preparation of bacterial cultures

Streptococcus uberis UT888 and UT366, *Staph. aureus* UT955, and *E. coli* DH5α stored at -70°C were thawed in a 37°C water bath, plated onto trypticase soy agar plates supplemented with 5% defibrinated sheep blood (BAP, Becton Dickinson and Company, Franklin Lakes, NJ), and incubated overnight at 37°C. After incubation, bacterial lawns were harvested and resuspended in 20 ml BactoTodd Hewitt broth (*S. uberis* and *Staph. aureus*) or Luria broth (*E. coli* DH5α) and incubated with shaking (150 rpm) for 2 h at 37°C. Bacterial suspensions were then washed three times by centrifugation (2500 x *g,* 15 min at 4°C) with phosphate buffer saline (PBS, pH 7.4), resuspended in Dulbecco's Modified Eagle's (DMEM Gibco, Grand Island, NY) to a bacterial concentration of approximately 10^7 colony forming units/ml (cfu/ml).

Coverslip preparation

A modification of the method described by Jones and Portnoy (1994) was used. Briefly, round glass coverslips (1x15mm) stored in 70% ethanol were dipped in 95% (v/v) ethanol and blotted. Eight sterile coverslips were placed into a sterile Petri dish avoiding overlapping to ensure uniform monolayer formation.

Mammary epithelial cells and culture conditions

A bovine mammary epithelial cell line (MAC-T) described by Huynh *et al.*(1991) was used. MAC-T cells were cultured in T75 cell culture flasks using cell growth media (CGM) described previously (Almeida *et al.,* 2000). MAC-T cell monolayers were harvested by trypsinzation, transferred into Petri dishes containing coverslips, and incubated at 37°C in 5% CO_2: 95%

air (vol/vol). For fluorescence microscopy studies, MAC-T cells were gown to confluence on 4-well polystyrene tissue culture LabTek chambers (Nunc, Inc., Naperville, IL).

Bacterial internalization assay

After removing the supernatant, a bacterial inoculum containing ~ 10^7 cfu in GCM was added to Petri dishes and incubated at 37ºC, ensuring that coverslips were not disturbed. At regular time intervals (1, 2, 4, 6 and 8 h), three coverslips per Petri dish were removed and transferred into 50 ml conical tubes containing 5 ml of sterile PBS (pH 7.4) for gentle washing. One ml of CGM containing penicillin (5 µg/ml, Sigma Chemical Co.) and gentamicin (100 µg/ml, Sigma) was added to monolayers cocultured with *S. uberis* and *E. coli* or one ml of CGM containing lysostaphin (1µg/ml, Sigma) was added to *Staph. aureus* UT 955 cocultured monolayers. Coverslips were incubated for 2 h at 37ºC and after removing CGM containing antibiotics, monolayers were washed three times with PBS and lysed with trypsin (0.25%, Sigma) and Triton X-100 (0.01%, Amersham, Arlington Heights, IL). Colony forming units/ml in lysate, monolayer supernatants and bacterial inoculum were determined by standard colony counting techniques. Extracellular bacterial killing activity of the antibiotics/lysostaphin suspension was monitored by culturing supernatants from MAC-T cells cocultured with bacteria treated with antibiotics or lysostaphin. Experiments were conducted in triplicate and repeated three times.

Long term survival of intracellular bacteria

Two methods were employed to determine intracellular survival of *S. uberis* in bovine mammary epithelial cells following internalization. The first method was conducted following the internalization protocol described above except that after coculturing with bacteria, bovine mammary epithelial cell monolayers were incubated with CGM containing antibiotics or lysostaphin for 120 h. Since gentamicin may slowly penetrate the eukaryotic membrane (Jones and Portnoy, 1994), its concentration was decreased 4-fold after the initial 2 h of incubation. The second method was conducted following the protocol described by Barker *et al.*(1997) with modifications. Briefly, fluorescent labeled bacteria (Live/Dead® *Bac*Light Bacterial Viability Kit, Molecular Probes, Inc., Eugene, OR) were cocultured with MAC-T cell cultures for 2 h at 37ºC in 5% CO2: 95% air (vol/vol), treated with antibiotics, and incubated for 96 h at 37ºC in 5% CO2: 95% air (vol/vol). At each sampling time, slides were washed with PBS (pH 7.4), mounted, and kept at 4°C. Slides were observed using a confocal epifluorescence microscope (Leica TCS SP2, Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Images were captured and analyzed using Leica Lite software (Leica Microsystems Heidelberg GmbH) and Image J software (www.rsb.info.nih.gov/ij/).

Statistics

Internalization and intracellular survival assays were performed three times with each condition in triplicate. Means from each experiment were analyzed by ANOVA and those showing statistically significant differences were further analyzed by Student's *t* test using ProStat (Poly Software International, Salt Lake City, UT) statistical software.

Results

Internalization of *S. uberis* into MAC-T cells

Results from the first hour of coculture showed that *S. uberis* UT366 internalized more efficiently than *S. uberis* UT888 ($P < 0.005$). The highest internalization was achieved by *S. uberis* UT366, however, internalized *S. uberis* UT888 survived better. Contrary to the steady loss of intracellular viability observed with *S. uberis* UT366, *S. uberis* UT 888 intracellular cfu/ml counts were constant during the assay. The progressive decline of *S. uberis* UT366 viability was so marked that at 8 h of incubation the percentage of intracellular *S. uberis* UT366 was lower than *S. uberis* UT888 and 66% lower than that observed at 1 h of coculture. When internalization values of the positive (*Staph. aureus*) and negative (*E. coli* DH2α) controls were compared with *S. uberis* strains, *Staph. aureus* UT955 showed the highest internalization values. Maximum internalization for *Staph. aureus* UT955 was seen at 6 h of incubation and was approximately 2.5 $\log_{10}$ higher than at 1 h of coculture, and 4.5 logs higher than *S. uberis* UT888 and UT366 for the corresponding point (6 h). No internalization of *E. coli* DH2α into MAC-T cells was detected at any time.

Intracellular persistence of *S. uberis* in MAC-T Cells

To assess the survival ability of internalized *S. uberis* stains over a 6 day period, MAC-T cells were cocultured with *S. uberis* strains (UT888 and UT366) for 1 h, washed and treated with antibiotic solution for 2 h, and incubated with 0.25 X diluted antibiotic solution for 144 h. Number of cfu/ml of internalized *S. uberis* recovered after 24 h of incubation was slightly higher (UT888 $P = 0.26$; UT366 $P = 0.04$) than that recovered at 8 h of coculture. After the initial 24 h of incubation, internalization of *S. uberis* UT888 into host cell was significantly higher than for UT366 ($P = 0.02$). This strain effect was observed throughout the remainder of the assay. Colony forming units/ml of internalized *S. uberis* increased 1.35 and 1.43 $\log_{10}$ for UT888 and 1.63 and 1.72 $\log_{10}$ for UT366 at day 2 and 3, respectively, compared with values observed at day 1. Beginning at day 4, counts of internalized *S. uberis* UT888 and UT366 decreased to values not significantly different from those of day 1. Although 3.0 $\log_{10}$ higher, *Staph. aureus* UT955 showed an almost similar increasing trend of internalized cfu/ml as described for *S. uberis*. However, after reaching maximum values at day 2, the number of internalized *Staph. aureus* decreased markedly reaching values lower than that observed for *S. uberis* UT888 and UT366 on day 6. This reduction in internalized *Staph. aureus* was probably caused by the loss of viability and death of host cells. Nevertheless, *S. uberis* UT888 values at 96 h were higher than *S. uberis* UT366 and *Staph. aureus* UT955. Results from fluorescent labeled *S. uberis* MAC-T cell cocultures showed that both *S. uberis* strains maintained intracellular viability at each sampling point. When numbers of intracellular viable bacteria were calculated, a trend similar to the classical internalization assays was observed. Of all bacteria tested, *Staph. aureus* UT395 showed the highest numbers of internalized and viable microorganisms, except at 96 h where numbers of internalized *S. uberis* UT888 were the highest. No internalized *E. coli* D5α was detected at any time. Results from these assays show the capacity of both *S.uberis* strains evaluated to survive intracellularly for extended periods inducing minimal damage to host mammary epithelial cells.

Discussion

Previous studies from our laboratory demonstrated the ability of *S. uberis* to attach to and internalize into bovine mammary epithelial cells exploiting host cell cytoskeleton and signal transduction pathways (Matthews *et al.,* 1994; Almeida *et al.,* 2000). Result of the present study showed that both *S. uberis* strains evaluated were capable of maintaining a low level of intracellular persistence. Results also showed that, different from *Staph. aureus*, *S. uberis* did not cause apparent extensive host cell damage and host cell destruction seemed to be the limiting factor for *Staph. aureus* "in vitro" intracellular persistence. When internalization and persistence patterns of *S. uberis* stains were compared, differences were noticed. Differences in the internalization profile between *S. uberis* strains were observed after 1 h of coculture. *Streptococcus uberis* UT366 internalization values were 2.6 times higher than UT888, suggesting that UT366 induced a better uptake signal than UT888. Over time, the initial UT366 difference was gradually lost and by 8 h of culture, internalization values of *S. uberis* UT366 was lower than UT888. These data suggest that the progressive loss of *S. uberis* UT366 viability is probably due to host cell intracellular killing mechanisms. These putative intracellular killing mechanisms appeared to be less effective against *S. uberis* UT888 or even *S. uberis* UT366 after a low level of internalization was reached. When long internalization values were analyzed, strain differences were also observed. *Streptococcus uberis* UT888 maximum internalization values were detected at 48 and 72 h of coculture. We were unable to confirm if increased internalization values observed at these sampling points were due to the single or combined effect of intracellular growth and/or decreased intracellular killing. However, data from this and from other studies conducted in our lab demonstrated that both *S. uberis* strains survived well inside mammary epithelial cells and preferential endocytosis intracellular trafficking uncoupled with host-cell killing mechanisms were employed by both *S. uberis* strains evaluated. Taken together, these results suggest that *S. uberis* have evolved mechanisms by which intracellular persistence is achieved after reaching a low level of internalization. Results from fluorescent assays confirmed results from classical internalization assays and showed the ability of *S. uberis* to survive intracellularly. Concerning strain differences, it is interesting to note that *S. uberis* UT366 typically produced acute IMI with severe local and generalized symptoms (Doane *et al.,* 1987), whereas *S. uberis* UT888 produced less severe clinical mastitis which typically resulted in chronic and persistent infections (Oliver *et al.,* 1998b).

In conclusion, results of this study suggest that *S. uberis* has mechanisms to survive intracellularly while maintaining a low level of viable microorganisms that may serve as a reservoir for persistent infections. Future research should define these mechanisms and confirm if these *in vitro* observations are linked to characteristics of natural *S. uberis* IMI such as persistent infections.

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Association between genotype of *Staphylococcus aureus*, recovered at the end of lactation, and cure of the organism in the dry period

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Abstract

The purpose of this study was to examine the association between the genotype of *Staphylococcus aureus*, as determined by pulsed-field gel electrophoresis (PFGE), and the cure of that infection in the dry period. Data from 121 subclinically infected quarters, representing 92 cows from 40 herds, were analyzed. Endonuclease digestion of DNA was performed with S*ma*I, fragments were separated by electrophoresis, and macrorestriction fingerprint patterns were analyzed using GelCompar II software. A total of 18 different macrorestriction DNA profiles were identified from these 121 isolates, and grouped into three distinct lineage groups. Isolates of PFGE lineage group D were more likely to cure than isolates of other lineage groups (group A and F) (P <0.05). However, there was a significant interaction of the association between lineage group and cure with the intramammary treatment administered at drying off. There was no difference in cure rates for any PFGE genotype when the novel intramammary antibiotic containing tilmicosin phosphate was administered; however, when benzathine cloxacillin (the positive control) was administered, 87% of lineage group D isolates were cured as compared to 46% group A and 33% of group F isolates (P < 0.05). Interpretation of the multivariable logistic regression model, controlling for important herd and cow factors, revealed the influence of somatic cell count on cure, and its significant interaction with PFGE genotype. This study indicates that identifying the genotype of *S. aureus* may provide useful information towards further understanding of the pathophysiology and probability of cure of *S. aureus* during the dry period.

Keywords: *Staphylococcus aureus*, genotype, dry period, PFGE

Introduction

Successful elimination of existing and prevention of new cases of intramammary infections (IMI) routinely involves the use of antibiotics. This is particularly true for management of udder health in the dry period. The use of dry cow antibiotic therapy (DCT) at the end of lactation has been extensively studied, and recent reports confirm DCT is effective towards reducing new IMI in the dry period and clinical infections in the subsequent lactation (Berry and Hillerton, 2002). However, despite widespread adoption of DCT, quarters still do become infected throughout the dry period, and numerous existing IMI at the end of lactation are not eliminated. Elimination of subclinical *Staphylococcus*

aureus during the dry period remains particularly difficult. Cure rates vary from 20-80% of quarters (Dingwell *et al.*, 2003; Nickerson *et al.*, 1999).

Epidemiological studies at the cow and quarter level have identified strong risk factors associated with the cure of subclinical *S. aureus* (Sol *et al.*, 1994). The age of the cow, the number of quarters that are infected prior to drying off, the number of times a quarter cultured positive, and the somatic cell count (SCC) of the cow can all be used to predict the probability of cure. However, considerable variation and many unknown factors influencing the success of DCT to cure *S. aureus remains*. As such, much attention has been paid to describing specific characteristics of this pathogen. Biotyping, phage typing, random amplified polymorphic DNA typing (RAPD), pulsed-field gel electrophoresis (PFGE), and binary typing (BT) are techniques that have been used to describe, understand, and to fingerprint *S. aureus* (Zadoks *et al.*, 2000; Lam *et al.*, 1996).

For purposes of this study, two databases were combined. The first contained the bacteriological and cow data derived from a field study which evaluated the efficacy of a new DCT to eliminate subclinical *S. aureus* IMI. Isolates from all positive cultures of *S. aureus* from that original field study were frozen and were later independently analyzed for molecular typing by PFGE. These PFGE profiles of the *S. aureus* isolates constituted the second database. The databases were combined in this experiment to test the null hypothesis that there was no difference in the proportion of natural subclinical *S. aureus* infections that cured during the dry period based on molecular type.

Materials and methods

The participation of herds from three geographical regions of Canada and specific enrolment criteria have been reported previously (Dingwell *et al.*, 2003). Enrolled cows had quarter milk samples aseptically obtained at four weeks, and again at two weeks, prior to their anticipated drying off date. If at least one of these quarter samples were culture positive for *S. aureus* they were considered to be subclinically infected, and were randomly assigned to receive one of two DCT treatments. All cows had quarter milk samples taken again on the day of drying-off. The intramammary treatments used were an approved benzathine cloxacillin DCT as a positive control (Dry-Clox; Ayerst Laboratories, Guelph, Ontario), or an experimental intramammary product being tested which contained tilmicosin phosphate (Provel, a division of Eli Lilly Inc. Canada, Guelph, Ontario). Following the dry period, all *S. aureus* cows had quarter milk samples collected by aseptic technique in the first, third and fourth week following calving.

Bacteriological procedures and definition of cure

All quarter milk samples were frozen and sent to a single laboratory. Collection of quarter milk samples and the laboratory procedures were conducted in accordance with NMC recommended procedures, and have been previously described (Dingwell *et al.*, 2003). A sample was defined to be contaminated if three or more different colonies were present after 48 hours of incubation. An infected quarter was considered to have cured during the dry period, only if all three milk samples from that same quarter were bacteriological negative for *S. aureus* in the first month of lactation. Isolates of *S. aureus* cultured from any quarter samples were re-incubated in broth solution and then transferred to TSA slants to be stored. The TSA slants in glass tubes were frozen and stored at -20^{o}C.

Pulsed-field Gel Electrophoresis (PFGE)

The stored *S. aureus* isolates were sent to Agriculture and Agri-Food Canada, Food Research Program where PFGE molecular typing was performed. Specifics of the PFGE methodology, as well as a detailed description of the molecular types identified and distribution among herds, has been previously reported (Sabour *et al.*, 2004). Endonuclease digestion was performed with *Sma*I (Promega, Madison, WI). Concatameric bacteriophage lambda DNA molecules (New England Biolabs, Mississauga, ON) and *Sma*I fragments of the cellular DNA from *S. aureus* NCTC 8325 was used as standards. Macrorestriction fingerprint patterns were analyzed using GelCompare II software (Version 2.4; Applied Maths, Kortrijk, Belgium) and dendrograms were created by using the Dice coefficient, unweighted pair group method with arithmetic means (UPGMA), and a position tolerance of 1%. Isolates with identical restriction patterns were assigned to the same type.

Statistical analysis

Descriptive statistics were generated with the frequency and univariate procedures and by sorting the data on various stratification levels using SAS statistical software. Simple differences in proportions of strains that cured or did not cure were tested using Fischer's exact test.

Logistic regression for the probability of a quarter to cure was modeled by fitting a generalized linear model with a logit link function and a binomial error distribution. Since quarters within a cow are not independent, this correlation was accounted for using generalized estimation equations The variance components at both the herd and the cow level were evaluated. (PROC VARCOMP, SAS v.8.02) to decide whether cow and herd effects would be considered as either random or fixed effects in the final model. As the data demonstrated very little clustering in herds once the clustering within cows had been adjusted for, it was decided to exclude the fixed effect of herd in the final model. A compound symmetry correlation structure (equal correlation between quarters within a cow) was used. A univariate model with each independent variable of interest was evaluated first, with all variables at $P < 0.20$ allowed to enter the multivariable model. A backwards selection of main effects was done and biologically meaningful two-way interactions were examined between significant variables.

Results

Complete bacteriological culture information to define cure, as well as molecular typing, were available for 121 individual quarters. Specifically, there were a total of 51, 65 and 5 quarters from 42, 45 and 5 cows in Ontario, Quebec and Prince Edward Island, respectively. The mean herd size was 64 cows, and the average length of the dry period was 66 days. The average number of *S. aureus* infected quarters per cow in the dataset was 1.3.

For the 121 isolates of *S. aureus* used in this analysis, 18 distinct macrorestriction profiles were identified which were arranged into 3 lineage groups (A, D and F) (Table 1). The majority of herds (88%) had only one lineage group isolated from all cows enrolled from that herd; however, there were 5 herds from which 2 different groups were isolated. PFGE lineage group D isolates cured significantly more (81.5%) than types A (56.9%) and F (54.5%) ($P < 0.05$). This effect of lineage group was influenced greatly by treatment received at drying-off. When separated out by DCT, there was no difference in cure among

all groups (A, D, F) for those that received intramammary tilmicosin (71%, 75%, 80%, respectively). However, group D isolates were more likely to cure than group A and F isolates when cloxacillin was administered (87%, 46% and 33%, respectively) ($P < 0.05$). Cows with quarters infected with lineage group D, had a higher LS than cows with isolates identified as lineage group A or F ($P < 0.05$).

The multivariable model (Table 2) revealed infected quarters of first and second lactation cows had a higher probability for curing as compared to third lactation and older animals ($P = 0.05$). The probability for cure was significantly decreased for cows that had a LS equal to or above the mean of 5.7 ($P = 0.05$). The main effects of lineage group for cure were non-significant with dry LS centered in the final model. There was still a strong tendency for isolates of lineage group D to more likely to cure compared to isolates of lineage group A. However, there was a significant interaction between lineage group and LS on last DHI test. When the LS of an infected cow was at least the mean value and the cow was infected with lineage group D, that infected quarter was significantly more likely to cure ($P < 0.001$).

Table 1. Descriptive data of the Pulsed-Field Gel Electrophoresis genotypes of the 121 S. aureus *isolates recovered from 92 cows subclinically infected at the end of lactation.*

Study Site	Herds (n)	Cows (n)	Lineage A	Lineage D	Lineage F	Total
P.E.I.	3	5	1	0	4	5
Quebec	16	45	40	21	4	65
Ontario	21	42	31	6	14	51
Total	40	92	72 (59.5%)	27 (22.3%)	22(18.2%)	121

Table 2. Final logistic regression model output determining the probability for quarters to cure subclinical S. aureus *infections.*

Variable	Strata	Estimate	Standard error	P value
Parity	1	1.12	0.58	0.05
	2	1.09	0.56	0.05
	3+	referent	referent	
PFGE Lineage	D	1.25	0.68	0.06
Group	F	-0.45	0.61	0.46
	A	referent	referent	
LS on last DHI		-0.56	0.29	0.05
LS last test* Group	D	1.77	0.46	0.001

Discussion

Several studies have examined risk factors at both the cow and quarter-level to predict the cure of *S.* aureus (Dingwell *et al.*, 2003; Sol *et al.*, 1994). However, none of these studies of risk factors for cure of *S. aureus* in the dry period have included genotypes. The influence of strain type for changes in SCC, and clinical mastitis in lactation has been studied more extensively. One of the most recent studies conducted concluded that no significant

differences were found among *S. aureus* strains and that the degree of parenchymal injury induced by *S. aureus* infections is affected by factors other than strain type (Middleton *et al.*, 2002). Similarly, strain information has been used more extensively to study the epidemiologic characteristics of *S. aureus* and characterize its spread and implement control practices (Joo *et al.*, 2001). Genotyping has also been helpful to demonstrate the contagiousness of the pathogen (Joo *et al.*, 2001; Zadoks *et al.*, 2000). Previous research has found only a limited number of *S. aureus* genotypes in any given herd, which might be attributable to quarter-to-quarter spread of the pathogen (Lam *et al.*, 1996). Mathematical modeling has also indicated that this is the most likely explanation for spread of subclinical *S. aureus* mastitis (Lam *et al.*, 1996).

This current study supports that finding. Overall, 87.5% of herds had only strains from a single lineage group identified from all cows subclinically infected with *S. aureus*. However, it should be noted that this finding is not as clear as being able to say that only one genotype was isolated from the same herd. There was considerable genetic heterogeneity among *S. aureus* isolates. From the complete PFGE research completed by the Agriculture Canada investigators (Sabour *et al.*, 2004), 288 isolates from 58 farms were analyzed of which 121 isolates from 40 farms were used in this analysis.

The current experiment tested the hypothesis the no difference existed in the natural cure of subclinical *S. aureus* infections among different genotypes. This experiment was conducted with purposively selected isolates of *S. aureus* and bacteriological information from existing databases. Interpretation of results should bare this fact in mind. Nontheless, the results demonstrate that the lineage group of *S. aureus* isolates, as determined by PFGE, significantly influenced the probability for cure of this pathogen during the dry period. Although there are contradictory reports on the pathogenicity of different *S. aureus* strains (Middleton *et al.*, 2002), it has been suggested that virulence of the organism may be strain dependent. Therefore targeting cows with virulent, and possibly predominant strains may lead to advances in the control and management of this pathogen (Middleton *et al.*, 2002; Zadoks *et al.*, 2000).

The only surrogate measure of virulence available in this current experiment, was the linear somatic cell count score (LS) of cows on the DHI tests prior to the dry period. The lineage group of *S. aureus* with the highest cure rate, also had a significantly higher LS on the last DHI test, which is contradictory to previous research findings. What this current study demonstrates is that genotypes of *S. aureus* within a particular lineage group may naturally cause a higher LS compared to other genotypes, but yet may still have a higher probability of cure. Studies on natural populations of *S. aureus* have identified considerable genetic heterogeneity, and there is data available to suggest that certain strain types are more likely to produce particular virulence factors (Fitzgerald *et al.*, 2000). This should be the subject of further research in this field.

Despite the unique findings of this research, it will remain a challenge to incorporate this information into applicable recommendations at the herd level. Even with the previous knowledge of known risk factors for cure of *S. aureus*, (such as age, LS, number of quarters infected) regular incorporation of this information in day to day herd management decisions has not been widely adopted. As such, expecting that further diagnostic tests and expense would be readily adopted by the industry is not feasible. However, this study offers more insight and understanding into the area of cure of subclinical *S. aureus*, and may allow more meaningful decisions to be made by those producers willing and prepared to make the most informed decision.

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Toxin genes of *Staphylococcus aureus* isolated from bovine intramammary infection of different clinical characteristics and outcome

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Abstract

The material comprised 161 *Staphylococcus aureus* strains isolated from 116 dairy cows with mastitis and treated with antimicrobials. The strains originated from pre- and post-treatment samples and represented different PFGE types. The aim of the study was to investigate the relationship between the toxin determinants of the strains and clinical characteristics of mastitis caused by them. A selection of 18 toxin determinants of the bacterial strains for haemolysins (*hla-hlg*), leukocidins (*lukE-D*, *lukM*) and superantigens (*entA-E*, *entH-J*, *tst*, *etA*, and *etB*), as well as penicillin and methicillin resistance genes (*blaZ* and *mecA*) was determined by PCR. Prevalence of the determinants and their combinations were compared with severity and outcome of mastitis. The genes *hla*, *lukE-D, hlg* and *hld* and were the most common genes present (in 95.7%, 95.7%, 87.1% and 87.1%), but *entE*, *etA* and *etB* were not found. The most frequent superantigen determinants *entG* (31.9%), *entI* (31.9%) and *entJ* (19.0%) were detected from several PFGE types, but the others were linked with the types. A combination of *hla, hlb*, *hld, hlg, lukE-D, lukM*, *entC*, *entG, entI,* and *tst* was unique to one PFGE type previously associated with severe signs but short persistence. For 40/45 isolates (88.9%), persistency of infection could be demonstrated by similarity of PFGE- and toxin gene patterns between pre- and post-treatment isolates. Isolates lacking superantigen determinants were more common ($P<0.05$) among cows that recovered than among chronically infected cows (53.7% vs. 28.2%). *entJ* was associated with persistent mastitis ($P<0.01$); in particular *entJ* increased the probability of chronic mastitis in *blaZ* positive strains ($P<0.05$).

Keywords: *Staphylococcus aureus,* virulence determinant, superantigen, antimicrobial resistance, persistent infection

Introduction

Staphylococcus aureus (S. aureus) is worldwide a common cause of bovine intramammary infection (IMI) which typically develops chronic mastitis. *S. aureus* is able to produce a wide variety of virulence factors. Certain factors are clearly involved in toxin-mediated diseases of humans, like enterotoxins in staphylococcal food poisoning and TSST-toxin in toxic shock syndrome. More recently, a selection of toxins was associated with invasive infections of humans (Peacock *et al*., 2002). Production of some toxins or presence of certain

virulence genes have also been linked to severity of bovine IMI (Matsunaga *et al*., 1993; Fitzgerald *et al*., 2000), but studies on this are scant.

S. aureus exotoxins may help the bacteria to combat host defense system and aid them to persist in the bovine udder. Enterotoxins, toxic shock syndrome toxin (TSST-1) and exfoliative toxins form a family of superantigens, which are considered to be involved in modulating the host immune response. The major effects of these toxins are to inactivate cells of the host immune system, through superantigen mediated release of cytokines or direct cytotoxicity. *S. aureus* also produces a number of haemolysins (α, β, γ, and δ), which are suggested to degrade host tissues into nutrients required for bacterial growth.

We have recently reported that certain widespread PFGE-determined clonal types of *S. aureus* isolated from bovine IMI were linked with the severity of clinical signs or persistence of infection (Haveri *et al*., 2005). In the present study we investigated the prevalence of toxin determinants in different PFGE types and evaluated their significance to the clinical characteristics and outcome of mastitis. The relationships between toxin genes and resistance genes *blaZ* and *mecA* were also determined.

Materials and methods

Bacterial isolates and clinical characteristics of mastitis

A total of 161 *S. aureus* strains isolated from 116 cows with mastitis (34 subclinical, 53 mild clinical, and 29 severe clinical) were included. Ninety of the isolates were from samples taken pre- and post-treatment from 45 cows, which remained infected after 4 weeks follow-up period. The infection was considered persistent if the paired isolates from pre- and post-treatment samples had identical PFGE and toxin gene patterns. Preliminary identification of *S. aureus* was based on colony morphology, gram staining and positive reaction in catalase, rabbit plasma coagulation, and clumping factor tests (Slidex Staph Kit, BioMérieux, France).

PFGE typing

PFGE typing was made as previously described (Haveri *et al*., 2005). PFGE fingerprints with one to three band shifts were considered closely related and of the same pulsotype, and these were assigned a capital letter; the closely related clones of the respective pulsotype were accordingly numbered with a numeric suffix. Of the 116 pre-treatment isolates, 104 (89.7%) represented pulsotypes A, B, C, D, or E, which were found from several cows. Pulsotypes F-I, K-O and U were sporadic.

Toxin gene detection

Genomic DNA for PCR amplifications was extracted from the bacterial isolates grown overnight in 0.5 ml of brain heart infusion broth by using the Qiagen kit (Promega, Hilden, Germany) with some modification. Species identification was confirmed by PCR amplification of thermonuclease (*nuc*) gene. The genes *blaZ* and *mecA* coding resistance to penicillin G and oxacillin were screened by PCR. Detection of genes *sea-e*, *seg-j*, *tsst-1*, *eta*, *etb*, *lukE-lukD*, *lukM*, *hla, hlb, hld, and hlg* encoding toxins SEA-E, SEG-J, TSST-1, ETA, ETB, LukE-LukD, LukM, HLA, HLB, HLD, and HLG were performed by PCR reactions in Peltier 200 Thermal Cycler (MJ Research, Waltham, Massachusetts, USA) using primers as described earlier

(SigmaAldrich, Germany; Jarraud *et al.*, 1999, McLauchlin *et al.*, 1999, Lina *et al.*, 1999, Mehrotra *et al.*, 2000). A positive control and a negative control were included.

Statistical analyses

Pearson's chi-square test or Fischer's exact test were used to study the associations of toxin genes with persistence of IMI. Effects of toxins on symptom severity were tested by ordinal logistic regression. SPSS Statistical Software, vs 10.0 was used.

Results

Of the 18 toxin genes determined by PCR, the most prevalent were those coding haemolysins α, γ, δ, and β (in 95.7%, 87.1%, 87.1% and 75.0%), and the leukocidin gene *lukE-D* (in 95.7%). One isolate harboured *entB*, and one was negative for all toxin genes. The genes *entE*, *eta* and *etb* were not detected.

At least one superantigen was found from 58.6% isolates, and 35.3% were positive for two or more such determinants. Isolates lacking superantigens were found more commonly ($P<0.05$) from cows that recovered than from cows with persistent mastitis (53.7% vs. 28.2%). Of the individual superantigens, the gene coding enterotoxin J, which was detected in 19.0% of the isolates among several PFGE types (A, D, E), was associated with chronic mastitis (Table 1). The gene *lukM*, in 26.7% of the isolates, was connected with non-persistent and clinical mastitis. *tst*-gene, always detected together with *entC*, was never found in isolates from chronic infections.

The genes for *entG* and *entI* were always present concomitantly and in 31.9% of the isolates. These genes tended to be associated with mastitis with higher cure rates compared with isolates lacking them ($P=0.059$). However, *entC*, *tst* and *lukM* were typical and *entG*

Table 1. Distribution of toxin genes of S. aureus isolated from bovine IMI according to severity and persistence of mastitis caused by these isolates.

	Severity of mastitis (n of cows=116)				Persistence of mastitis(n of cows=101)		
Toxin gene	*Subclinical (n=33)*	*Mild (n=54)*	*Severe (n=29)*	*P*	*Recovered (n=61)*	*Persistent (n=40)*	*P*
entA	3 (9.1%)	3 (5.6%)			2 (3.3%)	3 (7.5%)	
entB	1 (3.0%)					1 (2.5%)	
entC	2 (6.1%)	4 (7.4%)	3 (10.3%)		9 (14.8%)		*
entD		1 (1.9%)	1 (3.4%)		1 (1.6%)	1 (2.5%)	
entG	6 (18.2%)	21 (38.9%)	10 (34.5%)		23 (37.7%)	8 (20.0%)	
entH	5 (15.2%)	2 (3.7%)			4 (6.6%)	3 (7.5%)	
entI	6 (18.2%)	21 (38.9%)	10 (34.5%)		23 (37.7%)	8 (20.0%)	
entJ	6 (18.2%)	10 (18.5%)	6 (20.7%)		5 (8.2%)	16 (40.0%)	**
tst	1 (3.0%)	4 (7.4%)	3 (10.3%)		8 (13.1%)		*
lukE-D	31 (93.9%)	51 (94.4%)	29 (100%)		60 (98.4%)	36 (90.0%)	
lukM	3 (9.1%)	13 (24.1%)	15 (51.7%)	**	23 (37.7%)	5 (15.0%)	**
α	31 (93.9%)	52 (96.3%)	28 (96.6%)		57 (95.1%)	37 (92.5%)	
β	24 (72.7%)	38 (70.4%)	25 (86.2%)		47 (77.0%)	28 (70.0%)	
γ	27 (81.8%)	47 (87.0%)	27 (93.1%)		53 (86.9%)	34 (85.0%)	
δ	29 (87.9%)	48 (88.9%)	24 (82.8%)		53 (86.9%)	34 (85.0%)	

Table 2. Proportions of the most common toxin genes among all pulsotypes (116 isolates) and among the main pulsotypes A-E (104 isolates) of S. aureus isolated from bovine mastitis.

Toxin gene	All n=116 n (%)	A (n=50) n (%)	B (n=22) n (%)	C (n=8) n (%)	D (n=6) n (%)	E (n=18) n (%)
Hla	111 (96)	45 (90)	22 (100)	8 (100)	6 (100)	18 (100)
Hlb	87 (75)	43 (86)	22 (100)	2	0	12 (67)
Hld	101 (87)	41 (82)	19 (86)	8	3	18 (100)
Hlg	101 (87)	40 (80)	21 (95)	8	4	17 (94)
lukE-D	111 (96)	47 (94)	22 (100)	8	5	18 (100)
lukM	31 (27)	5 (10)	22 (100)	1	0	2 (11)
entA	6 (5)	1 (2)	0	3	0	2 (11)
entC, tst	8 (7)	0	8 (38)	0	0	0
entG, I	37 (32)	0	15 (68)	1	2	17 (94)
entH	7 (6)	0	0	7	0	0
entJ	22 (19)	20 (40)	0	0	1	1 (6)

and *entI* common in strains of PFGE type B (Table 2). When the B-type strains were removed from the analyses, *lukM*, *entG* and *entI* affected neither the severity nor the persistence of mastitis. Among PFGE type B strains, the presence of *entC* and *tst* was not associated with severity or higher cure rates, when compared with isolates without these genes. The PFGE type B had the highest number (n=10) of toxin genes (*entC*, *entG*, *entI*, *tst*, *hla-hlg*, *lukE-D*, and *lukM*), which were detected in eight isolates.

More than half of the *entG* and *entI* positive isolates, and all of those carrying *entD* or *entJ* also had the betalactamase gene *blaZ*. The *blaZ* positive isolates were typical for mastitis with low cure rate ($P<0.01$). The cure rate was further lower ($P<0.05$), when the infecting strain carried both *entJ* and *blaZ* (16/21; 76.2% remained infected) than *blaZ* only (9/20; 45.0% remained infected). When isolates with *entJ* and *blaZ* were excluded from the analyses, cure rates between IMIs caused by *blaZ* positive (25.0% persisted) and *blaZ* negative strains (45.0% persistend) were not significantly different but a trend was found ($P=0.09$). The genes *entA*, *entC*, *entH*, and *tst* were present only in isolates negative for *blaZ*. The gene for *mecA* was not detected.

The distribution of toxin genes was not different for the sporadic PFGE types F-I, K-O and U (n of isolates=12) compared with the main PFGE types. The toxins were not accumulated into certain clones of the PFGE types A-E either.

Discussion

Bovine IMI caused by *S. aureus* has a high tendency to persist in the udder. Certain toxins including superantigens, haemolysins and leukocidins are suggested to be involved in this process. The presence of gene coding staphylococcal enterotoxin J tended here to be associated with mastitis developing chronic. *entJ* has been associated with invasive infections in humans (Peacock *et al.*, 2003). *entJ* occurred always simultaneously with the betalactamase gene *blaZ*. *entJ* is consistently located in plasmid, which may also encode penicillin resistance (Zhang *et al.*, 1998; McCormick *et al.*, 2001). Penicillin-resistant

staphylococcal strains have been associated with lower cure rates compared with susceptible strains (Taponen *et al.*, 2003). Some virulence factors expressed together with resistance factors may have synergistic effect on the development of chronic IMI. In this study, the combination of *entJ* and *blaZ* increased the probability of persistent mastitis compared with isolates harbouring *blaZ* only.

The co-existence of the genes *entC* and *tst* (Fitzgerald *et al.*, 2000; Akineden *et al.*, 2001; Zshöck *et al.*, 2004), or the co-production of the respective toxins (Tollersrud *et al.*, 2000, Stephan *et al.*, 2001) has been found from *S. aureus* isolated from bovine mastitis. In an earlier study by Matsunaga *et al.* (1993), production of TSST-1 was associated with severe, peracute form of bovine mastitis. Isolates carrying *entC* and *tst* tended here to cause severe signs, but infections were always eliminated.

The majority of isolates carried haemolysin genes α-γ, which probably play a marked role in the pathogenesis of bovine *S. aureus* IMI. Nevertheless, the expression of these genes may not be essential for the infection. Akineden *et al.* (2001) found that 28/103 (27.2%) of *S. aureus* isolates from bovine mastitis did not show α- or β-haemolysin production. The gene *lukE-D* was detected in nearly all isolates, and it encodes bicomponent leukotoxin LukE-LukD that has been previously associated with impetigo in humans (Gravet *et al.*, 2001). The significance of superantigens in bovine IMI remains unclear. A high number of isolates was negative for these genes. Many of them, *entC, entH* and *tst*, were strongly linked to the PFGE type. The direct causality between severity and persistence of IMI and the presence or absence of most toxin genes remains to be shown. In the present study, one gene was connected with the presence of penicillin-resistance gene, and the synergistic effect of these genes on the prognosis of IMI should be further investigated. Many of the genes were genotype linked, which information can be epidemiologically useful.

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Changes in leucocyte surface receptor expression and functional responses in dairy cows with *Staphylococcus aureus* infection

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Abstract

Staphylococcus aureus (SA) is the major pathogen causing chronic mastitis in cows. The roles of host immune system components in the defence of the mammary gland against infection are not well understood. Expression of adhesion molecules and lymphocyte markers and the functional responses of leucocytes from SA-infected cows were determined to elucidate the association between decreased host defence and SA infection. Eight Holstein lactating cows infected with SA were used for this study. Expression of adhesion molecules such as CD18, L-selectin and lymphocyte receptors, CD4,8 and surface IgG, on blood and milk leucocytes from cows with SA infection were determined by immunofluorescent analysis. Matrix metalloproteinases (MMP)2,9, lactoferrin (LF), acetyl glucosaminidase and somatic cells were measured to monitor the inflammatory response. LF and IgG concentrations in SA-infected quarter milk were 668.7ug/ml and 0.53mg/ml, respectively. LF, albumin and IgG and acid glycoproteins in mastitic milk were detected by immunoblotting. Activities for MMP-2,9 were clearly detected in SA-infected milk by zymography. L-selectin expression on neutrophils from SA-infected quarter milk showed a significant decrease compared to blood levels. Expression of CD18 on blood neutrophils in SA-infected cows was slightly increased compared to normal cows. Fc-receptor(FcR)-stimulated chemiluminescence (CL) of blood neutrophils was apparently decreased. SIg expression on blood lymphocytes from SA-infected cows appeared to be decreased compared with normal cows. Lymphocyte responses and FcR-stimulated neutrophil CL were found to be decreased in dairy cows with SA infection and may be at least in part associated with decreased host resistance capability in chronic SA infection.

keywords: adhesion molecules, chronic mastitis, *Staphylococcus aureus*, functional response

Introduction

Staphylococcus aureus (SA) often causes infections of long duration compared to infections caused by environmental pathogens (Fitzpatrick 2000). Persistence of infection results from a number of pathogen or host factors or pathogen-host interactions (Park *et al.*, 1993; Sutra and Potrel 1993; Ferens *et al.*, 1998; Soltys and Quinn 1999). The role of the host immune system in the defence of the mammary gland against SA-infection is not

well understood. It is likely that the interaction of host defence capability with SA infection may be a detrimental factor affecting the changes in responsiveness of leucocytes in dairy cows infected with SA. Expression of adhesion molecules and lymphocyte markers and the functional responses of leucocytes from SA-infected cows were determined to evaluate the relationship between host defence capability and SA infection.

Materials and methods

Cows

Eight Holstein lactating cows infected with SA were used. Age, milk yield, lactational stage and the therapeutic history were recorded. SA isolates from 8 infected quarter milks were identified based on the NMC procedure (Hogan *et al.*, 1999).

Leucocytes

Peripheral blood (20 ml) and quarter milk samples (50 ml) were used for isolation of leucocytes according to the procedure described previously (Nagahata *et al.*, 2000). Opsonized zymozan (OPZ: 10mg/ml) and aggregated bovine IgG (Agg-IgG, ICN Biomedicals: 10mg/ml) were used as stimulants. Superoxide production and chemiluminescent response (CL): Superoxide production and CL responses of neutrophils stimulated with OPZ or Agg-IgG were measured. Immunofluorescent analysis of leucocytes using FITC-conjugated monoclonal antibodies: anti- CD18 (MHM23, Dako A/S), L-selectin (CD62L, Dako A/S) and bovine IgG (IBL Res.) was performed.

MMP

Activities of metalloproteinases 2 and 9, acid glycoprotein, IgG, and haptoglobin in milk from SA-infected cows were evaluated (Raulo *et al.*, 2002). Concentrations of lactoferrin (LF) and immnunoglobulin G (IgG) in milk samples were determined using commercially available kits (Kawai *et al.*, 1999). Data were compared using Student's t-test, and values of $P<0.05$ were regarded as significant.

Results

Expression of CD18 on blood neutrophils from SA-infected cows was slightly higher than in normal cows. The levels of CD18 on milk neutrophils were similar to those of blood neutrophils. SIg expression on blood lymphocytes from SA-infected cows appeared to be decreased compared with normal cows. L-selectin expression on neutrophils from SA-infected quarter milks showed a significant decrease compared to blood levels. The Agg-IgG-induced CL response of neutrophils from cows with SA infection was decreased.

LF and IgG concentrations in SA-infected quarter milk were 668.7 ug/ml and 0.53 mg/ml, respectively. Increased levels of LF, haptoglobin, IgG and acid glycoproteins in mastitic milk were detected by immunoblotting analysis. Activities for MMP-2 and 9 were clearly detected in SA-infected milk by zymography.

Discussion

SA has been shown to reduce the effectiveness of the immune response, including suppression of the effector mechanisms in the mammary gland; however, it is still unclear that whether the responsiveness of leucocytes is modulated when the mammary gland is infected with SA.

Decreased neutrophil and lymphocyte functions in dairy cows were found to be associated with intramammary gland SA infection in this study. Differences in lymphocyte and neutrophil responses between normal cows and cows with SA are considered to be related to the severity, duration and extent of inflammation of SA infection in the mammary glands.

Expression of L-selectin on neutrophils in mammary gland was markedly decreased compared with that of blood. This finding was consistent with previous studies (Soltys and Quinn 1999). FcR-induced neutrophil responses were found to be decreased in cows with SA infection. It is likely that suppression of B lymphocyte functions may be associated with the decreased humoral immunity and survival of SA in inflammed tissues (Park *et al*., 1993).

Increased levels of MMP, LF, IgG and acid glycoproteins in mastitic milk were detected. MMPs and cytokines may modulate the activation of surface receptors and cell signalling of migrated leucocytes in the mammary gland (Raulo *et al*., 2002). The LF binding site is a nucleolin, a non-histone nuclear phosphoprotein that is a ligand of L-selectin (Britigan *et al*., 2001). LF regulates the expression of adhesion molecules and recruitment of leucocytes at inflammatory sites (Srivastava and Pollard 1999). The modulation of migrated leucocytes in the inflammed mammary gland appeared to be induced by inflammatory products such as IL-1*f*À, TNF-*f*¿, LF and MMPs, depending on the inflammatory stage of SA infection of dairy cows. These results suggest that SA infection causes decreased host responses and this may be a contributing factor for latency of SA in the infected mammary gland. It is likely that virulence factors of SA may also be involved in decreased host defence capability and in misdirecting the immune cells to induce inadequate responses. Further studies are required to elucidate the relationship between SA and intramammary infection of dairy cows.

In conclusion, decreased leucocyte responses may at least in part be associated with the decreased host resistance capability that promotes the persistence of SA in dairy cows and contributes to chronic intramammary SA infection.

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Vitamin E supplementation and udder health: A meta-analysis

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Abstract

The objectives of this study were to conduct a meta-analysis on available literature to estimate the magnitude and significance of the weighted summarised effect of Vitamin E status on udder health. Inclusion criteria for studies were one or more groups of cows with given values of vitamin E status or supplemental levels, cow/herd plasma or serum vitamin status or ratios of these to cholesterol or total lipids, vitamin E dosage, route of dosage, period of administration and duration of study. Papers with an abstract in English published between 1984 and 2003 were located through a literature search based on examination of computerised scientific databases and cross-referencing citations in retrieved papers. Fourteen out of 34 papers that addressed the relationship between vitamin E and udder health were eligible for use in the meta-analysis. Vitamin E supplementation was on average associated with a 14% reduction in the risk of intra-mammary infection (IMI). Vitamin E supplementation was also associated with a reduction in milk somatic cell counts (SCC) by a factor of 0.70 and a 30% decrease in the risk of occurrence of clinical mastitis (CM). The observed heterogeneity between studies could be explained by control and supplemental vitamin E levels, their differences and concurrent Selenium supplementation. Publication bias was evident in the meta-analysis. In conclusion, results support the hypothesis that Vitamin E supplementation during the dry and early lactation periods is associated with lower IMI, SCC and CM. However for practicality, dosages, when to start and how to proceed with supplementation and costs need to be determined.

Keywords: meta-analysis, vitamin E, clinical trial

Introduction

Vitamin E is a fat-soluble, sub-cellular and cellular membrane antioxidant, which provides stability and prevents undesirable peroxidation of membrane lipids. Vitamin E's physiological status has been associated with susceptibility and response to infectious diseases like mastitis (Smith *et al.*, 1984, Weiss *et al.*, 1997).

Several studies have reported the relationship between vitamin E status and udder health indicators such as the incidence of clinical mastitis (CM), and Sub-clinical mastitis (SCM) as shown by somatic cell counts (SCC) and intra-mammary infection (IMI). The association between vitamin E status and udder health indicators from these studies has ranged from

protective (Smith *et al.,* 1984, Weiss *et al.,* 1997), through no-relation (Batra 1992 *et al.,* Le Blanc at al 2002,) to an unfavorable effect (Erskine *et al.,* 1987, Batra *et al.,* 1992). The goal of the current study was to carry out a meta-analysis on the available literature to study the size and significance of the effect of vitamin E on udder health indicators.

Methods

Inclusion criteria

Studies had to meet the following inclusion criteria:

1. one or more groups of Vitamin E levels were being compared of which one could be classified as the control group;
2. indicator of udder health status like SCC, IMI, CM;
3. plasma or serum vitamin E cow/herd status, or ratios of these to cholesterol or total lipids;
4. vitamin E per cow or herd dosage, route of dosage and period of administration;
5. duration of the study; and
6. ruminant studies.

Literature search strategy

We considered randomised clinical trials and field studies. Papers with at least an abstract in English and in German, Dutch, English, Chinese, Japanese, Turkish, Serbo-Croatian published between 1984 and 2003 were located through a literature search that was based on 1) examination of computerized scientific directories and 2) cross-referencing of citations in retrieved papers.

Databases used included Agricola, Beast CD, Vet CD, Medline and Web of Science. We examined each title generated from the searches and identified potentially eligible articles. Abstracts of these were then obtained and for those meeting the eligibility criteria full article texts were sought.

Record selection and entry

Data from studies that were originally recorded at quarter level were converted to animal level according to the assumption that each infected cow would on average harbor 1.5 infected quarters (National Mastitis Council (1996). When several vitamin level groups were compared, the comparisons were taken as independent trials (records) with the lowest level serving as the control for all the other vitamin levels of the same study.

Statistical analysis

For cohort or randomized clinical trial (RCT) designs, the relative risk (RR) was considered to be the best measure of effect while the prevalence rate ratio was used as an estimate of the RR in cross-sectional study designs. The mean difference (MD) was used to evaluate the effect of Vitamin E on SCC.

The inverse variance or the Maentel-Haenszel (MH) method and Hedge's adjusted g (Rosnow *et al.,* 2000) were used as weighting factors respectively for RR and MD meta-analyses. Heterogeneity between studies was tested using a χ^2 procedure. If heterogeneity was present, a random effect model was applied to summarize the data, else a fixed effect model. A weighted (inverse of the variance) univariable meta-regression of effect measures

(RR, MD) on each of the possible explanatory variables of heterogeneity was carried out using SPSS®. Funnel plots were used to check for publication bias.

Results

Search results

The search initially resulted in a total of 34 papers on the relationship between vitamin E and udder health indicators. Only 14 papers were eligible for use in various sub-analyses, with a total of 18 records (Table 1).

Relation between vitamin E and udder health indicators

The reported and subsequently used pooled estimates as shown in Forest plots (Figure 1, 2 and 3) are those computed from RCTs only.

Vitamin E supplementation was on average followed by a significant 14% reduction in the risk of IMI (RR= .86 (.51-.83)) with concurrent selenium supplementation explaining 49% of the variation (Figure 1).

The pooled estimate showed a significant .35 units reduction in ln(SCC) (MD= -.35 (-.69- -.01)) with vitamin E supplementation (Figure 2). On average vitamin E supplementation was associated with a reduction in SCC by a factor of .70. Vitamin E supplemental and control levels and their difference accounted, respectively, for 92%, 76% and 99% of the variation in ln(SCC).

Vitamin E supplementation led to a significant 30% reduction in the risk of getting CM (RR=.70 (.59-.83)) (Figure 3). Twenty-six percent and 15% of variation in RR of CM was explained by background (control) CM incidence and duration of supplementation respectively. The difference between supplemental and control vitamin E levels and concurrent Se supplementation each explained 11% of the total variation in RR of CM.

Significant heterogeneity was observed with IMI and SCC. No year trend was observed across records in all udder health indicators. Funnel plots (results not shown) revealed publication bias as indicated by an absence of smaller studies (high standard error) that did not indicate a "positive" effect of vitamin E supplementation for all udder health indicators.

Discussion

Surprisingly large effects of vitamin E levels on udder health indicators were observed in this meta-analysis, particularly in the light of the control vitamin E levels. The reported basic (control) dietary levels of vitamin E in all studies were way above the 1988 NRC recommended levels (15 IU/kg DIM), which are the minimum requirement to prevent overt signs of deficiency and guarantee reasonable animal performance. For proper immune function intake levels were suggested higher at 1000 IU/cow (Weiss *et al.*, 1998, Hogan *et al.*, 1993). Therefore, a RR not far different from unity could be interpreted as no effect of vitamin E, yet it could be the effect of the high control levels. Therefore the NRC and researchers have to come up with standard supplementation for control animals so that the true benefit of vitamin E can be accurately evaluated.

Vitamin E kinetics is largely unknown, more-so for different routes of administration. The same status of vitamin E as measured in plasma/serum or these as ratios to total lipids

Table 1. Record characteristics with name of first author, study design, number of cows in groups, vitamin E status and udder health indicators.

Record First author, year[STUDY DESIGN]	Numb. of cows Treatment/ Control{Duration of suppl.}	Vit. E Suppl. [Route]	Vit. E status (µg/Ml) Treatment/ Control	Vit. E: cholesterol Ratio(µg/Mg) Treatment/ Control	Udder health Indicator
Le Blanc, 2002 [RCT]	574/568 {21}	3000IUVit.EVs Placebo [Injection]	2.96/2.14	3.79/2.88	CM, SCC
Baldi, 2000 [RCT]	14/14 {28}	2000IU Vit.E Vs 1000IU [Diet]	4.85/3.25	5.61/3.92	SCC
Valle,2000 [RCT]	26/26 {60}	1000IUVit.E Vs ND [Diet]	–	–	CM,IMI
Valle,2000 [RCT]	26/26 {60}	1000IU& 3000IUVit.E Vs ND [Diet]	–	–	CM,IMI
Morgante,1999 [RCT]	25/25 {150}	250IU Vs placebo [Injection]			CM, SCC, IMI
Weiss,1997 [RCT]	21/22 {67}	1000&500IUVs 100IU [Diet]	2..35/2	3.7/2.24	CM,IMI
Weiss,1997 [RCT]	19/22 {67}	100,4000IU, 2000IUVs100IU [Diet]	3.84/2	6.57/2.24	CM, IMI
Erskine,1997 [RCT]	204/216 {44}	3000IU Vs Placebo [Injection]	3.94/2.98	5.01/3.69	CM
Liu Yu,1995 [RCT]	48/39 {300}	500IU Vs ND [Diet]	–	–	CM
Nizamlioglu,93 [CS]	14/20 {0}	–	0.71/0.68	–	SCC,IMI
Batra,1992 [RCT]	108/95 {305}	1000,500IUVs ND [Diet]	3.84/3.11	–	CM
Batra,1992 [RCT]	58/60 {224}	1000,500IUVs ND	3.84/3.11	–	SCC
Nizamlioglu,92 [CS]	15/25 {0}	–	0.70/0.68	–	SCC, IMI
Erskine,1987 [CS]	679/690 {0}	–	4.85/4.21	–	IMI, SCC
Atroshi,1986 [CS]	20/21 {0}	–	6.0/4.84	–	SCC
Smith,1985 [RCT]	27/28 {305}	2mg/kg,88mg/kg Vs ND [Diet]	4.95/3.77	–	IMI, CM, SCC
Smith,1984 [RCT]	21/20 {60}	740IU Vs ND [Diet]	–	–	IMI, CM
Smith,1984 [RCT]	20/20 {60}	740IU Vs ND [Diet]	–	–	IMI, CM

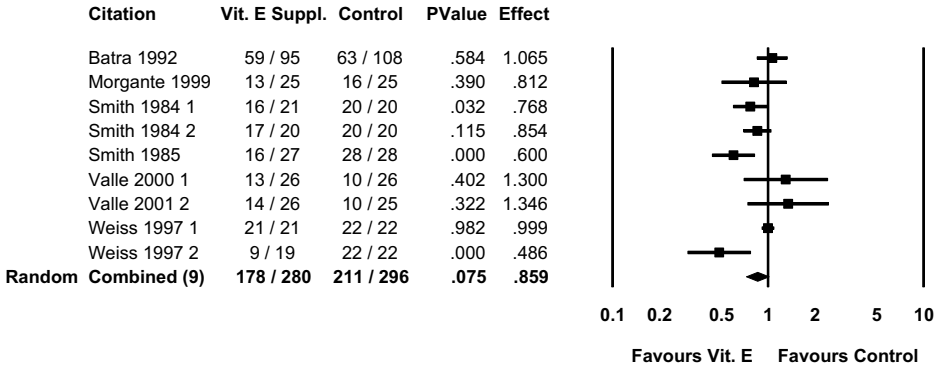

Figure 1. RR with 95% CI: Cows with IMI during vitamin E supplementation.

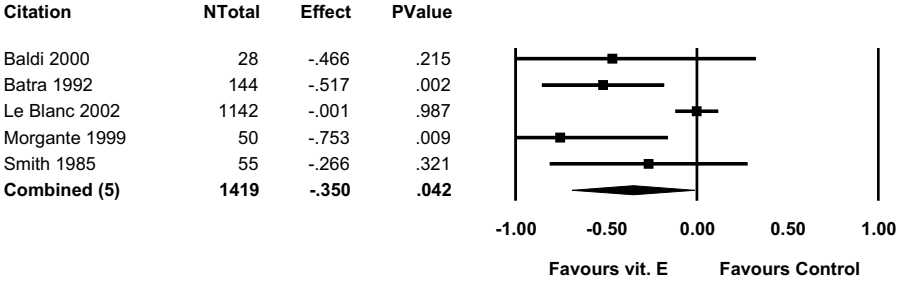

Figure 2. WMD with 95% CI: MD LNSCC of cow groups during vitamin E supplementation.

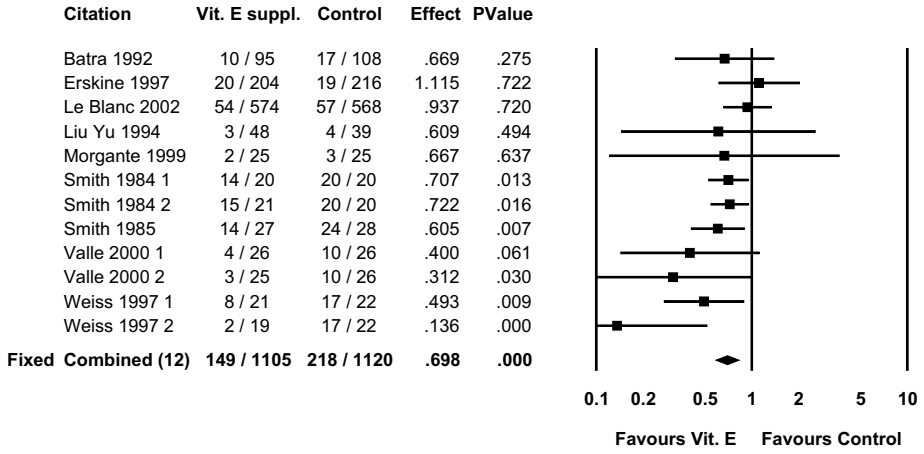

Figure 3. RR with 95% CI: Cows with CM during vitamin E supplementation.

may have different implications following parenteral versus oral administration. The scarcity of information on the kinetics of vitamin E has led to discrepancies between follow up periods in many studies. Most studies started recording udder health indicators at or around the commencement of supplementation, when it is not certain if the observed udder health parameters are in any way related to the supplemented Vitamin E and/or went on too long to still expect an effect.

Finally, as expected, publication bias was present in this meta-analysis, which could in contrast to all above points have caused an overestimation of the vitamin E effect.

Implications for dairy practice

Our results support the hypothesis that Vitamin E supplementation during the dry and early lactation periods is associated with lower IMI, SCC and CM. However for practicality, dosages and when to start and how to proceed with supplementation and costs need to be determined. Therefore more research is needed, possible outside the periparturient period, with use of robust indicators of vitamin E status, to establish vitamin E kinetics in its relation to udder health. Consideration of vitamin E alone would be folly, as all components of the anti-oxidant system appear to interact.

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α-Tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving

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Abstract

Vitamin E is an important antioxidant with positive effects on immune functions of dairy cows. A lowered blood concentration around calving has been associated with immune suppression, and increased disease incidence. Studies on supplementation of vitamin E in the periparturient period have exclusively been performed with synthetic vitamin E, but the natural form, RRR-α-tocopherol, has been shown to have a higher biological activity than the synthetic forms in several species. The aim of this study was to compare the effects of supplementing dairy cows with 1000 IU/day of all-*rac*-α-tocopheryl acetate (SynAc), RRR-α-tocopheryl acetate (NatAc), or RRR-α-tocopherol (NatAlc), from 3 weeks before to 2 weeks after calving, on the concentration of α-tocopherol and its stereoisomers in blood and milk. An unsupplemented group was also included (control). Blood samples were collected at 3, 2 and 1 weeks before calving, at calving, and 3, 7 and 14 days after calving, while milk samples were taken twice within 24 h after calving and at 7 and 14 days in milk. The plasma concentration of α-tocopherol was significantly higher in NatAc than in the other groups. A significant effect of time was observed with lowest values around calving. The α-tocopherol concentration was significantly higher in colostrum than in milk. In control cows and cows supplemented with natural vitamin E, the proportion of RRR-α-tocopherol was higher than 98% in plasma and milk. In cows fed SynAc, the proportion of RRR-stereoisomer in plasma and milk was significantly lower. In conclusion, daily oral supplementation of dairy cows with RRR-α-tocopheryl acetate gave the highest blood concentrations of α-tocopherol in the periparturient period. The α-tocopherol stereoisomer distribution further indicates that the conversion factor between all-*rac*- and RRR-α-tocopheryl acetate is higher in cows than the official ratio of 1.36 determined in rats.

Keywords: vitamin E, natural, synthetic, stereoisomers, calving

Introduction

Vitamin E is the generic name of a group of lipid-soluble compounds known as tocopherols. In nature they occur in different forms, i.e. α-, β-, γ-, δ-tocopherols and -tocotrienols, respectively. α-Tocopherol is normally the major form found in feedstuffs and

in cattle blood and milk (Pehrson and Hakkarainen, 1986). Vitamin E is an important biological antioxidant in mammalian cell membranes, preventing the oxidation of unsaturated fatty acids (Reddy and Frey, 1990). It has been found to increase cellular and humoral immunity, and during vitamin E insufficiencies the defence against infectious diseases is impaired (Ndiweni and Finch, 1996; Politis *et al.,* 1996).

The vitamin E status of dairy cows can easily be determined by blood analysis. The lowest concentration of vitamin E in blood is normally obtained around calving (Weiss *et al.,* 1990; Meglia *et al.,* 2001). Several factors have been implicated in the drop detected around calving, such as reduced feed intake and transfer of vitamin E from blood to colostrum (Goff and Stabel, 1990; Weiss *et al.,* 1990). The decrease in blood vitamin E around calving coincides with suppression of the immune system and increased disease incidence. Several reports indicate that the disease incidence is reduced when the animals were fed extra vitamin E (Weiss *et al.,* 1990; Weiss *et al.,* 1997). However, high amounts of extra vitamin E are needed to eliminate the drop in blood concentration at calving, which increases the feeding costs.

The relative vitamin E activity of synthetic vitamin E (all-*rac*-α-tocopheryl acetate), which consists of an equal proportion of all eight possible stereoisomers of α-tocopherol with four stereoisomers possessing the 2R configuration (RRR, RRS, RSS, RSR) and four the 2S configuration (SRR, SSR, SRS and SSS), compared to the relative vitamin E activity of the naturally occurring RRR-α-tocopherol has been questioned for decades. Studies in humans (Burton *et al.,* 1998) and sows (Lauridsen *et al.,* 2002a, b) suggest a higher biological activity of the natural isomer compared to the synthetic isomers. This has been ascribed to the presence of α-tocopherol transfer protein (α-TTP), which discriminates between forms of α-tocopherol (Hosomi *et al.,* 1998; Jishage *et al.,* 2001). Hidiroglou *et al.,* (1988), reported higher plasma concentrations of α-tocopherol after feeding beef cows equal amounts, on IU basis, of RRR-α-tocopheryl acetate compared to all-*rac*-α-tocopheryl acetate. However, no studies have compared the effects of supplementing natural and synthetic α-tocopherol in periparturient dairy cows, or studied the distribution of α-tocopherol stereoisomers in blood and milk. Therefore, the aim of the present study was to compare the concentrations of α-tocopherol and its stereoisomers (RRR-, RSS-, RRS-, RSR- and the sum of the 2S-forms) in blood and milk when feeding synthetic or natural vitamin E to cows around calving.

Material and methods

The study was performed using thirty-six Holstein-Friesian cows from the herd at Research Centre Foulum, Denmark. All cows were dried off 2 months before expected calving, and were assigned to the experiment from 3 weeks before estimated calving to 14 days after calving. The animals were housed in individual tie stalls and fed a total mixed ration (TMR) according to Danish recommendations for dry and lactation periods. The cows were equally distributed in four experimental groups with nine cows in each. Vitamin E supplementation was given daily, from three weeks before estimated calving to 14 days after calving, as top dressing on the feed. Three groups were supplemented with 1000 IU per day of all-*rac*-α-tocopheryl acetate (SynAc, Rovimix E50-Ads, Roche A/S, Denmark), RRR-α-tocopheryl acetate (NacAc, Natur-E granulate 40%, Pharmalett A/S, Denmark) or RRR-α-

tocopherol (NatAlc, Natur-E micelle, Pharmalett A/S, Denmark), respectively, while one group was not supplemented (Control).

Blood samples were taken from the jugular vein of each animal at 3, 2 and 1 weeks before estimated calving, within 12 h after calving, and 3, 7 and 14 days after calving into heparinized tubes. Blood plasma was collected after centrifugation and stored at -20 °C until analysed. From each cow, composite colostrum samples were taken once within 12 h and once between 12 to 24 h after calving. Composite milk samples were also taken at 7 and 14 days. The samples were stored at -20 °C until analysed. Representative feed samples were taken two weeks, two months and four months after the start of the experiment, and were frozen at -20 °C until analysis of α-tocopherol.

Feed samples were freeze dried and milled prior to analysis, and 1 g of feed sample, 1000 mg of milk and 500 µl of blood plasma were suspended in a mixture of ethanol, methanol, ascorbic acid and KOH. The mixture was saponified and the tocopherols were extracted with heptane, as previously described by Jensen and Nielsen (1996). Stereoisomers of α-tocopherol were analysed by HPLC as described by Drotleff and Ternes (2001).

Repeated measures of α-tocopherol and the stereoisomers in blood and milk were analysed with the MIXED procedure (SAS, 1999). One-way analysis of variance (ANOVA) was used to compare groups within days, and time points within groups.

Results

Treatment had a significant (P<0.001) effect on the content of α-tocopherol in plasma with higher concentration in the NatAc group than in the other groups (Table 1). Time did also have a significant (P<0.001) effect on the plasma concentration. The concentration decreased as parturition approached, reaching a nadir at calving or 3 days after calving (Table 1).

The α-tocopherol concentration in milk varied significantly over time (P<0.001) with higher amounts in colostrum than in milk (Table 2). Overall, treatment did not have a significant effect on the α-tocopherol concentration in milk. However, according to the one-way ANOVA colostrum from cows fed NatAlc had a higher α-tocopherol concentration than colostrum from SynAc cows.

Table 1. Plasma concentrations (mg/l) of α-tocopherol in 36 dairy cows fed daily supplementation of no vitamin E (Control), all-rac-α-tocopheryl acetate (SynAc), RRR-α-tocopheryl acetate (NatAc), and RRR-α-tocopherol (NatAlc) from 24 days prepartum to 14 days postpartum.

Days relative to calving	Control	SynAc	NatAc	NatAlc	P-values
-24	2.24±0.26	2.48±0.31	2.56±0.21	2.16±0.26	0.67
-16	2.54±0.24	3.33±0.26	3.76±0.41	3.06±0.47	0.13
-9	2.40±0.28^{b}	2.86±0.29^{ab}	3.92±0.35^{a}	3.24±0.37^{ab}	0.01
0	1.92±0.23	2.52±0.34	2.89±0.24	2.39±0.29	0.13
3	1.95±0.18^{b}	2.11±0.28^{ab}	2.83±0.19^{a}	2.60±0.37^{ab}	0.08
7	2.15±0.21^{b}	2.54±0.28^{ab}	3.39±0.33^{a}	2.62±0.37^{ab}	0.05
14	2.62±0.25	3.36±0.41	4.00±0.52	2.90±0.48	0.14

^{a,b} Values within a row with different letters are significantly different

Table 2. Composite milk concentration (mg/l) of α-tocopherol day 1, 7 and 14 postpartum in 36 dairy cows fed daily supplements of no vitamin E (Control), all-rac-α-tocopheryl acetate (SynAc), RRR-α-tocopheryl acetate (NatAc), and RRR-α-tocopherol (NatAlc) from 24 days prepartum to 14 days postpartum.

Day	Control	SynAc	NatAc	NatAlc	P-values
1	5.9±0.7^{ab}	4.7±0.6^{b}	7.2±0.9^{ab}	7.4±0.7^{a}	0.05
7	0.73±0.12	0.85±0.08	1.00±0.13	1.13±0.20	0.21
14	0.67±0.08	0.78±0.09	0.90±0.09	0.77±0.05	0.26

^{a,b}Values within a row with different letters are significantly different

The distribution of stereoisomers of α-tocopherol in plasma and milk from cows in the SynAc group was significantly different from the NatAc, NatAlc and Control groups. RRR-α-tocopherol was the dominant isomer in all blood and milk samples, but blood and milk from cows fed SynAc had a significantly lower proportion of RRR-α-tocopherol compared to cows in the other groups.

Discussion

In the present study, a higher blood concentration of α-tocopherol was detected in periparturient cows supplemented with NatAc compared with the other groups. In milk, the α-tocopherol concentration was higher in colostrum from cows fed NatAlc compared to cows fed SynAc. In control cows and cows supplemented with the natural forms of vitamin E, the proportion of RRR-α-tocopherol in plasma and milk was higher than 98%. In cows fed SynAc, the proportion of the RRR-stereoisomer was significantly lower than in the other groups, but above 85% throughout the study despite the fact that this stereoisomer only made up approximately 35% of the total amount of α-tocopherol fed. This indicates that periparturient cows to a greater extent than humans (Burton *et al.*, 1998) and pigs (Lauridsen *et al*., 2002a,b) have a preferential uptake of RRR-α-tocopherol compared with the other stereoisomers of α-tocopherol. This is most likely due to a strong preferential by the α-TTP to incorporate RRR-α-tocopherol into chylomicrons rather than the other isomers (Burton *et al*., 1998; Brigelius-Flohé and Traber, 1999). Thus, the bioavailability of RRR-α-tocopheryl acetate was higher relative to all-*rac*-α-tocopheryl acetate for periparturient dairy cows than what was determined using the rat fetal-resorption assay (Weiser and Vecchi, 1982).

In previous experiments with beef (Hidiroglou *et al*., 1988) and lambs (Hidiroglou *et al*., 1992) the alcohol form of α-tocopherol was superior to the acetate form, but that was not the case in the present experiment. The reasons for the discrepancy between studies are not clear. However, in contrast to the acetate form, the alcohol forms act as an antioxidant in the feed and the intestine (Halliwell *et al*., 2000), which may result in a loss prior to its absorption by the cow.

The stereoisomer composition in milk was similar to blood within treatment, which suggests that mammary cells do not have a preferential uptake of RRR-stereoisomer, but take up what is available in plasma. These findings agree with studies in pigs (Lauridsen *et al*., 2002b). The placenta transfer of vitamin E is very low in ruminants, as well as in pigs, making colostrum the most important source of vitamin E for the calf (Van Saun *et al.*, 1989). It may be speculated that a high RRR-stereoisomer proportion in colostrum/milk

is better for the calf due to a higher biological activity of this isomer (Burton *et al*., 1998; Lauridsen *et al*., 2002a, b).

In conclusion, the results indicate that daily oral supplementation of dairy cows with RRR-α-tocopheryl acetate (NatAc) in the periparturient period is preferential in terms of increased plasma vitamin E status of cows. Furthermore, the conversion factor between all-*rac*-α-tocopheryl acetate and RRR-α-tocopheryl acetate seems to be higher in cows than the official value of 1.36 determined in rats (United State Pharmacopeia, 1985). Moreover, irrespective of dietary treatment, dairy cows had a high preferential uptake of the RRR-stereoisomer compared to the other stereoisomer forms of α-tocopherol, which lead to an enrichment of the RRR-form of α-tocopherol in milk to more than 85% of the total concentration.

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Comparison of blood and milk non-specific immune parameters in heifers after calving

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Abstract

A practical protocol to study udder immune status in field conditions was planned to assess different non-specific immune parameters in blood and milk samples from dairy heifers during the periparturient period. Five herds located in Northern Italy were selected and overall 39 heifers were enrolled in the trial. The parameters assessed were SCC, N-acetyl-β-glucosaminidase (NAGase), lysozyme, nitric oxide, superoxide dismutase, haptoglobin, respiratory burst, and serum proteins profile. After calving, a significant decrease in blood PMN respiratory burst (RB) and in serum nitric oxide concentration was observed in comparison with the pre-calving values but not with the post-calving samplings. Total proteins, β- and γ-globulins showed a progressive and significant increase in concentration after calving, in comparison with pre-calving values. In milk, we did not observe a significant decrease in RB, post-calving; milk PMN from healthy cows showed low RB level, while the values from infected quarters were significantly higher. Significant differences between healthy and infected animals were also observed for milk NAG, lactoglobulin and albumin. The results of the study confirm that a decrease of immune functions can be observed in commercial dairy herds in the first four weeks after calving. The amplitude of this phenomenon is not common to all animals and all herds, suggesting the possibility to reduce the impairment by improving management and genetic selection.

Keywords: heifers, periparturient period, blood milk, non-specific immunity

Introduction

Most of the information available on cow immune defences are based on blood parameters, while data on milk immune defences are fewer, even if their number is increasing (Paape *et al.*, 2003, Rainard, 2003, Sordillo and Streicher, 2002).

To evaluate the possibility to develop a practical protocol to study the immune status of dairy cows under field conditions, a study was planned assessing several non-specific immune parameters in blood and milk from dairy heifers during the periparturient period.

Materials and methods

Animals and samplings

Five herds located in Northern Italy, were selected. The herds can be considered as representative of Italian dairy herds, all of them are free-stall herds, herd size is in the range 80-350 Holstein cows; total mix ratio, dry-cow therapy and post-dip are regularly

applied. Overall 39 heifers, free from clinical diseases, were enrolled in the trial (7 from herd A, 12 from herd B, 7 from herd C, 4 from herd D, 9 from herd E).

Quarter milk samples (QMS) were collected before milking by an aseptic procedure, starting 7±1 days after calving, then at 14±1, 21±1, 28±1, 45±1, 60±1, and 75±1 days after calving. Blood samples were taken at the same intervals as milk samples, but starting 2 weeks before expected parturition.

Milk bacteriological analysis

At the laboratory, an aliquot (0.01 ml) of each QMS was spread on blood agar plate. Colonies were isolated and identified by proper methods according to National Mastitis Council (N.M.C., 1999). Somatic cells were counted on a Bentley Somacount 150 (Bentley USA). The quarter status was defined following the scheme described by Pyorala (Pyorala, 2003). Samples with SCC ≥200 000 cells/ml were defined as *positive* (IMI); a bacteriologically positive sample with SCC <200 000 cells/ml as *latent IMI*; a bacteriologically negative sample was defined as *healthy* if SCC were <200 000 cells/ml.

Serum, whey and cells isolation

Whey was obtained from skimmed milk by centrifugation at 60 000 x g for 40 min at 4°C, serum was obtained from centrifugation of blood at 1 500 x g for 15 min at room temperature, stored and processed as described for serum.

Polymorphonuclear leukocytes (PMN) were isolated by density gradient separation on Ficoll-Paque Plus (AmeshamBiosciences, Sweden), after centrifugation at 500 x g for 15 min at 4 °C for milk and after hypotonic lysis for blood, following the procedure described by Carlson and Kaneko (Carlson and Kaneko, 1973) and immediately delivered for respiratory burst assay.

Respiratory burst

Respiratory burst (RB) was assessed by luminol-enhanced chemiluminescence. The assay was performed adding 100.000 viable PMN/ml to two wells of a microplate, in one well PMN were stimulated with phorbol myristate acetate, PMA (Sigma-Aldrich, USA) at a concentration of 1 mg/ml, while PMN in the other well were not stimulated (control).

Biochemical assays

Lysozyme was assessed in duplicate by the procedure described by Metcalf *et al*. (Metcalf *et al.*, 1986). The lyses of bacteria was measured by changes in optical density at 450 nm after 2 min on a microplate spectrophotometer (Spectramax 340, Molecular Devices, USA). Concentration of unknown samples, in µg/ml, was calculated by a standard curve obtained by adding standard amount of lysozyme in each plate.

N-Acetyl-b-glucosaminidase (NAG) was assessed in duplicate by the procedure described by Kitchen *et al*. (Kitchen *et al.*, 1978), and expressed as units defined as pmol of 4-methylumbelliferon released per min at 25°C catalysed by 1 µl of milk, on a microplate fluorimeter at 355 ex and 460 em (Ascent, ThemoLabsystem, Finland).

Superoxide dismutase (SOD) was assessed in duplicate with a commercial kit (SOD Assay Kit- WST, Doijndo, Japan) based on the reaction with a tetrazolium salt on a microplate spectrophotometer at 440 nm (Ukeda *et al.*, 2002).

Nitric oxide (NO) was assessed in duplicate by a commercial kit (Nitrite/Nitrate Fluorometric Assay Kit, Cayman, USA), based on the conversion of all the nitrate in nitrite, by nitrite reductase. Then the nitrite fluorescent compound was measured by microplate fluorimeter at 375 ex and 415 em (Misko *et al.*, 1993) .

Haptoglobin (HP) concentration was assessed in duplicate with a commercial kit based on its binding affinity to haemoglobin (Haptoglobin, Tridelta, Ireland) on a microplate spectrophotometer at 630 nm (Saini *et al.*, 1998).

Serum protein electrophoresis

Serum proteins were assessed by agarose gel electrophoresis, applying the standard kit for blood serum proteins (Hydragel 30, Sebia, France), whey proteins were assessed with Hydragel 15 HR (Sebia, France).

Total proteins (TP) were assessed in duplicate by bicinchoninic acid assay (Smith *et al.*, 1985) with a commercial kit (BCA Protein Assay Kit, Piece, USA) on a microplate spectrophotometer at 562 nm.

Statistical analysis

Data were collected in a database, and the differences among sampling times were analysed by the general linear model for repeated measures procedure on SPSS 11.5 (SPSS, 2002). The between-subjects factor was represented by herd (5 levels) and the within-subjects factor was represented by sampling time (7 levels) and the model applied was a full factorial, with polynomial contrasts for within-subjects factor. To compare the differences in immune parameters between healthy and infected cows a general linear model procedure on SAS software was applied with the appropriate error terms for within- and between-subject factors as suggested by Hatcher and Stepanski (1994).

Results

Blood parameters

Only heifers that fulfilled the sampling schedule were included and therefore the study involved 39 heifers from five herds. The heifers considered did not show signs of clinical diseases or acute distressful conditions during the whole follow-up period.

After calving, a significant decrease of respiratory burst could be observed in comparison with the pre-calving values until 75 d, while the differences among the post-calving samplings were significant only in the 21 d sampling (lowest level). Significant decreases in NAGase activity and in NO concentration could also be observed, in comparison with pre-calving values. Moreover, NO showed a progressive and significant decrease after calving with differences among the samplings up to 28 d. No other parameter showed any significant difference among samplings, out of the SOD values in the first two samplings and in the last two samplings after calving in comparison with 14 d pre-calving samples.

Total proteins, albumin, β-globulins (βG) and γ-globulins (γG) showed a progressive and significant increase in concentration after calving, in comparison with pre-calving values. Albumin and βG concentrations were significantly lower at 7 d after calving in comparison with the following samplings, while γG concentrations showed significant differences only in comparison with samples taken at 14 days after calving.

Milk parameters

The first sampling after calving was the highest for SCC, NAG and γG. Serum ALB and lactoferrin (LF) were rather stable, lactoglobulin (LG) and RB values were higher after the first sampling, whereas lactoalbumin (LA) showed an increasing trend as days in milk (DIM) progressed. The statistical analysis for repeated measurements confirmed that mean values at first sampling for NAG, SCC, LG and γG were statistically different from the values observed in the following samplings. Lactoalbumin showed significantly higher values in samples taken from 28 DIM until the end of the follow-up period, when compared with the samples taken in the first 3 weeks of lactation. In all the other cases, some statistically significant changes can be observed, however a trend could not be identified.

Herd influence on non-specific immune status

The analysis of the influence of the herd, sampling and their interaction on the blood immune parameters considered showed that sampling was significant for all parameters out of aptoglobin and lysozyme. Herd showed a significant influence on NO, TP and βG,while their interaction was significant for SOD, NO and RB.

The analysis of the influence of the different factors on milk immune parameters showed that the variance of all parameters, out of RB, was influenced by the interaction of herd and sampling. Herd was statistically significant only for ALB and γG, while sampling was statistically significant for all parameters out of lysozyme, ALB and LF.

Blood and milk comparison

The most challenging results were observed when blood and milk immune parameters were compared in healthy and IMI animals. NAGase in blood showed similar patterns in both samples taken from healthy and unhealthy cows, with a decrease in the first month of lactation. A different pattern was observed in milk. Indeed, milk samples showed an overall decline in NAGase activity as lactation advanced. However, mean levels showed values significantly higher than in negative ones in infected quarters. In the second and third months of lactation, negative quarters showed rather stable values around 40 units, while the levels in IMI quarters progressively declined, even if they were higher than in negative quarters, until the last sample.

Lysozyme showed very large variation in blood, when stratified by mammary gland health status, while milk lysozyme showed very low mean levels independently from sampling and udder health. Gamma-globulins showed a completely different pattern between IMI and negative quarters both in blood and milk. Blood γG was significantly lower at first sampling in IMI quarters, to increase significantly at 45 DIM, when compared with negative quarters. In milk, γG were significantly higher in IMI quarters in comparison to negative quarters, and the values declined in both cases from 7 to 45 DIM. Milk PMN respiratory burst levels were significantly higher in IMI samples when compared with healthy ones, throughout the follow-up period. In the first 3 weeks after calving, RB in blood PMN, when stratified by health status, showed levels lower than in the following samplings.

Milk serum albumin showed a different pattern from the other parameters considered. The mean values were similar during the follow-up period in both healthy and IMI quarters, except for samples taken at 21 and 28 DIM when IMI quarters showed significantly higher levels of ALB in comparison with healthy quarters. This pattern is the opposite of the one observed in blood, where infected cows showed lower ALB concentration in comparison with

healthy cows, with a significant difference at 28 d. During the first month of lactation IMI quarters showed significant lower levels of LG in comparison with negative quarters. In blood, globulins showed lower values in the first month of lactation, both in IMI and negative cows, in comparison to the remaining part of follow-up period.

Discussion

This trial shows that different immune parameters can in field conditions. Particularly, NAGase, respiratory burst, nitric oxide, and serum/whey protein profile showed to be useful markers in assessing immune status of the cow.

In this field study, non-specific humoral defences, such as LF and milk γG, showed higher mean values in the first week after calving, when compared with the following samplings. However, the differences were statistically significant only for γG, as expected, being this latter component one of the most important milk constituent for newborn calf.

SCC and consequently milk NAGase, a lysosomial enzyme, were higher in the first sampling after calving. Unexpectedly, the levels of RB in milk PMN observed in the first two weeks after calving, even if lower, were not statistically different from the values observed in samples taken in the following weeks.

The results of the study confirm that a decrease of immune functions can be observed in commercial dairy herds during the first four weeks after calving in similar but non-equal management conditions. The amplitude of this phenomenon is not common to all animals and all herds, suggesting the possibility to reduce the impairment by improving management and genetic methods. These data suggest that a decrease in non-specific immune defences can be observed during periparturient period at milk level, but in a lesser extent in comparison to what observed at blood level in the same cow (Piccinini *et al.*, 2004). Cellular enzymes and RB showed different values in comparison to blood ones, but a significant decrease in the first samplings after calving could not be demonstrated. The interaction with herds and with IMI prevalence, suggests that udder immune response could be influenced both by cow immune status and by external factors such as pathogens and management. Therefore, the reduction in immune defences, particularly in heifers, is not unavoidable, as already suggested under experimental conditions (Mehrzad *et al.*, 2002), and methods to boost particularly PMN activity should be investigated.

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Influence of resident milk neutrophils chemiluminescence and viability on the severity of bovine coliform mastitis

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Abstract

Few early lactation-related infections in dairy cows are as important as mastitis. This complex inflammatory disease has been the past, current and future focus of many bovine immunologists. Although the impact of blood neutrophils on the pathogenesis of mastitis is clear, the influence of milk neutrophils on the outcome of mastitis is unclear. It has been shown that increased quantity of neutrophils in milk protects udder from invading pathogens. However, little attention has been paid on the qualitative role of milk neutrophils in mastitis severity. We hypothesised that when E. coli invade the udder, the pre-existing milk neutrophils would be critical for mastitis severity. In our study the cows were classified as moderate and severe responders according to milk production loss in the non-infected quarters at post-infection hour (PIH) 48. We observed that higher pre-infection milk neutrophils viability correlated with bacterial clearance, which was accompanied by faster recovery. There was a good correlation between milk neutrophils chemiluminescence and their viability. There was also an inverse relationship between pre-infection milk neutrophils chemiluminescence and colony-forming units at PIH 6. The probability of severe response increased with decreasing pre-infection milk neutrophils chemiluminescence and viability. High pre-infection milk neutrophils chemiluminescence and viability and the immediate increase of milk neutrophils chemiluminescence and viability after infection limited bacterial growth, thereby facilitating recovery of E. coli mastitis in moderate responders. Our study strongly supports the idea that pre-existing milk neutrophils acts as a cellular antibiotic before and during infection, and low milk neutrophils chemiluminescence and viability can be a risk factor for bovine coliform mastitis.

Keywords: chemiluminescence, neutrophil, severity, viability

Introduction

Bovine milk polymorphonuclear neutrophils (PMN) have a potential to produce substantial amount of reactive oxygen species (ROS) to kill engulfed bacteria (Weber *et al.*, 1983; Mehrzad *et al.*, 2001a). Acute *Escherichia coli* (*E. coli*) mastitis in newly calved cows is accompanied by severe clinical symptoms (Hill *et al.*, 1979; Vandeputte-Van Messom *et al.*, 1993). The PMN are pivotal to protect cow's udder from E. coli infection (Kehrli *et al.*, 1994; Paape *et al.*, 2002; Burvenich *et al.*, 2003). Intramammary innate defense against invading pathogens heavily relies on the number of circulating PMN before infection and

their capacity to produce ROS (Heyneman *et al.*, 1990), the rate of PMN diapedesis into the infected quarters (Hill *et al.*, 1979; Vandeputte-Van Messom *et al.*, 1993) and on the activity of bone marrow to provide active PMN (Van Merris *et al.*, 2002; Burvenich *et al.*, 2003). Furthermore, milk PMN viability, which evidently reflects the pathophysiological condition of the mammary gland (Piccinini *et al.*, 1999; Mehrzad *et al.*, 2001a; b), could play a role in udder's innate defense mechanism. PMN ROS quantification after stimulation with soluble agents and/or particles using chemiluminescence (CL) assay is a reproducible technique to investigate the phagocytosis or bactericidal system of PMN (Allen *et al.*, 1972; Piccinini *et al.*, 1999; Mehrzad *et al.*, 2001c). Retrospectively, there is a big gap between the knowledge on bovine blood PMN function versus milk PMN's, and study on milk PMN functions might lead to pinpointing some novel contributing host factors to the severity of coliform mastitis. Furthermore, rarely have the milk PMN viability versus CL been investigated. The milk PMN viability, which represents their quality, could reflect the dynamic of PMN recruitment through blood-milk barrier. Throughout the lactation cycle, milk PMN CL in healthy cows varies from 47 to 78% of their blood counterparts (Mehrzad *et al.*, 2001a); similar variation might exist for milk PMN viability. These variations can be due to the impact of only parturition and lactation (Mehrzad *et al.*, 2001a; Van Oostveldt *et al.*, 2002). Undoubtedly, there is a deep change on milk PMN functions (CL and viability) during mastitis (Mehrzad *et al.*, 2001b). The concomitant assessment of PMN viability and CL in milk would, therefore, provide insight in the cytochemical, cytoskeletal, phagocytosis and bactericidal status of the PMN; this could mimic the most complex part of PMN-E. coli interaction in the udder during phagocytosis. We speculate that the resident milk PMN CL and viability could provide an efficient phagocytosis capacity for the mammary gland at the start of the E. coli infection, and the outcome of mastitis may also link to pre-infection milk PMN CL and viability. The impact of pre-infection milk PMN functions on bactericidal capacity in the gland, and on milk yield (as indicator for mastitis severity) was therefore investigated.

Materials and methods

Animals and experimental procedures

In total one hundred and six clinically healthy Holstein-Friesian cows in their first to third lactation from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected. In the first study, 20 cows were investigated for experimentally induced *Escherichia coli* (E. coli) mastitis to assess the impact of pre-infection milk PMN viability on the E. coli growth in the mammary gland at post infection hours (PIH) 6 and on the milk production (MP) loss at PIH 48; to prepare the inoculum, the bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON) at 37°C. The bacterial suspension, quantification and inoculation were done according to (Mehrzad, 2002). Immediately after the morning milking E. coli mastitis was induced into the left udder half by a single intramammary inoculation of 10 mL of 10^4 cfu of E. coli solution per quarter. In the second study, twenty healthy cows were selected for mastitis induction as explained above; the MP loss of non-infected quarters and cfu of infected quarters were measured at PIH 48 and 6, respectively. Based on their MP loss of non-infected udder halves at PIH 48 of infection compared to the pre-infection MP (Shuster *et al.*, 1996), the cows were divided into two different severity groups: moderate (M) with MP loss <50% and severe (S) with MP loss ≥50%. The relationship between pre-infection milk PMN CL and cfu at PIH 6 was also

examined. To investigate the relationship between milk PMN viability and CL, in a third study 66 healthy cows (22 ± 8 to 215 ± 38 d of lactation) were selected; sterile milk samples (500 mL) were aseptically collected, and the milk PMN viability values were simultaneously compared with their CL values. In all studies, quantification, isolation and preparation of PMN from milk were carried out according to previously described (Mehrzad *et al.*2001a; b; c) methods, which gives 70 - 95% pure PMN.

Chemiluminescence (CL) and viability assays in milk PMN

The CL of milk PMN from *E. coli*-infected and non-infected quarters was performed using luminol-amplified PMA (phorbol 12-myristate, 13-acetate)-and-latex beads (polystyrene 0.76 µm diameter, 4×10^{11} particles/mL; Sigma)-stimulated cellular assay according to (Mehrzad *et al.*, 2001b;c). The viability of isolated milk PMN was determined in duplicate by means of flow cytometry, using propidium iodide exclusion (Mehrzad *et al.*, 2001a). The percentage of PI positive PMN was calculated for measuring necrotic PMN and eventually calculated for the percentage of viable PMN.

Relation between milk PMN Viability and CL

In the third study, to assess the correlation between milk PMN viability and latex-stimulated CL, in non-mastitic cows it was further tested whether there is any connection between milk PMN CL and viability.

Statistical analyses

In the first study, the effect of pre-infection milk PMN viability on E. coli growth at PIH 6 and MP loss at PIH 48 was analyzed using regression analysis and it was tested whether the slope expressing the linear effect of pre-infection milk PMN viability was significantly different from zero. In the second study, the relationship between CL immediately before challenge and reduction of milk production 48 hours after challenge was first studied by a linear regression model, and the null hypothesis of the slope being equal to zero was tested. Alternatively, logistic regression analysis was performed to investigate whether CL immediately before challenge can predict whether a cow will be a S responder (reduction of milk production 48 hours after challenge >50%) or not. Again, the null hypothesis of the slope in the logistic regression model being equal to zero was tested. Furthermore, cfu at 6 hours after infection was linearly regressed on the CL (area under the curve; AUC) immediately before challenge and the null hypothesis of the slope being equal to zero was tested. In the third study, the Spearman correlation coefficient was used to assess the correlation between milk PMN viability and CL. It was further tested whether this correlation coefficient was significantly different from zero.

Results and discussion

The link between resident milk PMN viability and the inhibition of bacterial growth in the milk after induction of E. coli mastitis was evaluated. There was a strong negative correlation between pre-infection milk PMN viability and bacterial growth at PIH 6 ($P<0.01$; Figure 1a). There was also an inverse relationship between pre-infection milk PMN viability and MP loss at PIH 48 ($P<0.05$; Figure 1b). The PMN are both activated and activating cells; they are also the most susceptible cells in milk compartment (Paape *et al.*, 2002; Burvenich

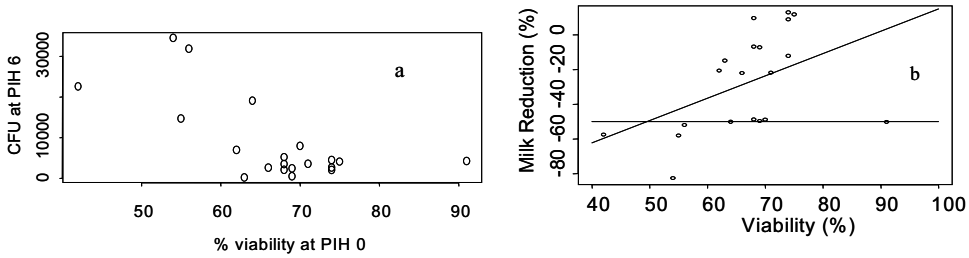

Figure 1. Correlation between pre-infection milk PMN viability and bacterial growth in the milk at PIH 6 (a) and between pre-infection milk PMN viability and milk production loss at PIH 48 (b). This indicates the milk PMN effectiveness towards killing of E. coli and the outcome of mastitis. There is a strong negative correlation between pre-infection milk PMN viability and bacterial growth at PIH 6 and milk production loss (P = 0.0012 and P = 0.041, respectively; n = 20).

et al., 2003). Theoretically, a low viability of resident milk PMN may compromise the total phagocytic and bacteriostatic capacity of teat cistern (Burvenich *et al.*, 2003), thus facilitating E. coli growth, especially during lag phase of the growth. A higher pre-infection milk PMN viability enhances the static phase of the innate defense. Necrotic PMN, as opposed to PMN viability, are not promptly cleared by phagocytes; their chromatin proteins may also boost prolonged inflammation (Scaffidi *et al.*, 2002). Studies reveal that milk samples of a severe coliform mastitic cow do not yield bacterial growth; such samples contain a lot of extra-cellular ROS, autolytic enzymes and many necrotic PMN that cause mammary tissue damage. To shorten inflammatory reactions, improving milk PMN viability immediately before infection can be beneficial, and would prevent extensive mammary epithelial damage. The level of pre-infection CL (AUC) in milk PMN had a significant influence on the severity of mastitis. Both MP loss and the probability of a severe response decreased significantly with increasing values of pre-infection CL (AUC) in milk PMN with both PMA and latex stimulation (Figures 2 and Table 1). Pre-infection milk PMN CL significantly influenced another parameter of mastitis severity, cfu at PIH 6 (Table 2); this inverse relationship was significant both for latex and for PMA stimulated CL.

We found that the extent of MP loss (an index for severity and mammary tissue damage) depends, in part, on the pre-infection milk PMN CL activity: inflammation was less severe with less mammary tissue damage at higher PMN CL activity; this was the case for both phagocytosis and non-pahgocytosis dependent CL. This relationship emphases the pivotal role of the pre-existing milk PMN in the udder's innate defense; this boosted bacteriostatic properties of the gland, enhancing rapid bacterial clearance at PIH 6. Therefore, the impact of milk PMN CL on mastitis severity was crucial; e.g., every unit increase in pre-infection

Table 1. Relationship between milk production loss (linear regression)/severity (logistic regression) and milk PMN CL stimulated with PMA and latex beads during experimentally induced E. coli mastitis. The slope and its standard error (S.E.) are based on the data of 20 cows.

Stimulator	Statistical analyses			
	Slope (S.E.) linear	P-value linear	Slope (S.E.) logistic	P-value logistic
PMA	0.0185(0.0077)	0.029	0.0041(0.0022)	0.048
Latex	0.036(0.014)	0.022	0.0062(0.0031)	0.043

Table 2. Relationship between pre-infection PMA- and -latex- stimulated milk PMN CL and cfu at PIH 6 (linear regression) during experimentally induced E. coli mastitis. The slope and its standard error (S. E.) are based on the data of 20 cows.

Stimulator	Statistical analyses	
	Slope (S.E.)	P-value
PMA	-7.91 (2.63)	0.0013
Latex	-16.78 (4.5)	0.0001

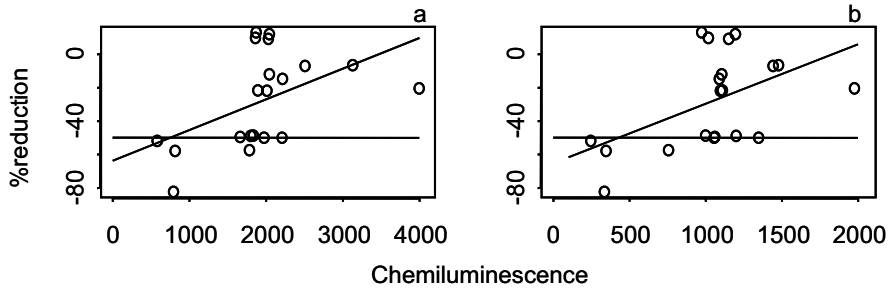

Figure 2. Relationship between PMA (a)-and-latex (b) stimulated milk PMN CL (AUC of 1000 viable PMN) before the induction of E. coli infection and the milk production loss at PIH 48 (n=20). Circles represent individual cows through which the regression line was fitted. The horizontal line corresponds to the 50% milk production loss (severity threshold).

milk PMN CL (AUC) resulted in roughly 20 mL gain in MP loss at PIH 48, which coincided with a decrease of 0.5% in the probability of developing severe E. coli mastitis (see Table 2 and Figure 2). This is consistent with the finding of Zecconi *et al.*, (1999).

There was a significant positive correlation between milk PMN viability and latex-stimulated CL ($\rho = 0.94$; $p = 0.0001$; Figure 3). The two parameters, viability and CL, in milk were closely interrelated. During early lactation, the milk PMN CL was lower and their

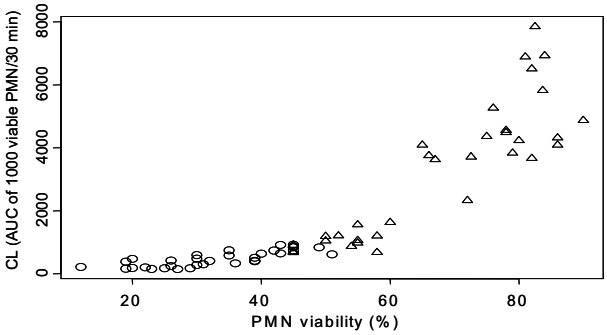

Figure 3. Correlation coefficient between milk PMN latex-stimulated CL and viability. A significant correlation between the PMN CL and their viability ($\rho = 0.94$; $p = 0.0001$; $n = 66$) during physiological conditions. The low values of CL and viability were fully related to early lactating (circles) cows, compared to mid lactating (triangles) cows.

survival in milk was also lower; however, these values were significantly high during mid lactation. Prevention of milk PMN necrosis during early lactation can be essential and may boost bactericidal capacity of PMN in the gland and diminish the severity of E. coli mastitis in dairy cows.

In conclusion, high milk PMN viability and CL at the start of the infection are crucial for phagocytosis and killing of pathogens in the infected quarters. The pre-existing milk PMN is a strong parameter for alleviating severity of E. coli mastitis. The present study indicates that PMN viability and CL in milk are inextricably interrelated. We also demonstrate that the pre-existing milk PMN viability and CL are involved in the underlying mechanism of the udder's innate defense against bacteria. It is conceivable that the resident milk PMN are strong parameter for alleviating the mastitis severity. To shorten inflammatory reactions in the udder, boosting resident milk PMN intracellular ROS and viability would be beneficial for dairy cows.

Acknowledgements

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Potential association of polymorphisms in the bovine CXCR2 gene with neutrophil survival and killing ability

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Abstract

Recent research in our lab has demonstrated a significant association between the incidence of subclinical mastitis and specific polymorphisms of the CXCR2 gene in Holstein dairy cows. This gene encodes the interleukin-8 (IL-8) receptor, a key regulator of neutrophil migration, killing and survival. Because of the importance of this gene in neutrophil regulation, we hypothesized that differences in neutrophil killing and survival may exist among the CXCR2 genotypes and potentially contribute to the observed variation in intramammary infections. To test this hypothesis, neutrophils were isolated from cows representing each CXCR2+777 genotype (GG, GC, CC; n = 10 or 20 each) and tested for reactive oxygen species (ROS) generation, bactericidal activity, and suppression of apoptosis. A significant reduction in neutrophil ROS generation in response to phorbol-13-myristate-12 acetate (PMA) was observed in cows with a CC genotype when compared to those with a GG genotype. However, no differences in bactericidal activity were observed. In contrast to the observed ROS and bactericidal activities, a significant increase in survival was observed for neutrophils from cows with CC genotype when compared to those with a GG genotype in response to IL-8, but not dexamethasone. The functional activity of neutrophils from cows heterozygous for this polymorphism was intermediate between those with homozygous genotypes. In summary, our preliminary results indicate that neutrophils from Holstein cows with different CXCR2 genotypes vary in their ability to produce ROS and suppress apoptosis. These differences have the potential to influence overall neutrophil function and may partially explain the variation observed with respect to mastitis in vivo. These results provide a solid foundation for future research aimed at better understanding the basic differences between dairy cows genetically more or less susceptible to mastitis and has the potential to provide novel preventive and therapeutic measures against inflammatory diseases such as mastitis.

Keywords: neutrophil, reactive oxygen species, bactericidal, apoptosis, polymorphisms

Introduction

Effective elimination of bacterial infections such as mastitis and bacterial pneumonia in dairy cattle requires neutrophil migration to the site of infection, the generation and release of bactericidal agents, as well as programmed cell death in order to minimize tissue damage (Hill 1981; Kehrli *et al*. 1989; Burg and Pillinger 2001). The effectiveness of this response varies with an animals' genetic background and subsequently influences its ability to resist disease. Recent research in our lab has demonstrated a significant association between the incidence of subclinical intramammary infections and polymorphisms in the

CXCR2 gene in Holstein dairy cows (Youngerman *et al*. 2004). More specifically, cattle with a CC genotype at nucleotide position +777 (Genbank accession number U19947) had a greater incidence (~35%) of subclinical mastitis than those with a GG genotype at this position (~20%). The polymorphism at this position, +777, causes an amino acid change from histidine to glutamine within the third intracellular loop of the receptor where G-protein binding occurs (Damaj *et al*. 1996; Youngerman *et al*. 2004). Thus this amino acid change has the potential to influence neutrophil function and subsequent disease resistance because the product of the CXCR2 gene is a receptor for interleukin-8 (IL-8) - a key regulator of neutrophil migration, killing, and survival (Chertov *et al*. 2000; Glynn *et al*. 2002). Because of the importance of IL-8 and its receptor CXCR2 in neutrophil regulation, we hypothesized that differences in neutrophil killing and survival may exist among the CXCR2 genotypes and potentially contribute to the observed variation in intramammary infections. To test this hypothesis, neutrophils were isolated from Holstein cows representing each CXCR2+777 genotype (GG, GC, CC; n = 10 or 20 each) and tested for reactive oxygen species (ROS) generation, bactericidal activity, and suppression of apoptosis.

Results

Reactive oxygen species generation

One of the means by which neutrophils kill invading organisms is the generation of free radicals or reactive oxygen species (ROS) through activation of NADPH oxidase and myeloperoxidase (Klebanoff 1992). A common means of assessing the ability of neutrophils to generate ROS is through stimulation of the protein kinase C (PKC) signal transduction pathway by treatment of neutrophils with phorbol-12-myristate-13 acetate (PMA) (van Eeden *et al*. 1999). Therefore, to determine if there were any differences in the ability of neutrophils collected from cattle with different genotypes to generate ROS, isolated neutrophils were stimulated with 0, 10, or 100 nM PMA for 15 min and evaluated for ROS levels by determining the fluorescence of dihydrorhodamine, a dye that fluoresces only after it is oxidized. Approximately 100% of the cells stimulated with PMA were capable of generating ROS, regardless of the cows' genotype. However, comparison of the median level of fluorescent intensity following stimulation with 10 nM PMA revealed that neutrophils collected from cattle with a CC genotype produced significantly less ROS than neutrophils from cows with a GG genotype ($P < 0.05$; Figure 1). A similar trend was observed following stimulation with 100 nM PMA ($P=0.16$). These findings suggest that neutrophils are capable of generating ROS in response to PMA; however, the overall effectiveness differs with respect to a cow's genotype.

Bactericidal killing

The observed variation in ROS generation may alter the ability of neutrophils from cows with different genotypes to kill bacteria. Therefore, the ability of neutrophils collected from cows with different genotypes to kill either unopsonized or opsonized *Streptococcus uberis* and *Staphylococcus aureus* was evaluated (Aarestrup *et al*. 1994). No differences were observed in the ability of neutrophils to kill either of these types of bacteria (Table 1). This finding suggests that there is no difference in the baseline capability of these cells to kill Gram positive organisms such as *Strep uberis* and *Staph aureus*. This was somewhat unexpected in light of the variation observed in ROS generation. However, there are a variety

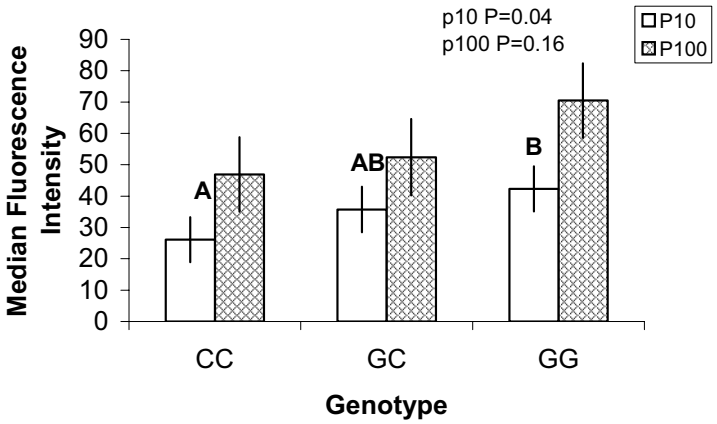

Figure 1. Neutrophil generation of reactive oxygen species following stimulation with PMA. Neutrophils from cattle with different genotypes were activated with 10 nM PMA (P10) and 100 nM PMA (P100). Reactive oxygen species were detected by increasing fluorescence of dihydrorhodamine, an oxidant sensitive dye. Data presented as the mean of the median fluorescence intensity ± SE for 20 cows within each genotype. [A,B] Different letters represent significant differences between the means at P<0.05.

Table 1. Bactericidal activity[1] of neutrophils isolated from Holstein cows with different CXCR2 genotypes[2].

Genotype	% bacteria killed (± SEM)			
	Strep. uberis non-opsonized	*Strep uberis* opsonized	*Staph aureus* non-opsonized	*Staph aureus* opsonized
GG	23.7 ± 2.6	53.7 ± 3.5	5.8 ± 1.9	29.9 ± 5.8
GC	21.5 ± 2.6	54.4 ± 3.6	4.5 ± 1.9	31.2 ± 5.8
CC	19.3 ± 2.6	51.1 ± 3.4	6.6 ± 1.9	32.4 ± 5.8

[1]Ratio of bacteria:neutrophils = 10:1

[2]Ten cows with each genotype were evaluated

of factors that could contribute to the lack of differences observed with respect to bactericidal activity: an alternative bactericidal pathway compensates for reduced levels of ROS, bacterial numbers (10 bacteria per neutrophil) were low enough that the ROS generated were sufficient for killing, or neutrophils were not 'primed' for killing. This last possibility may have considerable impact as in vivo neutrophils are 'primed' for killing through pre-activation by chemokines such as IL-8 (Mitchell *et al*. 2003). Future experiments will begin to evaluate these possibilities.

Neutrophil survival from spontaneous apoptosis

Neutrophils normally begin apoptosis within hours of leaving the bone marrow (Lee *et al*. 1993). Therefore, successful resolution of bacterial infections also requires neutrophils to simply survive until they reach the site of infection. Interleukin-8 plays a key role in this process by promoting neutrophil survival. Based upon our observations that neutrophil migration, adhesion molecule expression, and ROS generation were impaired in cattle with a CC genotype relative to those with a GG genotype, we expected neutrophil survival to be impaired in cows with a CC genotype (poster presentation at this meeting). However, we

observed the opposite. Neutrophils from cows with a CC genotype survived better than neutrophils from cows with a GG genotype after stimulation with IL-8 in vitro (Figure 2). No significant differences in survival were observed following treatment with dexamethasone (data not shown), suggesting this effect of genotype was related to IL-8 or events downstream in that signaling pathway.

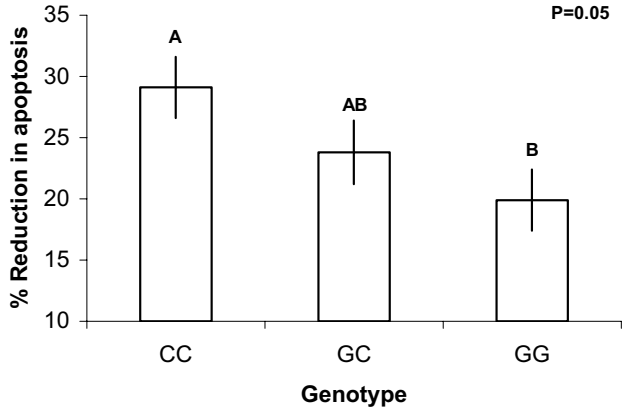

Figure 2. Neutrophil survival from spontaneous apoptosis following incubation with IL-8. Neutrophils from cattle with different genotypes were incubated with HBSS or IL-8 for 24 hours at 37°C. Apoptosis was determined by fluorescent detection of annexin V. The percent reduction in apoptosis (annexin V staining) was relative to control samples that did not receive IL-8. Data presented as the mean reduction in apoptosis ± SE for 10 cows within each genotype. ^{A,B} *Different letters represent significant differences between the means at P<0.05.*

Conclusions

In summary, our preliminary results indicate that neutrophils from Holstein cows with different CXCR2 genotypes vary in their ability to generate ROS and suppress apoptosis. These findings offer a possible functional mechanism for differences in subclinical mastitis observed among cattle with different genotypes at CXCR2 +777. Future research aimed at better understanding these basic differences has the potential to provide novel preventive and therapeutic measures against inflammatory diseases such as mastitis.

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Differential cell count and interdependence of udder quarters

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Abstract

For many years, there have been reports of indicators for the interdependence of udder quarters within one cow, i.e. compensation of milk yield after loss of one quarter. Despite these reports many studies are nevertheless still based on the hypothesis of quarter independence when using within cow controls. The possibility of communication between udder quarters in an immunological crisis (i.e. mastitis) has not yet been systematically assessed.

The aim of this study was to evaluate the interdependence of udder quarters by microscopic and flow cytometric cell differentiation in milk in light of the fact that somatic cells are agents of the immune system.

The physiological reference comprised 52 quarter milk samples from 13 normally secreting cows monitored from calving until the time of sampling. The samples from 26 mastitic cows were grouped according to their somatic cell counts.

After cell isolation, differential cell counts were conducted both microscopically and by flow cytometric analysis. The latter detected antigens on lymphocytes and on polymorphonuclear neutrophil granulocytes (PMN).

The percentages of lymphocytes (25%), CD4-positive cells (20%), and macrophages were significantly ($P < 0.0001$) higher in the reference group than in all others. On the other hand, the percentages of PMN (microscopy, 34%) and Bo116-positive cells (flow cytometry, 5%) were significantly lower ($P < 0.0001$) in the reference group than in all others except one.

These data indicate that the infection and inflammation of one quarter do indeed influence the differential cell counts of the other quarters within the same udder. Therefore, the quarters of one mammary gland cannot be regarded as four isolated units since they communicate with each other and are thus interdependent.

Keywords: udder quarter interdependence, somatic cell count, differential cell count, flow cytometry

Introduction

The anatomical structure of the bovine udder forms four mammary glands divided by septa of connective tissue. Furthermore, it is often the case that only a single udder quarter becomes clinically ill. These facts have probably led to the assumption that each udder

quarter acts as an independent unit. Various udder health parameters are assessed in research and in everyday veterinary practice on the basis of this assumption. However, there is evidence for the interdependence of udder quarters. It has for example been reported that the loss of milk production in a mastitic quarter is compensated for by the other, healthy quarters (Woolford, 1985; Hamann and Reichmuth, 1990). Furthermore, this compensation is accompanied by a collateral growth of glandular tissue (Knight and Peaker, 1991). An early indication that inflammation-related parameters are also subject to interdependence was reported by Hamann and Gyodi (1994), who found that leaving two udder quarters unmilked led to an increase in the somatic cell count (SCC) in the milk of the other two quarters. In a more recent study, Hamann *et al*. (2002) observed a significant increase in various inflammatory parameters at the onset of mastitis not only in the newly affected quarters but also in the (apparently unaffected) neighbouring quarters. It has been firmly established that leukocytes, especially polymorphonuclear granulocytes (PMN) play a crucial role in the udder defence system (Paape *et al*., 1979, Burvenich *et al*., 1995). There is thus good reason to believe that determination of the differential cell count might help to reveal the interaction between udder quarters and so lead to a better understanding of mammary gland immunity and ultimately to the development of modern strategies for the prevention and treatment of mastitis.

Materials and methods

Animals and milk sampling

Thirty-nine high-yielding primi- and multiparous cows in midlactation were sampled either after cytobacteriological screening according to the recommendations of the German Veterinary Society (DVG, Hamann and Fehlings, 2002) or as part of continuous surveillance at three-week intervals since calving. Quarters were sampled during morning milking according to National Mastitis Council standards (NMC, 1999). After collection of 10 ml of milk in sterile glass tubes (quarter foremilk sample) for cytobacteriological analysis, 500 ml were then hand milked into a glass bottle (milk sample).

Cytobacteriological analysis

The quarter foremilk sample was used for the determination of SCC with a Fossomatic 360 disk cytometer (Foss Electric, Hillrod, Denmark) according to International Dairy Federation standards (IDF, 1984); the microbiological status was evaluated according to National Mastitis Council standards (NMC, 1999).

N-acetyl-β-D-glucosaminidase activity (NAGase)

NAGase was measured fluoroscence spectroscopically in nmol x ml^{-1} x min^{-1} with the method described by Kitchen *et al*. (1978).

Cell isolation

The milk sample was diluted 1:2 with phosphate-buffered saline (PBS) and centrifuged for 5 min at 1000 *g*. The cell pellet was washed three times in PBS and the cell suspension adjusted to a concentration of 4 x 10^6 cells/ml. This cell suspension was used for both microscopic and flow cytometric cell differentiation.

Differential cell count

As described elsewhere (Schroeder and Hamann, 2005), the smears were prepared using a modified coffee grinder. For microscopic evaluation (magnification x1000, oil immersion), the smears were stained with Hemacolor (Merck Eurolab GmbH, Darmstadt, Germany) before 100 cells per smear were classified as PMN, lymphocytes (LYM) or macrophages (MAC).

Monoclonal antibodies

The following antibodies were used for flow cytometric analysis

CC8[a)] against CD4 and Bo116[b)] against an as yet unclassified structure on PMN.

The secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated F(ab')2 rabbit anti-mouse IgG, crossreacting with IgM[a)]

a. Serotec GmbH, Düsseldorf, Germany
b. Immunology Unit, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

Statistics

The data were grouped according to the udder health status of the cows as indicated by the quarter foremilk samples (Table 1). Statistical tests were performed with the SAS 8e 2002 software (SAS Institute GmbH, Heidelberg, Germany). The data were tested on a normal distribution and a two-factor analysis of variance was conducted. The levels of significance used were * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

Table 1. Definition of udder health groups.

Cows						
	All quarters	At least one quarter		At least one quarter		
SCC*	<100	100-400		> 400		
Group	A[1]	B		C		
n	13	9		17		
Quarters						
SCC*	<100	<100	100-400	<100	100-400	>400
Group	A	$B_{< 100}$	$B_{100-400}$	$C_{< 100}$	$C_{100-400}$	$C_{> 400}$
n	50	21	15	15	20	33

*SCC = somatic cell count $x10^3$/ml milk; [1]milk samples of group A were free of mastitis pathogens.

Results

Even though the same definition was applied to three groups on the quarter level (A, $B_{<100,}$ $C_{<100}$), the SCC and the NAGase activity in healthy quarters of mastitic cows ($B_{<100}$ and $C_{<100}$) tended to be higher than in the reference (A).

The significant difference in the SCC between the reference (A) and the healthy quarters of cows with severe mastitis ($C_{< 100}$) is confirmed statistically ($P < 0.05$). The difference becomes even more distinct in view of the differential cell count, where a marked difference is seen between the reference group A and all other udder health categories. In group A the lymphocyte content was significantly highest (25%), while the proportion of $CD4^+$

lymphocytes (T-helper lymphocytes) was significantly lowest (20%). On the other hand, group A contained the fewest PMN (34%). Furthermore, only 5% of the cells in the reference expressed Bo116. Even in the healthy udder quarters of mastitic cows ($B_{<100}$ and $C_{<100}$) there was a drop in the number of lymphocytes and a rise in the number of PMN and in the expression of CD4 and Bo116; this development was most evident in the mastitic udder quarters ($B_{100 - 400,}$ $C_{100 - 400}$ and $C_{>400}$). Table 2 shows the differential cell counts in detail.

Discussion

The SCC of the healthy udder quarters of cows with mastitis ($B_{<100}$ and $C_{<100}$) remained below the physiological threshold of 100,000 cells per ml as defined by the German Veterinary Society (DVG, 1994). It was in fact even lower than 50,000 cells per ml, which is well within the physiological range described by Doggweiler and Hess (1983). However, the SCC was slightly higher in the healthy quarters of mastitic cows than in the reference (A). Experimental udder infections with *Staphylococcus (S.) aureus* or *Escherichia coli* also led to a rise in SCC in the adacent quarters (Wever and Emanuelson, 1989; Burvenich *et al.*, 1994; Leitner *et al.*, 1995).

The pattern of NAGase activity was similar to that of SCC, i.e. the lowest values were measured in group A, while here, too, none of the values in the non-inflamed udder quarters (A, $B_{<100}$ and $C_{<100}$) were above the physiological range of 0.31 defined by Grabowski (2000). These findings confirm the results of Schüttel (1999) and Ebeling *et al.* (2001), who also found parallel reactions in healthy and mastitic quarters within a single cow, again on different levels. A similar development was observed not only for SCC and NAGase activity,

Table 2. Means and standard deviations of somatic and differential cell count, NAGase activity and expression of CD4 and Bo116.

	A^1	$B_{<100}{}^1$	$C_{<100}{}^1$	$B_{100 - 400}{}^1$	$C_{100 - 400}{}^1$	$C_{>400}{}^1$
log SCC/ml	4.35^{a}	$4.45^{a,b}$	4.54^{b}	5.36^{c}	5.36^{c}	5.89^{d}
	± 0.27	± 0.32	± 0.29	± 0.13	± 0.17	± 0.24
NAGase[2]	0.24^{a}	0.37^{a}	0.25^{a}	0.56^{b}	0.59^{b}	0.68^{b}
	± 0.15	± 0.25	± 0.22	± 0.22	± 0.39	± 0.32
% PMN[5]	33.03^{a}	$43.15^{a,b}$	$66.42^{b,c}$	$68.18^{c,d}$	$67.00^{c,d}$	79.91^{d}
	± 20.17	± 21.84	± 17.35	± 14.50	± 26.20	± 16.07
% LYM[4]	24.82^{a}	19.20^{b}	$11.23^{b,d}$	$7.20^{c,d,e}$	$11.05^{b,d,e}$	$6.33^{c,e}$
	± 15.35	± 9.75	± 10.99	± 3.00	± 10.13	± 9.42
% MAC[3]	39.05^{a}	34.95^{a}	19.84^{b}	24.53^{b}	21.75^{b}	12.94^{b}
	± 17.60	± 18.54	± 8.45	± 14.55	± 16.65	± 8.36
% CD4	20.43^{a}	28.77^{b}	36.80^{c}	$37.90^{c,d}$	$32.94^{b,c,d}$	33.18^{d}
	± 9.05	± 07.09	± 10.57	± 11.71	± 7.40	± 7.27
% Bo116	5.29^{a}	$10.13^{a,b}$	12.61^{b}	23.64^{c}	20.07^{c}	24.23^{c}
	± 5.36	± 7.50	± 11.53	± 8.45	± 10.86q	± 13.83

[1]Definitions: see Table 1; different superscript letters mark significant ($P < 0.05$) differences;

[2]measured in nmol/ml^{-1} x min^{-1}, shown as log_{10}; [3]MAC = macrophages; [4]LYM = lymphocytes;

[5]PMN = polymorphonuclear neutrophils

but also for electrical conductivity and galactose concentration in a study of mastitis caused by natural infections with various pathogens (Hamann *et al.*, 2002).

The tendencies observed here for the established parameters of udder health were confirmed by the development of the differential cell count. The pathological alterations in the differential cell count as well as those in the expression of CD4 and Bo116 were statistically significant in groups $B_{<100}$ and $C_{< 100}$. Although the striking increase in PMN in infected quarters within hours after infection has been well documented (Paape, 1979), neighbouring quarters have been investigated in only a single study, in which, in contrast to our own findings, they were found to be unaffected (Wever and Emanuelson, 1989).

The expression of CD4 on lymphocytes has already been shown to be a sensitive indicator of udder health. Physiologically, between 8 % and 33 % of milk lymphocytes are T-helper cells (Park *et al.*, 1992, Riollet *et al.*, 2001). In udder quarters infected experimentally with *S. aureus,* the number of $CD4^+$ lymphocytes and density of CD4 reacted even when there was no sign of infection according to SCC (Rivas *et al.*, 2000). The rise in T-helper cells due to mastitis was confirmed in another study (Taylor *et al.*, 1997), which also showed that infections with *Escherichia coli* lead to a greater increase in $CD4^+$ lymphocytes than streptococcal infections. No data have yet been published regarding the effect of mastitic quarters on the expression of CD4 on milk lymphocytes of an adjacent healthy mammary gland.

The results of our study can only be explained by the impact of diseased quarters on healthy quarters of the same mammary gland, and the force of this impact is greater than the well known compensation by increased milk production and auxiliary growth of mammary gland tissue documented in various studies (Woolford, 1985; Hamann and Reichmuth, 1990, Knight and Peaker, 1991). Our findings thus contradict the results of Wever and Emanuelson (1989), who found no evidence of the interdependence of udder quarters. The lack of such evidence in that study may have been due to the fact that the healthy reference used there would have been classified in our study as pathologically affected. It must be noted that we also did not find significantly different differential cell counts indicating different levels of pathology. The interdependence of udder quarters may be the reason why Emanuelson and Wever (1989) concluded that the differential cell count was a less suitable tool for the identification of infected udder quarters than the SCC.

Because the differential cell count indicates an interaction between the quarters of a single udder, we conclude that it is a more sensitive parameter for the detection of mastitis than SCC or NAGase activity. Due to this interaction, mastitis provokes changes in the milk constituents of the cow's healthy quarters, a fact which should be kept in mind in the development of online techniques for monitoring udder health.

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Response of rhlf-transgenic dairy cows to expermentally induced *escherichia coli* mastitis

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Abstract

Lactoferrin (Lf), an antibacterial glycoprotein, contributes to the defence mechanisms of the mammary gland. Generating recombinant human Lf (rhLf) transgenic dairy cows expressing high concentrations of rhLf (mean 2.9 mg/ml) in the milk, could increase their resistance to intramammary infections. The aim of this study was to investigate the response of rhLf-transgenic dairy cows to experimental *Escherichia coli* mastitis. Seven first-calving, early-lactating Holstein-Friesian cows expressing rhLf in their milk and six normal cows were used. One udder quarter of each cow was inoculated with 1700 cfu of *E. coli in vitro* susceptible to Lf. Clinical signs were recorded during the experimental period, and human and bovine Lf concentrations, bacterial counts and SCC were determined from milk and acute phase proteins from serum and milk. All cows became infected and developed clinical mastitis, which was mostly moderate in transgenic cows but more severe in control cows. Systemic signs, serum amyloid A, haptoglobin, cortisol and clinical recovery rate significantly differed between the groups. We conclude that it is possible to augment the nonspecific defence mechanism in the mammary gland by the endotoxin neutralizing effect of Lf, and to decrease the severity of the inflammation and thus to enhance the recovery from *E. coli* mastitis.

Keywords: transgenic cows, recombinant human lactoferrin, bovine lactoferrin, *Escherichia coli*

Introduction

Lactoferrin (Lf), an iron-binding protein, is found in the secondary granules of the polymorphonuclear leucocytes and in mucosal secretions, such as milk. Lf has a broad spectrum of antimicrobial properties and is widely considered to be important for the non-specific host defence on the mucosal surfaces (Brock 2002). The bacteriostatic effect of Lf is mainly based on its ability to sequester iron (Weinberg 1978). Bovine Lf has shown marked bacteriostatic activity against coliform bacteria (Bishop *et al.* 1976). The iron-independent effect of Lf is binding lipopolysaccharide (LPS) of the cell wall of *E. coli* and other Gram-negative bacteria (Appelmelk *et al.* 1994). The Lf content is high in the colostrum and dry-period secretion. In lactating healthy cows, bLf concentration in the milk varies from 0.02 to 0.45 mg/ml. In mastitis, the concentration of bLf increases.

The coliform mastitis, especially in early lactating dairy cows, is often associated with severe clinical signs, serious tissue damage and losses in milk yield. Transgenesis has been

proposed as a potential approach to increase the efficiency of host defence mechanisms of the mammary gland against infections. The first recombinant human lactoferrin (rhLf) bull was produced by Pharming Group NV in The Netherlands in 1990. The hLf gene was under control of the bovine αS1-casein promoter of the udder. Transgenic cattle were produced by microinjection (Brink *et al.* 2000). The aim of the present study was to investigate the mastitis resistance of rhLf- transgenic cows in experimentally induced *E. coli* mastitis.

Material and methods

Animals and experimental design

Seven primiparous, early-lactating Holstein-Friesian transgenic cows which expressed rhLF in milk, were used as experimental animals. The mean concentration of rhLf in milk of the cows was 2.9 mg/ml (Hyvönen *et al.* 2003). The control group consisted of six normal primiparous, early-lactating Holstein-Friesian dairy cows. The experimental cows were on the average 14 days from parturition and the control group 20 days. All cows were clinically healthy in the beginning of the experiment. The mean basic level of somatic cell count (SCC) in the milk was 34 200/ml in the transgenic cows and 99 600/ml in the control cows. One cow from the transgenic group and one from the control group were excluded from the trial because of abnormally high pre-trial values of acute phase proteins which could indicate some concomitant disease. One udder quarter of each cow was infused via the teat canal with *E. coli* FT238 (mean dose 1700 cfu), isolated from clinical mastitis and *in vitro* sensitive to bLf. The Ethics Committees of University of Kuopio and University of Helsinki had approved the study protocol and The Board for Gene Technology in Finland the use of transgenic animals.

Blood and milk samples

Blood and milk samples from the challenged and contralateral quarter were collected 12 hours before and every four hours post challenge (PC) during the first 24 hours, thereafter daily during the first week and finally 14 days after the challenge. Serum amyloid-A (SAA), haptoglobin (Hp) and cortisol were determined from serum. Human and bovine Lf, bacterial count, SCC, SAA and Hp were determined from milk.

Clinical observations

Systemic and local clinical signs were monitored throughout the experiment and were scored on a three point scale (1= no signs to 3= severe signs) using also half numbers (Pyörälä *et al.* 1994). Cows showing average scores ≤1.5 were recorded as having mild mastitis, those with scores >1.5 but ≤2.5 as having moderate mastitis and those with scores from >2.5 to 3 as having severe mastitis.

Analytical and statistical methods

Milk bacterial counts, SCC and hLf were determined as described earlier (Pyörälä *et al.* 1994, van Berkel *et al.* 1996). bLf was measured using Bovine Lactoferrin ELISA Quantitation KIT (Bethyl Laboratorioes, Inc. Montgomery, USA). Concentration of SAA and Hp in serum and milk were determined as described earlier (Hirvonen *et al.* 1999) and cortisol in serum by a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation, Helsinki, Finland. The effects of time post-challenge on concentrations of measured parameters and

clinical signs were analyzed statistically using mixed-model ANOVA (SPSS 11.0, SPSS Inc., Chicago, IL).

Results

All cows became infected and developed clinical mastitis within 8-12 hours after inoculation. The clinical response was seen 4 hours earlier in the control cows, which developed systemic signs 8 hours after challenge and started to recover about 4-8 hours later than the transgenic group (Figure 1a). This difference was significantly different ($p=0.008$). Systemic signs of all cows in the transgenic group returned to normal by 24 hours, while the recovery of the control cows was slower and lasted over 48 hours. Transgenic cows developed significantly less severe clinical signs compared with the normal cows ($p=0.020$). In the local signs no statistically significant differences between groups were found, but a trend towards a milder reaction was seen in the transgenic cows.

Expression of rhLf remained quite constant during the experiment (Figure 1b). The mean concentrations of bLf in the milk of challenged quarter of the transgenic cows is presented in Figure 1b. In the milk of the control quarters, mean concentrations of bLf were slightly elevated and peaked at 36-40 h PC. Bacterial counts in the milk of the challenged quarter peaked in both groups at 8 hours PC and bacteria were eliminated within 3.5 days PC (Figure 1c). No significant differences were observed. Milk SCC and SAA did not significantly differ between groups. Concentration of SAA in the serum was significantly lower in the transgenic group ($p=0.006$) and returned to basic levels within 7 days ($p=0.059$) (Figure 1d). In concentrations of Hp in the serum the same trend was observed, but the difference was significant only at 8 and 168 hours PC ($p=0.035$, $p=0.029$) (Figure 1e). All cows responded to the challenge with an increased serum cortisol concentrations (Figure 1f). The difference of serum cortisol concentrations between the groups was significant at 8, 36 and 168 hours ($p=0.003$, $p=0.031$, $p=0.048$).

Discussion

This is the first report to describe experimentally induced *E. coli* mastitis in transgenic cows expressing rhLf in their milk. The same approach has previously been tested by Kerr *et al.* (2001) in a study on mice, where mice expressing lysostaphin in their milk were found to be more resistant against intramammary *Staphylococcus aureus* infection than normal mice. Our aim was to test if it is possible to increase the resistance of dairy cows against mastitis by improving the defense of the mammary gland by rhLf. All experimental and control animals developed clinical mastitis, and bacterial counts and elimination of bacteria were similar in both groups, but the systemic signs of the transgenic group were less severe and they recovered faster than the normal cows. Lf-transgenesis thus did not protect the cows from the infection but the reaction was less severe. The rhLf concentrations in milk of transgenic cows have been reported to be constant during lactation (mean 2.9 mg/ml) as they were during this experiment (mean 2.63 mg/ml). The complete sequences for bovine and human lactoferrin show that bLf is homologous to other lactoferrins, and the homology at the protein level of hLf and bLf is 69 % (Pierce *et al.* 1991). rhLf in milk of transgenic cows and natural hLf are structurally and functionally highly similar *in vitro* (van Berkel *et al.* 2002).

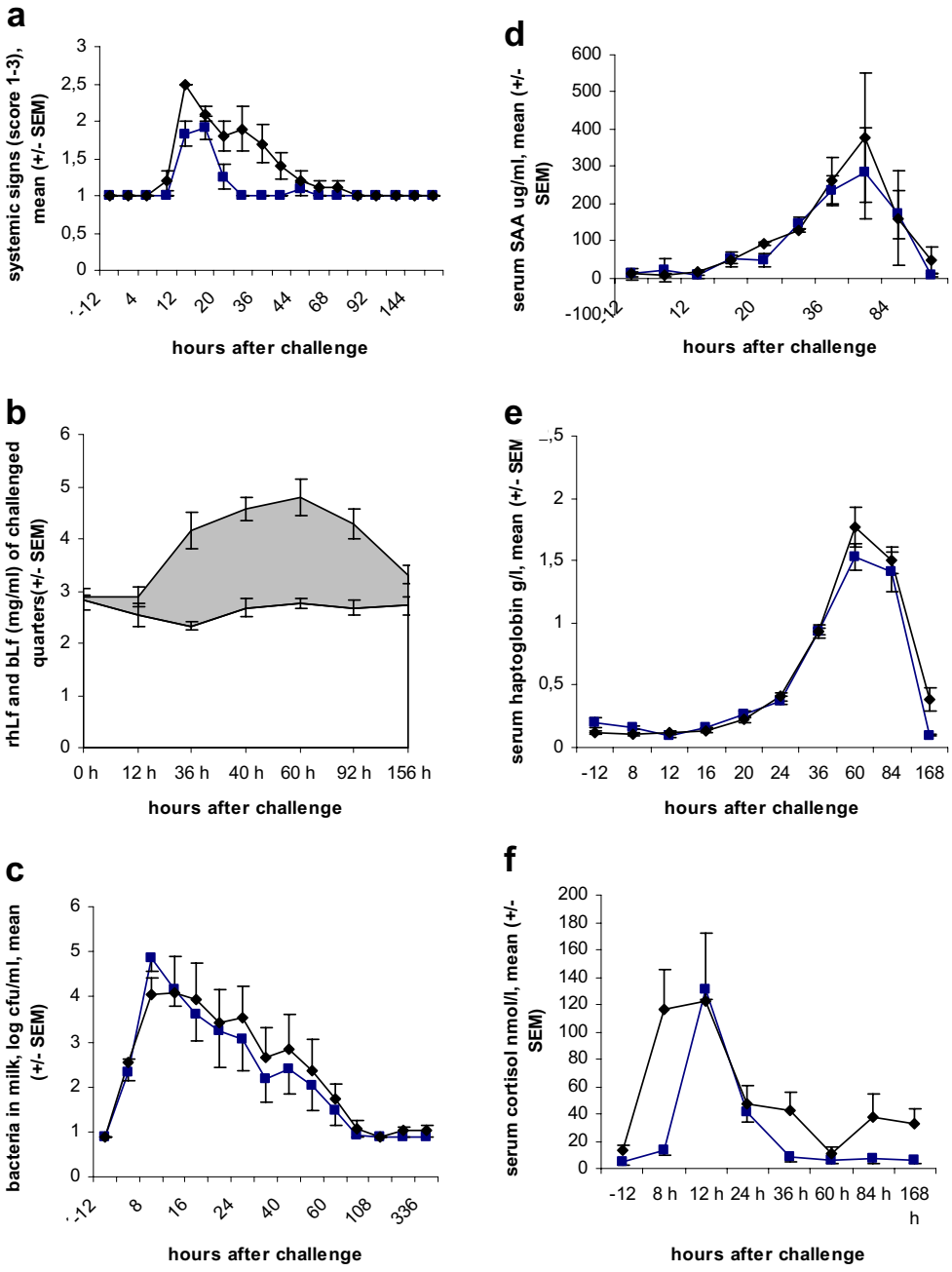

Figure 1. a) Changes in the response of systemic signs of the transgenic (■) and control (♦) cows after an intramammary administration of 1700 cfu of E. coli into single udder quarter and b) mean (± SEM) concentrations of rhLf (white) and bLf (grey) (mg/ml) of challenged quarters in the milk of transgenic cows. c) Mean (± SEM) bacterial counts (log cfu/ml) in the milk of the transgenic (■) and control (♦) cows during the experiment. Mean (± SEM) serum d) SAA, e) serum Hp and f) cortisol of the transgenic (■) and control (♦) cows during the experiment.

The concentration of Lf mRNA in the cisternal region and in the ducts near the teat is higher than that in the epithelial ducts of the mammary parenchyma, which may affect the antibacterial effect of Lf (Molenaar *et al.* 1996). The parenchymal Lf perhaps could not function here efficiently enough and the recombinant hLf in the milk did not compensate the lack of parenchymal bLf. The inoculum size and challenge via teat canal in an experimental mastitis model differ from natural infection where bacterial invasion to the mammary gland may progress slower, and the protective effect of mik additional Lf could be better in natural conditions.

Kutila *et al.* (2003) found that *E. coli*, the same strain as in our experiment, was totally inhibited *in vitro* by bLf concentration of 1.67 mg/ml. In our study the bacterial growth rate in the transgenic cows and normal cows was similar, suggesting that the bacteriostatic function of Lf *in vivo* is not only caused by its iron-chelating ability but is more complex. In the study of Kutila *et al.* (2004) bLf administered intramammarily did not inhibit the infection or bacterial growth but Lf perhaps neutralized the effects of LPS. In bovine coliform mastitis the clinical signs are mainly caused by LPS and our findings about the milder clinical reactions in the transgenic animals could be caused by the LPS neutralizing effect of Lf.

bLf is released from specific granules of neutrophils and the concentration of bLf in milk is related to SCC, stage of lactation and amount of milk. Increased SCC was found to be associated with reduced susceptibility to *E. coli* mastitis (Green *et al.* 2004). In our study, milk SCC did not differ between the groups during the experiment, but the mean pre-challenge SCC of the control cows was almost three times as high as that of the transgenic cows. In the present study SAA and Hp, the most sensitive acute phase proteins in cattle were lower in the transgenic cows than in the normal cows. Increase of SAA and Hp in serum indicates that both groups responded to infection, but in the transgenic cows the response was milder. SAA has been reported to be a sensitive inflammatory marker for acute *E. coli* mastitis correlating with the severity of the cow's response and Hp has been found to have prognostic value of predicting the final outcome from mastitis (Eckersall *et al.* 2001). A previous study of Jacobsen *et al.* (2004) showed that approximately one-fourth of the variation of serum concentrations of acute phase proteins depends on the individual animal. In our study the transgenic cows were genetically more homogenous, being offspring of the same bull. This may partly explain our results.

Our Lf-transgenesis model did not provide protection against *E. coli* mastitis in dairy cows, in contrast to the findings on experimental *S. aureus* challenge in lysotaphin-transgenic mice (Kerr *et al.* 2001). It is clear that mouse udder differs from the udder of dairy cows, and in addition lysostaphin is specifically targeted towards staphylococci, which explains the different results. The host responses also differ in mastitis caused by *E. coli* and *S. aureus* (Bannermann *et al.* 2004). The hLf-transgenic cows were not resistant against *E. coli* mastitis but the systemic signs were milder and the recovery was faster in this experimental study, but the results may give promise of some potential of transgenesis for mastitis resistance which could be further studied.

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Cell function in the bovine mammary gland: Interdependence of healthy and infected udder quarters

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Abstract

Current mastitis research has not completely explained udder defence mechanisms. In consideration of the anatomical construction of the udder, infection of one udder quarter is not considered to influence the immune status of neighbouring quarters.

In order to test this hypothesis, we compared the early immune reactions of individual udder quarters in response to microbiological attacks. Such reactions are characterised by polymorphonuclear leucocytes (PMN) which release oxygen radicals, which can be determined by chemiluminescence (CL). Milk from 140 udder quarters of 36 cows was thus analysed for somatic cell count (SCC), differential cell count, viability and CL activity. Three cow groups were classified cytologically: a healthy reference group A (11 animals, SCC limit <100,000/ml); group B (moderate mastitis, 8 cows, SCC ≥100,000 and <400,000/ml in at least one quarter) and group C (severe mastitis, 17 cows, SCC ≥400,000/ml in at least one quarter).

Infected and not infected quarters in groups B and C were analysed separately. Viability was significantly lower (P <0.0001) in group A (72.6%) than in the healthy quarters of group C (84.0%). Lowering the SCC limit of healthy quarters to <50,000/ml (group A: all quarters within the udder) revealed striking differences between samples of groups B and C: In addition to varying differential cell counts and viabilities, the CL activity of group $B_{<50}$ (2,929 CL units/million PMN) was remarkably lower than that of the other groups (5,616 in group $A_{<50}$ and 6,445 CL units/million PMN in group $C_{<50}$).

These results show that the infection of one udder quarter does influence the cell activity of neighbouring quarters. When the SCC threshold for healthy quarters was reduced to 50,000 cells/ml, much greater differences in cell activities were detected between healthy udders and healthy quarters of infected udders.

Keywords: udder defence activity, bovine PMN, chemiluminescence

Abbreviation key: CL = chemiluminescence, PMN = polymorphonuclear leucocytes, SCC = somatic cell count

Introduction

Research on subclinical mastitis has also led to investigations on udder defence mechanisms (Allen *et al.*, 1972; Jain, 1976; Burvenich *et al.*, 1995).

Immune cells occurring in milk are polymorphonuclear leucocytes (PMN), lymphocytes and macrophages (Paape *et al.*, 1981; Wever and Emanuelson, 1989). These immune cells differ in their distribution in normal and mastitic milk. The main task of PMN is the defence against bacteria at the beginning of an acute inflammation process (Jain, 1976; Paape *et al.*, 1979), when not only the number, but also the defence activity of PMN increase enormously (Targowski, 1983; Craven and Williams, 1985). The immune system responds in a highly differentiated way to a bacterial attack, and researchers need to learn to interpret information about cell differentiation and cell activity correctly.

One way to measure PMN activity is the chemiluminescence (CL) reaction. Reactive oxygen species like O_2^- and H_2O_2 (hydrogen peroxide) are catalyzed by chemical reactions called the respiratory burst, whereupon the radicals attack bacteria and nearby cells (Rossi and Zatti, 1964). The CL reaction is characterised by the oxidation of hydrogen peroxide under emission of photons which are detected in a luminometer (Allen *et al.*, 1972). The higher the CL activity per million PMN, the stronger is the defence reaction.

CL is used in human and veterinary medicine and in dairy research, but in the latter mainly for the determination of blood PMN (Lohuis *et al.*, 1990; Hoeben *et al.*, 2000a, 2000b). Some researchers also use PMN isolated from milk to measure CL activity (Dosogne *et al.*, 1999; Mehrzad *et al.*, 2001).

This study details the udder-health relevant cell activity of PMN from infected quarters, from neighbouring quarters and from those of healthy udders. In addition to the determination of CL activity, we evaluated SCC, viability and differential cell count from milk samples of healthy, moderately affected and severely diseased mammary glands.

Material and methods

Animals and milk sampling

Thirty-six cows from two farms were included in this study. Animals were selected on the basis of results of a three-week (farm B) pilot survey and on continuous surveillance data (farm A).

After cleaning and disinfection of the teat, 10 ml milk were collected in sterile glass tubes for cytobacteriological analysis. Afterwards 500 ml milk were sampled in glass bottles for cell isolation.

Cell isolation

Somatic cells from 500 ml milk were isolated according to Schroeder *et al.* (2004). In brief, the samples were diluted in phosphor-buffered saline (PBS) and centrifuged three times with decreasing speed (400, 300, 200*x*g). The resulting cell pellet was stored on ice in 0.5 ml PBS and, if necessary, diluted to 4.0×10^6 cells/ml.

Preparation of a smear and microscopic differential cell count

The smear was prepared with the aid of a modified coffee grinder for smooth distribution of the cells (Schroeder *et al.*, 2004). After air drying, the smear was stained with Hemacolor (Merck Eurolab GmbH, Darmstadt, Germany). Using oil immersion, 100 cells were differentiated into PMN, lymphocytes, macrophages and epithelial cells.

Viability

Viability of PMN was analysed flow cytometrically (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using propidium iodide (Sigma Aldrich Chemie GmbH, Steimheim, Germany), which binds to the DNA of membrane-defective cells.

Chemiluminescence

Chemiluminescence was determined in a Luminoscan Ascent luminometer (Labsystems, Helsinki, Finland), which has a sensitivity of < 1 fmol ATP per well. A reaction solution was prepared of Dulbecco's PBS containing 666 nmol/ml luminol (5-amino-2,3-dihydro-1,4-phthalazindion, Sigma Aldrich Chemie GmbH, Steimheim, Germany) and 1000 ng/ml PMA (Sigma Aldrich Chemie GmbH, Steimheim, Germany) and adjusted by use of NaOH to a pH of 10.0. A white microtiter plate (Nunc GmbH, Wiesbaden, Germany) containing 50 µl cell suspension per well was introduced into the luminometer. The dispenser of the luminometer added 150 µl reaction solution to each well immediately before the detection of light emission was started at 38 °C. The values were recorded at 90-second intervals as relative light units (RLU). After 45 minutes of measurement, the area under the curve (AUC) was taken as the final result. The study included 12 blind samples without cells and multiple (5 to 7) samples of each cell suspension, the means of which were determined (standard deviation below 5.0%). The results were documented as CL activity/1 million PMN.

Statistical analysis

Statistical tests were conducted with SAS 8e 2002 software (SAS Institute Inc., Cary, NC, USA). Different SAS procedures were applied to run Student's t-tests and tests based on the Pearson correlation coefficient.

Study design

Three cow groups were classified cytologically: a healthy reference group A (11 animals, SCC limit <100,000/ml, n =44 quarters); group B (moderate mastitis, 8 cows, SCC ≥100,000 and <400,000/ml in at least one quarter); and group C (severe mastitis, 17 cows, SCC ≥400,000/ml in at least one quarter.) (In group C, there were three animals with only three quarters that secreted milk, as one quarter was missing.) Infected and not infected quarters of groups B and C were analysed separately.

The unaffected quarters of groups B and C (SCC <100,000 cells/ml) were redefined as group $B_{<100}$ (n =17) and $C_{<100}$ (n =12). Groups $B_{100\text{-}400}$ (n =15) and $C_{100\text{-}400}$ (n =19) contained the samples with SCC of 100,000 - 400,000 cells/ml, while group $C_{>400}$ (n =33) contained those from the quarters with SCC >400,000 cells/ml.

Furthermore, those quarters of groups $B_{<100}$ and $C_{<100}$ that had an SCC below 50,000 cells/ml were redefined as group $B_{<50}$ (n =11) and group $C_{<50}$ (n =10). In group $A_{<50}$, all quarters of the udder contained fewer than 50,000 cells/ml (n =28).

Results

A comprehensive review of current udder health data and differential cell counts is found in Hamann *et al*. (2005), from which our data are taken. Data for the different groups are shown in Table 1. There was a significant difference in viability between group A and groups $C_{<100}$ and $C_{100\text{-}400}$ (P =0.0012 and P <0.0001, respectively). CL activity was significantly

Table 1. Means ($\bar{x}$) and standard deviations (sd) of data of the quarter groups A, $B_{<100}$, $B_{100-400}$, $C_{<100}$, $C_{100-400}$ and $C_{>400}$.

Group		A	$B_{<100}$	$B_{100-400}$	$C_{<100}$	$C_{100-400}$	$C_{>400}$
log SCC		4.31	4.39	5.30	4.55	5.31	5.91
	sd	0.28	0.36	0.18	0.29	0.18	0.27
PMN		27.2	45.8	58.7	57.4	71.7	81.9
	sd	21.9	22.2	24.6	20.9	24.5	13.9
Lym		23.8	19.1	9.6	17.2	8.5	4.8
	sd	18.6	10.2	8.1	12.0	9.0	5.3
Mac		46.3	32.3	31.2	22.5	19.5	12.9
	sd	23.2	15.7	21.8	10.1	15.8	9.3
Viability		72.6	77.3	82.7	84.0	83.2	82.0
	sd	12.1	10.3	12.1	6.1	9.1	14.2
CL		5652	4081	9290	7068	10110	10541
	sd	6397	3238	3952	6090	5845	5566

PMN: in %; Lym: lymphocytes in %; Mac: macrophages in %; Viability: in %; CL: CL activity/1 million PMN

lower in group $B_{<100}$ than in group $B_{100-400}$ (P =0.0004). There were no significant differences in CL activities of the groups A, $B_{<100}$ and $C_{<100}$, but standard deviations were high. The correlation coefficient between SCC and PMN is 0.70, that between SCC and macrophages is -0.53, that between SCC and CL activity is 0.40, and that between SCC and viability, 0.32.

Differences in groups $A_{<50}$, $B_{<50}$ and $C_{<50}$ were mainly found between groups $A_{<50}$ and $C_{<50}$ (Table 2). It has to be stressed that the SCC differs between those groups despite the selection conditions. The percentage of PMN was significantly higher both in groups $B_{<50}$ and $C_{<50}$ than in group $A_{<50}$, but the differences in the percentage of macrophages and viability are statistically significant only between group $A_{<50}$ and group $C_{<50}$. Furthermore, it should be noted that the mean CL activity was lowest in group $B_{<50}$ and highest in group $C_{<50}$.

Table 2. Means ($\bar{x}$) and standard deviations (sd) of data of groups $A_{<50}$, $B_{<50}$ and $C_{<50}$.

Group	log SCC	PMN	Lym	Mac	Viability	CL
$A_{<50}$	$\bar{x}$4.14	22.3	24.5	49.6	69.2	5616
	sd0.17	14.6	20.0	23.0	11.7	7070
$B_{<50}$	$\bar{x}$4.15	37.3	21.3	36.6	77.1	2929
	sd0.16	14.5	8.4	14.3	10.1	1565
$C_{<50}$	$\bar{x}$4.44	56.7	19.3	23.1	83.6	6445
	sd0.24	24.4	13.3	10.9	6.2	5880
P ($A_{<50}$:$B_{<50}$)	n. s.	**	n. s.	n. s.	n. s.	n. s.
P ($A_{<50}$:$C_{<50}$)	**	**	n. s.	***	***	n. s.

Lym: lymphocytes in %; Mac: macrophages in %; Viability: in %; CL: CL activity/1 million PMN

n. s.: not significant, $P \geq 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Discussion

The cell functionality was determined for milk of healthy (group A), moderately (group B) and severely diseased (group C) udders, and the results for healthy and infected quarters were interpreted separately.

There were high percentages of PMN in the differential cell count of samples with SCC of more than 100,000 cells/ml (groups $B_{100\text{-}400}$, $C_{100\text{-}400}$, $C_{>400}$). CL activity was also high in these quarters (almost twice as high as in group A), but the low correlation between SCC and CL activity indicates that these two parameters act rather more independently.

There were striking differences between healthy quarters of the groups $B_{<100}$ and $C_{<100}$ and neighbouring diseased quarters, not only in SCC and differential cell count but also in functional properties like viability and CL activity. The CL activity of group $B_{100\text{-}400}$ was significantly higher than that of group $B_{<100}$ (P =0.0004). This could be seen as support for the common assumption that the quarters within an udder react independently.

However, comparison of data from groups $B_{<100}$ and $C_{<100}$ with those from group A show that the percentage of PMN in the differential cell count was significantly higher in groups $B_{<100}$ and $C_{<100}$ than in group A (P =0.0058 and P <0.0001, respectively). Viability was lowest in group A, while the CL activity was about the same in all three groups despite a broad range of variation. It must be pointed out that the mean SCC in group $C_{<100}$ was significantly higher than in group A (P =0.0090), although those groups were selected on the basis of the same SCC limits.

This phenomenon persisted even in respect to the smaller SCC range of the groups $A_{<50}$, $B_{<50}$ and $C_{<50}$. Therefore the significant differences of macrophages and PMN between group $A_{<50}$ and group $C_{<50}$ must be attributed to correlations between the SCC and those parameters.

Nevertheless, both PMN content as well as viability were higher in groups $B_{<50}$ and $C_{<50}$, indicating the presence of defence activities. The lowest mean CL activity was found in group $B_{<50}$, which - in spite of the very high variation - allows some speculation about the general defence situation of these animals. Perhaps group B mainly consisted of chronically diseased cows, while group C consisted of animals with acute infections and a very effective immune system. Further research is necessary to confirm this interpretation of the data in.

This study showed that milk from healthy udders and milk from infected and not infected quarters differ in their cell functionality. A similar effect has been reported for milk components in case of a mastitic event (Hamann, 2002). These results indicate that the infection of one udder quarter does influence the cell activity of neighbouring quarters. When the SCC threshold for the definition of a healthy quarter is reduced to 50,000 cells/ml, the effects observed here became even clearer.

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Delayed neutrophil apoptosis in bovine subclinical mastitis: Possible involvement in persistent accumulation of cells in milk

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Abstract

Bovine subclinical mastitis can be defined as a moderated inflammatory disease characterized by a persistent accumulation of neutrophils in milk. As granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated delay of neutrophil apoptosis contributes to the accumulation of inflammatory cells at the inflammation site in many human diseases, we sought to determine whether subclinical mastitis in cows is also associated with a GM-CSF-dependent increase in milk neutrophil survival. We first addressed the hypothesis that GM-CSF delays bovine neutrophil apoptosis by activation of members of the signal transducer and activator of transcription (STAT) family, which are critical regulators of the expression of various Bcl-2 family proteins. GM-CSF significantly delayed apoptosis of blood neutrophils obtained from healthy cows. In these cells, GM-CSF activated STAT5 and induced an increase in the mRNA of the anti-apoptotic Bcl-2 member, Bcl-x_L. This pathway was blocked by the Jak inhibitor, AG-490. This inhibition was associated with abrogation of the prosurvival effect of GM-CSF, demonstrating a key role for STAT5 in delayed neutrophil apoptosis. We further found that GM-CSF expression was increased in milk cells from subclinical mastitis-affected cows. Neutrophils from these cows demonstrated a significant delay of apoptosis as compared with neutrophils obtained from healthy cows. Active STAT5 complexes were detected in these neutrophils. Finally, in the presence of AG-490, apoptosis in milk neutrophils from mastitis-affected cows was induced. These results indicate that neutrophil survival is enhanced in milk of subclinical mastitis-affected cows and suggest a role for a GM-CSF-activated STAT5 signaling pathway in this phenomenon. This pathway could thus represent a target for the control of persistent accumulation of neutrophils in the bovine mammary gland.

Keywords: neutrophil, apoptosis, GM-CSF, Bcl2 family

Introduction

Bovine subclinical mastitis can be defined as a moderated inflammatory disease resulting from an imbalance between the bacteria virulence and host defense mechanisms, which consequence is a fluctuating increase in somatic cell count (SCC). This SCC increase is mainly due to the migration of polymorphonuclear neutrophils. In the course of eliminating pathogens, neutrophils can also be detrimental to tissues by releasing intracellular products

which exacerbate the inflammatory process resulting in damage to alveolar tissue, reduction in milk yield and deterioration in milk composition (Murphy *et al.*, 1989; Harmon, 1994; Klei *et al.*, 1998).

Persistent accumulation of inflammatory cells at the inflammatory site requires both continuous neutrophil influx and increased survival of extravasated granulocytes. Indeed, delayed neutrophil apoptosis has been reported to be a general phenomenon contributing to the development of neutrophilia (Dibbert *et al.*, 1999). Numerous inflammatory mediators are able to regulate the life span of neutrophils. However, it has been established that granulocyte-macrophage colony-stimulating factor (GM-CSF) crucially contributes to inhibition of granulocyte apoptosis at the site of inflammation (Brach *et al.*, 1992), and therefore may contribute to the accumulation of inflammatory cells at the site of inflammation.

As we previously found increased GM-CSF levels in milk cells of mastitis-affected cows (Boulanger *et al.*, 2003), we sought to determine whether subclinical mastitis in cows is also associated with a GM-CSF-dependent increase in milk neutrophil survival. We first addressed the hypothesis that GM-CSF delays bovine neutrophil apoptosis by activation of members of the signal transducer and activator of transcription (STAT) family, which are critical regulators of the expression of various Bcl-2 family proteins. As this family contains both anti-apoptotic proteins such as Bcl-2, Bxl-x_L and Mcl-1, and pro-apoptotic proteins such as Bax, Bad and Bcl-x_S (Reed, 1997), we also investigated the potential role of Bax, Mcl-1 and Bcl-x_L in the mammary gland inflammation. We first determined the involvement of this pathway in blood neutrophils and then we investigated its potential role in milk neutrophils.

GM-CSF blood pathway in bovine neutrophils from healthy cows

GM-CSF prevents apoptosis and induces activation of STAT5 with up-regulation of Bcl-x_L mrna in bovine blood neutrophils

If many studies have demonstrated that GM-CSF delays the apoptotic cell death of human neutrophil, the question of whether GM-CSF prevents the apoptosis of bovine neutrophil has never been addressed. To solve this issue, blood neutrophils (isolated by density centrifugation) from healthy cows were cultured in the presence or absence of GM-CSF (10ng/mL) for 6, 24, 48h before apoptosis and necrosis detection using dual color annexin-V-FITC/PI staining and flow cytometry analyses. From 24h, GM-CSF-stimulated blood neutrophils showed a significant apoptosis delay compared with untreated cells (Figure 1).

To investigate the potential involvement of the STAT family in the mechanism of GM-CSF-induced neutrophil survival, blood neutrophils of healthy cows were stimulated with GM-CSF and protein extracts were analyzed by Electrophoretic Mobility Shift Assay (EMSA) for STAT5 DNA-binding activity (Figure 2). Active STAT5 complexes were detected 20min after stimulation. This activity was still detectable at 60min but declined.

To determine whether GM-CSF mediates bovine neutrophil survival activity through the control of Bcl-2 members, levels of mRNA expression of 3 representative members of this family, namely Bax, Mcl-1 and Bcl-x_L, were assessed by semi-quantitative RT-PCR in untreated and GM-CSF-treated blood neutrophils for 2, 5 and 24h (Figure 3). Bax and Mcl-1 mRNA levels remained constant throughout the protocol in both treated and untreated cells. By contrast, Bcl-x_L levels were increased after GM-CSF stimulation (Figure 3).

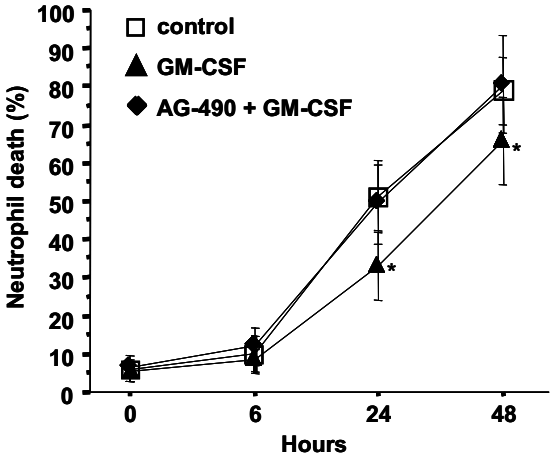

*Figure 1. GM-CSF anti-apoptotic activity abrogated by AG-490 in blood neutrophils. Neutrophils from healthy cows were cultured in the absence or presence of GM-CSF, or with a combination of GM-CSF and AG-490 (200 µM). Results are presented as means ± SD (n = 6). * P< 0.05*

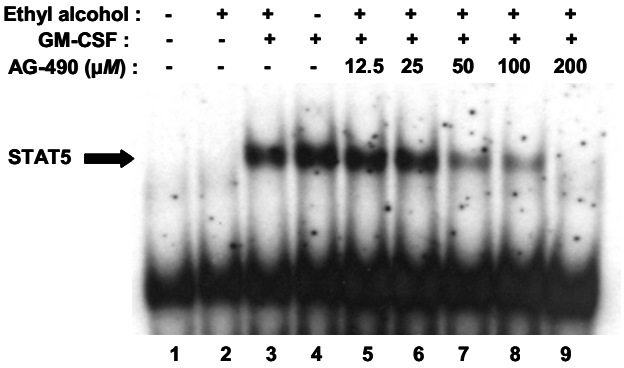

Figure 2. GM-CSF-dependent STAT5 activation blocked by AG-490. Blood neutrophils isolated from healthy cows were treated for 1h with medium (lane 1) and drug solvent alone (Ethyl alcohol absolute, vehicle control) (lane 2) or with the indicated concentrations of AG-490 (lanes 5-9). Cells were then stimulated with GM-CSF for 20min before protein extracts were performed and STAT5 DNA-binding activity analyzed by Electrophoretic Mobility Shift Assay.

Reversal of GM-CSF anti-apoptotic activity by AG-490 in blood neutrophils

The role of STAT5 activation in GM-CSF-mediated delay in apoptosis and in Bcl-x_L mRNA up-regulation was evaluated using the Jak-selective tyrphostin pharmacologic inhibitor, AG-490 (Levitzki, 1999). AG-490 was demonstrated to be a selective inhibitor of both Jak2 and Jak3 and thereby reduces STAT activation (Kirken *et al.*, 1999). Increasing doses of AG-490 were used to determine the appropriate STAT5 inhibiting dose (Figure 2). We found that STAT5 DNA-binding activity was attenuated by doses of 50 and 100 µ*M* and completely inhibited by a dose of 200 µ*M* (Figure 2). To confirm the role of STAT5 activation in Bcl-x_L expression, healthy blood neutrophils were treated with GM-CSF alone or in combination

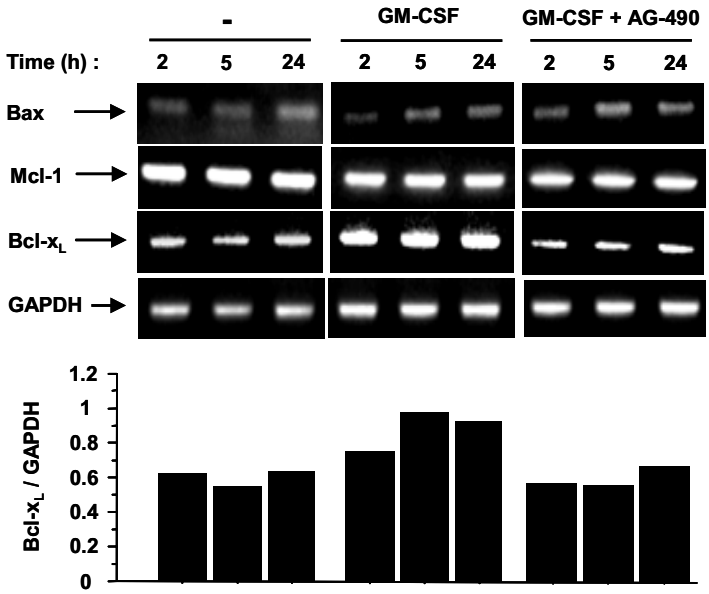

Figure 3. GM-CSF up-regulation of Bcl-x_L mRNA blocked by AG-490. RNA was prepared from blood PMN, cultured for 2, 5 and 24h in the presence and absence of GM-CSF or a combination of GM-CSF and AG490 (200 µM), and analyzed by RT-PCR for expression of Bax, Mcl-1 and Bcl-x_L. As a control for quantification GAPDH was also amplified. Filled columns show the ratio between Bcl-x_L and GAPDH mRNA, as determined by densitometry analyses. These results are representative of 3 comparable experiments.

with a dose of 200 µ*M* AG-490 and RT-PCR were performed (Figure 3). We observed that GM-CSF-dependent up-regulation of Bcl-x_L mRNA was blocked by the Jak inhibitor, AG-490.

Finally, blood neutrophils from healthy cows were exposed to GM-CSF in the presence of AG-490 and apoptosis assays were performed (Figure 1). Apoptosis rates were compared with those in untreated and GM-CSF-treated cells from the same donor. We found that AG-490 treatment prevented the GM-CSF-mediated protection from apoptosis in neutrophils. At 24 and 48h, the amounts of apoptosis in medium-cultured cells and in AG-490-treated cells were similar and statistically higher than those from GM-CSF-treated neutrophils.

GM-CSF milk pathway in bovine cells from mastitis-affected cows

Milk neutrophils from subclinical mastitis-affected cows present a significant delay in apoptosis

To determine whether subclinical mastitis is associated with an increase in milk neutrophil survival, milk cells from healthy and subclinical mastitis-affected cows with SCC higher than 10^6 cells/mL were cultured for different times before apoptosis assays (Figure 4). After 24h, neutrophil apoptosis of healthy cows reached 97.9%, while the rate of neutrophils from affected cows was 44.8%. Then, to establish the involvement of GM-CSF in enhanced survival of milk neutrophils from subclinical mastitis-affected cows, these cells were cultured in the presence or absence of GM-CSF (Figure 4). At the different times tested, apoptosis levels of GM-CSF-treated and untreated milk neutrophils were comparable.

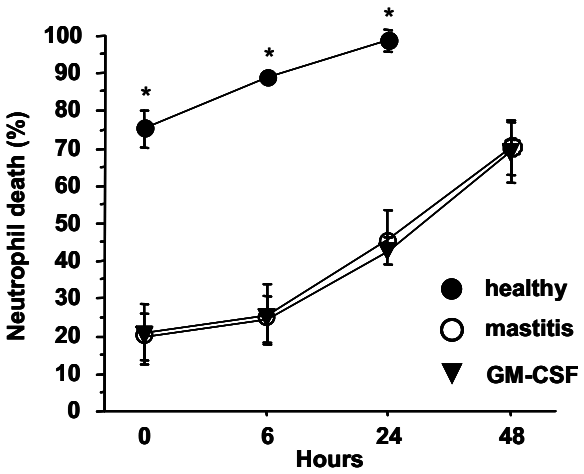

*Figure 4. Apoptosis assays in milk cells. Kinetic analysis of apoptosis in milk neutrophils from healthy and subclinical mastitis-affected cows. Cells from mastitis cows were cultured in the presence or absence of GM-CSF. Data are presented as means ± SD (n = 6). *Significantly different from values obtained with milk neutrophils from subclinical mastitis-affected cows.*

These results indicate that (1) anti-apoptotic mechanisms enhance the survival of neutrophils that have migrated from blood to udder of subclinical mastitis-affected cows, and that (2) these cells are unresponsive to GM-CSF stimulation, suggesting that they are maximally stimulated by GM-CSF at the site of inflammation.

STAT5 activity profile in milk cells

Protein extracts prepared from milk cells of 25 cows with different SCC were tested for STAT5 DNA-binding activity (Figure 5). No active STAT5 complex was detected in milk cells from healthy cows (Figure 5, lanes 1-3). However, STAT5 activity and SCC simultaneously increased in milk from subclinical mastitis-affected cows (Figure 5, lanes 4-9). Microscopic analyses confirmed that neutrophil was the predominant cell type with percentages increasing as SCC rose (data not shown).

AG-490 Induces apoptosis in mastitis milk neutrophils with a down-regulation of Bcl-x_L mRNA

To investigate the potential role of STAT5 in the apoptosis delay of neutrophils observed in milk from subclinical mastitis-affected cows, neutrophils from milk with SCC higher than 10^6 cells/ml were cultured in the presence or absence of AG-490 for different times before apoptosis assays (Figure 6). We found that the inhibitor was able to induce apoptosis. From 24h, the apoptosis rate of AG-490-treated neutrophils was significantly higher than the rate of untreatred cells. RT-PCR showed that Bax and Mcl-1 mRNA levels were maintained in both treated and untreated cells (Figure 6). By contrast, Bcl-x_L mRNA was decreased 2h after treatment and was almost totally down-regulated at 5h.

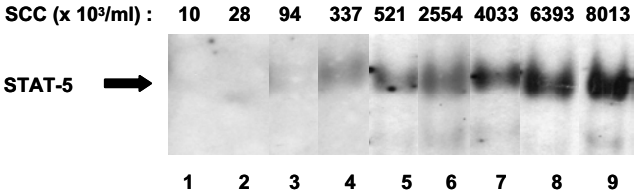

Figure 5. STAT5 activity in milk cells. Representative EMSA performed with protein extracts prepared from milk cells of healthy cows (lanes 1-3) and subclinical mastitis-affected cows (lanes 4-9). The arrow indicates specific STAT5 complexes.

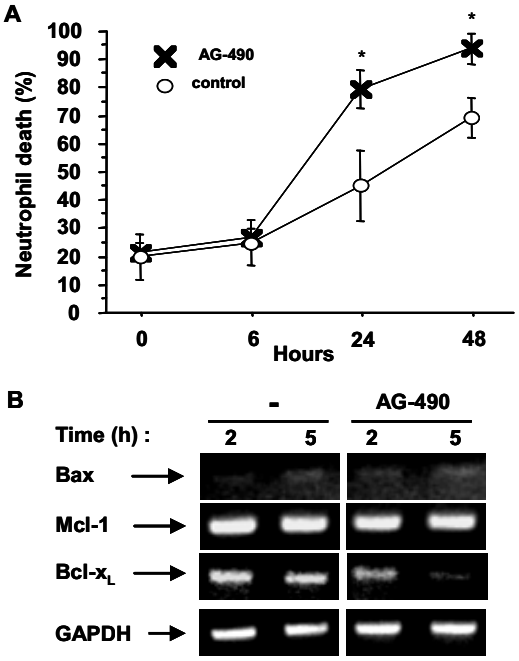

*Figure 6. AG-490 induces apoptosis and Bcl-x_L down-regulation in milk neutrophils from subclinical mastitis-affected cows. Cells were obtained from cows with SCC>10^6cells/ml and were cultured in the presence or absence of AG-490 (200 µM). (A) Kinetic analysis of apoptosis of AG-490-treated neutrophils. Data are presented as means ± SD (n = 6). *Significantly different from values obtained with untreated cells for P<0.001. (B) mRNA levels assessed by RT-PCR for expression of Bax, Mcl-1 and Bcl-x_L. As a control for quantification, GAPDH was also amplified. Filled columns show the ratio between Bcl-x_L and GAPDH mRNAs, as determined by densitometry analyses. These results are representative of at least 3 comparable experiments.*

Discussion and conclusion

Persistent accumulation of neutrophils in the udder is a characteristic feature of subclinical and chronic mastitis in dairy cows. In many bacterial inflammatory diseases, delayed apoptosis is an important mechanism for neutrophil accumulation. Therefore, a reduction in inflammatory cell apoptosis is a central concept in the maintenance of inflammation and a potential target in the resolution of inflammation (Haslett, 1999). In

the present study, we have demonstrated for the first time that an anti-apoptotic mechanism enhances the survival of milk neutrophils from subclinical mastitis-affected cows, which could be implicated in the persistent accumulation of these cells in the udder. We suggest a role for a GM-CSF-activated STAT5 signaling pathway in this phenomenon.

Neutrophils contribute to the elimination of pathogens and also amplify the inflammatory response by the production of cytokines (Cassatella, 1999). Thus, we may hypothesize that the apoptosis delay observed in milk neutrophils from subclinical mastitis-affected cows contributes to generate autoregulatory feedback loops perpetuating inflammation. Then, the induction of neutrophil apoptosis may represent a target for the control of persistent accumulation of cells in the udder. Our data demonstrated that AG-490 did not accelerate neutrophil apoptosis in blood but only at the site of inflammation, a finding consistent with results indicating that *in vivo* AG-490 treatment does not affect the survival of normal cells and does not block the function of immune cells (Burdelya *et al.*, 2002). Further studies are needed to determine whether induction of neutrophil apoptosis in milk from subclinical mastitis-affected cows could have therapeutic application in the resolution of persistent inflammation of the bovine mammary gland. In addition, the questions of whether such a treatment could affect host defense mechanisms and/or enhance the virulence of the pathogen have to be elucidated.

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Economics of mastitis and mastitis management

Use of partial budgeting to determine the economic benefits of antibiotic treatment during lactation of chronic subclinical mastitis caused by *Staphylococcus aureus*

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Introduction

Staphylococcus aureus mastitis is a highly prevalent and costly disease. Mastitis prevention through good management is usually cost effective (Zepeda *et al.*, 1998), but treatment of subclinical *S. aureus* mastitis during lactation is generally considered to be ineffective (Fox and Gay, 1993) and economically not justified (Allore *et al.*, 1998). Treatment of *S. aureus* mastitis is often postponed until a clinical episode occurs or until dry-off. This increases the duration of infection leading to a lower probability of cure (Sol *et al.*, 1997, 2000), more clinical mastitis and transmission to other cows (Lam *et al.*, 1996; Zadoks *et al.*, 2002) and ultimately, a higher probability of culling. Treatment of subclinical *S. aureus* mastitis during lactation could be a viable alternative to delayed treatment. It could result in a higher probability of cure (Bramley and Dodd, 1984) and has an often overlooked indirect effect, i.e. prevention of clinical mastitis and transmission to other cows (St Rose *et al.*, 2003) (Figure 1).

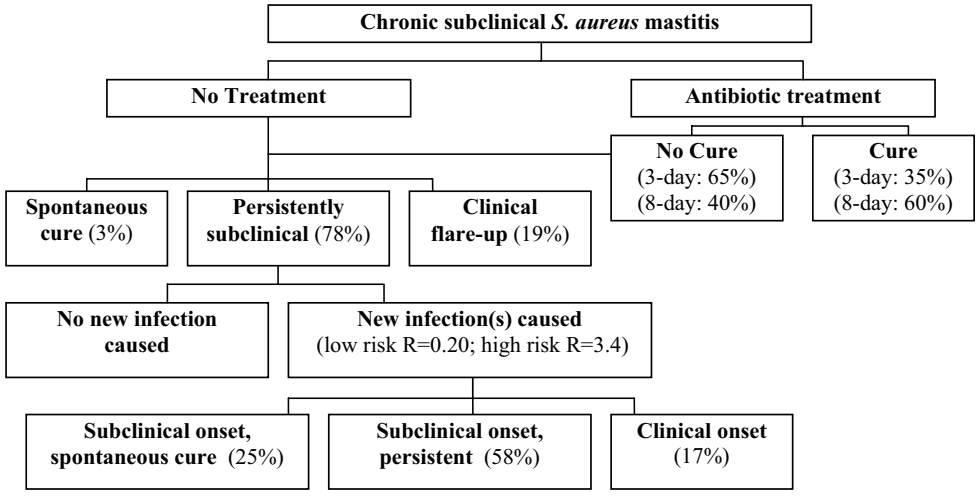

Figure 1. Deterministic model for effect of no, 3-day or 8-day treatment on outcome of chronic subclinical Staphylococcus aureus mastitis in low and high transmission scenario. R = reproductive ratio (number of new infections caused, (Lam et al.*, 1996)*

Chances of cure can be improved by extended treatment (Deluyker *et al.*, 2005) and by selecting the right cows for treatment using current knowledge of risk factors for cure (Deluyker *et al.*, 2005; Sol *et al.*, 1997). To determine the profitability of cure, potential benefits of treatment must be weighed against several other factors such as the price of antibiotics and loss of milk due to withholding times. Surprisingly, economical profitability of treating subclinical mastitis during lactation has been shown for *Streptococcus agalactiae* (Yamagata *et al.*, 1987) and for non-agalactiae streptococci (Swinkels *et al.*, 2005), but not for *S. aureus*.

We explored the economic benefits of treating chronic subclinical *S. aureus* mastitis during lactation using partial budgeting. In this analysis, effects at the cow level, such as bacteriological cure and prevention of clinical mastitis, and effects at the herd level, such as reduced transmission potential, will be taken into account.

Material and methods

Partial budgeting was used to develop a deterministic simulation model to estimate the net cost or benefit of treatment of subclinical *S. aureus* mastitis during lactation. Input variables are based on literature, if available, and on dairy prices of 2003-2004 in The Netherlands. Costs and benefits were calculated at the cow level during one lactation. Three-day or 8-day treatment was compared to no treatment (Table 1).

Sensitivity analysis was performed to determine the impact of input variables (Table 2). The risk of transmission depends on herd management (Lam *et al.*, 1996; Zadoks *et al.*, 2002) and strain factors (Smith *et al.*, 1998; Zadoks *et al.*, 2002). Therefore, four scenario's were analysed: 3-day treatment or 8-day treatment combined with low (R=0.20) and high (R=3.35) risk of transmission. Because cow and strain factors influence the probability of cure (Deluyker *et al.*, 2005; Sol *et al.*, 1997, 2000) impact of these factors on economic benefits were analysed in each scenario (Table 3).

Results

When contagious transmission of *S. aureus* is likely (R=3.4), 3 and 8-day treatment result in a very high positive net profit of €112,68 and €166,01, respectively (Table 2). When the probability of *S. aureus* transmission is low (R=0.2), the average economic benefit of a 3 or 8-day lactational treatment of chronic subclinical *S. aureus* mastitis was € -2.65 and €-31.71, respectively (Table 1). On low-transmission farms, a net profit was achieved for cows with more than 37% chance of cure (Table 2). When treatment is extended to 8 days, a positive net profit could only be reached on low-transmission farms in the exceptional situation of a predicted bacteriological cure of 90% (Table 2). On high-transmission farms or in an outbreak situation (R=3.35), 3-day and 8-day treatment already reached a positive net result when the probability of cure for an individual cow was higher than 11 and 25%, respectively (Table 3). Sensitivity analysis showed that the five most influential input variables were bacteriological cure, Reproductive Ratio (R), probability of culling, retention pay off and antibiotic costs.

Table 1. Partial Budgeting: net profit (€) of 3- or 8-day lactational treatment of chronic subclinical S. aureus *mastitis compared to no treatment when risk of contagious transmission is low. Net profit is calculated as (extra revenue + reduced costs) minus (reduced revenue + extra costs). References as listed or from Swinkels* et al. *(2005).*

Contribution to economic effect	Reference	Treatment	
		3-day	8-day
Extra revenue			
Increase in milk production after cure (kg milk)	St. Rose *et al.*, 2003	0	0
Total extra revenue	Calculated	0	0
Reduced costs			
Reduction in probability of clinical mastitis after treatment (%)	Lam, 1996; Zadoks, 2002; Calculated	6.7%	11.4%
Costs of clinical flare-up of pre-existing subclinical mastitis	De Vos and Dijkhuizen, 1998	187	187
Reduced costs due to prevention of clinical flare-ups (€)	Calculated	12.44	21.32
Reduction in probability of persistent subclinical mastitis (%)	Calculated	43.7%	61.7%
Number of new infections that is prevented	This paper	0.15	0.15
Probability that new infection results in spontaneous cure (%)	Zadoks, 2003	24%	24%
Probability that new infection results in clinical mastitis (%)	Lam, 1996; Zadoks, 2002	17%	17%
Probability that new infection results in chronic subclinical mastitis (%)	This paper	59%	59%
Costs of spontaneous cure (€)	This paper	4.2	4.2
Cost of clinical mastitis(€)	De Vos and Dijkhuizen,1998	233	233
Costs of subclinical mastitis(€)	This paper	113.75	113.75
Reduced costs due to prevented transmission (€)		7.48	12.81
Reduction in probability of persistent subclinical mastitis (%)		27.3%	4.7%
Retention pay off (€)	AdAdapted from De Vos and Dijkhuizen, 1998	506	506
Culled animals (%)	Esslemont and Kossaibati, 1997; Whitaker, 2001; NRS, 2003;this paper	12%	12%
Reduced costs due to prevented culling (€)		16.58	28.40
Reduced costs due to prevented penalties for high scc		0	0
Reduced costs due to prevention of decreased fertility		0	0
Total reduced costs (€):		36.49	62.55
Reduced revenue:			
Milk discard because of antibiotic residue (kg/day)	NRS, 1998	25.3	25.3
Duration of milk withhold (days)	This paper	6	11
Total discarded milk (liters)		150	275
Balanced profit milk (€/kg)		0.08	0.08
Total reduced revenue (€):		12.14	22.26
Extra costs:			
Antibiotics (€)	This paper	27	72
Labour (€)	This paper	0	0
Costs penalties antibiotic residues in milk (€)		0	0
Total extra costs (€):		27	72
Net profit (€):		-2.65	-31.71

Table 2. Sensitivity analysis: Effect on net profit of the five most influential input variables (proportional change in output higher than proportional change in input) in the scenario of 3-day or 8-day treatment on a farm with a low probability (R=0.20) of contagious transmission of Staphylococcus aureus.

	Net profit 3-d treatment (€)	Net profit 8-d treatment (€)
Bacteriological cure (%)		
10	-28.71	-83.83
20	-18.29	-73.41
30	-7.86	-62.98
40	2.56	-52.56
50	12.99	-42.13
60	23.41	-31.71
80	44.26	-10.86
90	54.69	-0.43
100	65.11	9.98
Number of new infections caused by an existing infection = R (Lam *et al.*, 1996)		
0.20 (low risk of contagious transmission)	-2.65	-31.71
3.35 (high risk of contagious transmission)	112.68	166.01
Proportion (%) culled for subclinical mastitis		
0	-21.71	-64.39
12 (This paper)	-2.65	-31.71
20	10.06	-9.92
Retention pay off (€)		
0	-21.71	-64.39
506 (De Vos and Dijkhuizen, 1998)	-2.65	-31.71
1000	15.96	0.18
Antibiotic costs (€)		
15	23.62	35.17
27 (This paper; 3-day treatment)	11.62	23.17
38	0.62	10.93
50	-11.38	0.17
72 (This paper; 8-day treatment)	-33.38	-21.83

Discussion

It is widely believed that treatment of chronic subclinical *S. aureus* mastitis during lactation is ineffective and uneconomic. Even so, antibiotics that are specifically registered for lactational treatment of subclinical mastitis are being marketed and promoted in Europe, where the maximum acceptable level of BMSCC is 400.000 cells/ml. Treatment of chronic subclinical streptococcal mastitis during lactation can be profitable when indirect effects of cure, i.e. prevention of clinical flare-ups and prevention of transmission to other animals, are taken into account (Swinkels *et al.*, 2005). In the cost-benefit analysis presented here, we show that the profitability of lactational treatment of *S. aureus* mastitis depends strongly on host, pathogen and herd factors and that treatment is economically justified in many situations.

Table 3. Sensitivity analysis of host, pathogen and treatment factors on net economic result of treatment of subclinical Staphylococcus aureus mastitis during lactation on farms with low (R=0.20) and high pathogen transmission(R=3.4).

Treatment Strain	Predicted Cure (%)	Cow factors (Sol *et al.*, 1997)				Net profit (€)	
		parity	ln CSCC[1]	LS[2]	QL[3]	R=0.20	R=3.4
3-day	0,48	>2	>6,9	0-100 d	hind	-38	-37
PS[4]	1,24	>2	>6,9	100-200d	hind	-37	-33
	3,46	≤2	>6,9	100-200d	hind	-35	-24
	11,11	≤2	<6,9	100-200d	hind	-27	9
	24,42	≤2	<6,9	>200d	hind	-13	66
	36,59	≤2	<6,9	100-200d	front	-1	119
	59,87	≤2	<6,9	>200d	front	23	220
3-day	0,30	>2	>6,9	0-100 d	hind	-38	-37
PR[4]	0,76	>2	>6,9	100-200d	hind	-38	-35
	2,15	≤2	>6,9	100-200d	hind	-36	-29
	7,11	≤2	<6,9	100-200d	hind	-31	-8
	16,53	≤2	<6,9	>200d	hind	-21	32
	26,13	≤2	<6,9	100-200d	front	-11	74
	47,77	≤2	<6,9	>200d	front	10	168
8-day	1,96	>2	>6,9	0-100 d	hind	-92	-85
PS	4,90	>2	>6,9	100-200d	hind	-89	-73
	12,84	≤2	>6,9	100-200d	hind	-80	-38
	33,96	≤2	<6,9	100-200d	hind	-58	-53
	57,08	≤2	<6,9	>200d	hind	-34	153
	70,37	≤2	<6,9	100-200d	front	-20	211
	86,00	≤2	<6,9	>200d	front	-4	278
8-day	1,21	>2	>6,9	0-100 d	hind	-93	-89
PR	3,06	>2	>6,9	100-200d	hind	-91	-80
	8,28	≤2	>6,9	100-200d	hind	-85	-58
	23,97	≤2	<6,9	100-200d	hind	-69	9
	44,91	≤2	<6,9	>200d	hind	-47	100
	59,28	≤2	<6,9	100-200d	front	-32	162
	79,01	≤2	<6,9	>200d	front	-11	248

[1]ln CSCC: Logarithm of Cow Somatic Cell Count;

[2]LS: Lactation Stage;

[3]QL: Quarter Location;

[4]PS: Penicillin Sensitive, PR: Penicillin Resistant.

On farms where contagious transmission of *S. aureus* is likely to occur as a result of suboptimal udder health management, antibiotic treatment is nearly always profitable. On such farms, improvement of management is the first priority in an udder health program, but this can go hand in hand with antibiotic treatment of subclinically infected cows. Risk factors for cure of *S. aureus* mastitis are well known and consistent across studies (Deluyker *et al.*, 2005; Sol *et al.*, 1997, 2000; Ziv and Storper, 1985). When the right cows, the right *S. aureus* strain (penicillin sensitivity) and the right treatment duration are selected on the right farm, lactational treatment of chronic subclinical *S. aureus* mastitis can reach a

remarkable cure which is economically profitable. Cows with induration of udder tissue, multiple infected quarters or other indicators of chronic infection should not be selected for treatment, but early detection and treatment of infected cows, especially young animals, can result in high chances of cure and profitability.

On farms with low risk of *S. aureus* transmission, treatment of an average cow with chronic subclinical *Staphylococcus aureus* mastitis is not profitable. Even in this situation 3-day treatment is profitable when cows are selected with an expected cure probability of approximately 35-40% or higher (Table 3). For example, 1st or 2nd parity cows infected with a penicillin sensitive strain, with CSCC < 1million cells/ml and >100 days in lactation, are eligible for treatment. Extended 8-day treatment is only profitable when the predicted bacteriological cure is approximately 90% or higher. Although a longer treatment will lead to a higher cure, this benefit does usually not outweigh the higher costs of antibiotics and discarded milk. In practice, this means that the preferred treatment duration on well managed farms should not exceed 3 days. Very low risk of transmission can be achieved through strict milking time hygiene, and often needs to be supported by routine testing of milk samples for SCC or bacteria (Lam *et al.*, 1996; Smith *et al.*, 1998; Zadoks *et al.*, 2002). The cost of such testing is not accounted for in the current model. Even with good udder health management, transmission of highly contagious *S. aureus* strains may occur (Smith *et al.*, 1998). Thus, it is very important that cow factors, strain factors and management factors are taken into account when making treatment decisions.

Conclusion

When transmission of *S. aureus* is likely, antibiotic treatment of cows with chronic subclinical *S. aureus* mastitis is often profitable, provided that the right cows are selected for treatment using known risk factors for cure. On farms where contagious transmission is unlikely, extended treatment is nearly always uneconomical, but 3-day antibiotic treatment can be economically profitable when young animals with recent infections with penicillin sensitive strains are selected for treatment.

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Impact of epidemiological, zootechnical, managerial and price factors on the economic efficacy of subclinical mastitis treatment during lactation

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Abstract

A partial budget model was designed to assess the main parameters, not linked to the drug, influencing the economic interest for the farmer to treat subclinical mastitis with antibiotics during lactation. The model assumes that a treatment curing subclinical infections consequently reduces the herd infection prevalence, the bulk milk SCC, the incidence of new infections and clinical mastitis by contagious pathogens, the rate of incurable infections and increases the incidence of environmental mastitis. These effects on the herd mastitis status result in an improved milk quality for SCC, an extra milk production, less culled cows and globally less clinical mastitis treatments. On the other hand, the new treatment generates an extra expense of antibiotics and more unmarketable milk. More than 30 epidemiological, zootechnical, managerial and price parameters were hierarchically ordered according to the amplitude of variation of the economic result when each single parameter varied between extreme values, the other parameters remaining fixed at medium. The three most influencing parameters were the initial CMT scores of the treated quarters, the initial bulk milk SCC and the SCC penalty scheme. The treatment benefit reached a maximum for a BMSCC value situated between the penalty thresholds and decreased towards negative figures when SCC moved away below the lowest or above the highest threshold. The other main influencing parameters were the parity of the treated cows (negative), the rates of spontaneous cure (negative), the on-farm valorisation of the unmarketable milk, the relative risk of new infection in cured quarters (negative), the price of freshening heifer, the treatment cost of a clinical mastitis by contagious pathogens, the cow depreciation time for milk production. Unexpectedly, the proportion of treated infections due to *Staphylococcus aureus* relative to streptococci and the shortage of milk to produce the farm quota had few influence.

Keywords: subclinical mastitis treatment, lactating cows, economic efficacy, partial budget model, parameter hierarchy

Introduction

In the current strategy of mastitis control, the elimination of subclinical infections is performed by dry cow therapy that generally allows better cure rates than treatments during lactation, without milk to discard. On the other hand, dry cow therapy does not reduce the milk SCC during the current lactation and increase the risk of incurability of some infections remaining without treatment until drying off. So, the economical rationality of this strategy,

which was indisputable 30 years ago, has become more and more questionable as and when the dairy context changes especially as regards farm milk quota, milk price penalties according to BMSCC, increasing cow replacement rate in the dairy herds (Seegers *et al.*, 2002).

In other respects, different products injected by the local or systemic route have been recently assessed for their curative efficacy in lactating cows against subclinical mastitis. (Abric and Sérieys, 2001; Deluyker, 2005; Durel *et al.*, 2005).

Basically, the economical interest for the farmer to treat subclinical mastitis during lactation depends for a minor part on few factors linked to the drug, i.e. bacteriological cure rate, milk withholding time, price, and for a major part on a lot of other factors related to the treatment strategy, the epidemiological mastitis status, the herd management, the zootechnical performances and the prices of input and output products. So, the relative interest of the available drugs may be rather easily assessed by comparing them on 3 criteria whereas the situations in which the treatment is economically justified are much more difficult to determine. The main purpose of this study is to identify the main factors not linked to the drug which influence the economical result of the treatment of subclinical mastitis during lactation in the prospect of advising the farmer about the economical interest of this operation and the choice of a pertinent strategy in the particular context of his farm.

Materials and methods

Model

A partial budget model designed to assess the effects of curative or preventive actions incorporating specific products and strategies, was used for this study. This model integrated three modules in cascade corresponding to three steps of effects.

In a first step, the model calculated the health effects. It assumed that the treatment by curing subclinical infections in some quarters consequently reduced the herd infection prevalence, the incidence of new infections and of clinical mastitis by contagious pathogens, the bulk milk SCC, the rate of incurable infections, whereas it increased the incidence of environmental mastitis in the cured quarters which are particularly sensitive to new infections. It was postulated that the treatment had no direct effect on the quarter milk SCC independently of the bacteriological cure.

In a second step, these changes in the herd mastitis status generated zootechnical effects, i.e. gains in kind relative to the milk quality for SCC, the herd milk yield, the number of treatments for clinical mastitis, the number of incurable cows culled.

In a final step, these zootechnical effects resulted in economical effects by converting the former gains in kind to gains in money. The subtraction of the treatment cost, including the expense in products and the loss in discarded milk, gave the final economical result.

The calculation of the different health, zootechnical and economical effects, was performed by periods of ten days for the whole lactations corresponding to the number of cows present all the year. It uses data tables regarding: the milk yield by ten days periods according to the lactation yield, parity and calving month; the relative risk of new infection by ten days periods of lactation according to parity; the monthly repartition of calving when grouped in spring, in autumn or staggered all the year; the positive predictive value of the different CMT scores for the diagnosis of infection; the correspondence between CMT scores

Table 1. List of the model parameters and their medium, low and high values.

Parameters	Values		
	Medium	Extreme low	Extreme high
1 Strategy of treatment			
Parity of the treated cows	30%, 40%, 30% [1]	50%, 30%, 20%	20%, 30%, 50%
Stage of lactation at time of treatment	60%, 30%, 10% [2]	80%, 20%, 0%	30%, 40%, 30%
Age of infection at the time of treatment	80%, 20%, 0% [3]	100%, 0%, 0%	60%, 30%, 10%
CMT scores of the treated quarters	70%, 20%, 10% [4]	90%, 10%, 0%	30%, 50%, 20%
% of quarters treated twice	0%		20%
% of overlaying of intercurrent treatments	10%	0%	20%
Overlaid withholding time	4 days	2 days	6 days
2 Epidemiology			
Staphylococcal / other treated infections	40%, 40%, 20% [5]	20%, 60%, 20%	60%, 20%, 20%
Cure rates correction for infection age	+3%, -3% (6)	0%, -0%	+5%, -5%
Cure rates correction for parity	+5%, +5%, -10% [1]	+3%, +3%, -6%	+10%, +5%, -15%
% cure rates reduction if 2^{d} treatment	70%	50%	90%
Spontaneous cure rates	10%, 20%, 30% [5]	5%, 10%, 20%	15%, 30%, 50%
Clinicity rates of subclinical infections	30%, 50%, 10% [5]	20%, 40%, 5%	40%, 60%, 20%
Interval infection - clinical signs	90 days	60 days	120 days
Bacteriological cure rates of clinical mastitis	50%, 80%, 65% [5]	40%, 80%, 50%	70%, 90%, 80%
Contagion rate during lactation	50 %	30 %	70 %
Prevalence of infected quarters	25%	15%	35%
Relative new infection risk in cured quarters	2	1	3
Contagious /environmental new infections	50%, 50 %	30%, 70 %	70%, 30 %
Environmental new infection clinicity rate	70%	50%	90%
Bacterial cure rates during the dry period	60%, 85%, 70% [5]	50%, 70%, 60%	70%, 90%, 80%
3 Herd management and performance			
Annual milk yield per cow	7000 l	5600 l	8400 l
Marketable milk relative to the farm quota	No shortage	Shortage	
% unmarketable milk given to calves	25%	0%	50%
Annual average BMSCC of marketed milk	300	150	450
Calving period	Autumn	Spring	all the year
Incurable quarters in mastitis culled cows	2	1.5	2.5
Cow depreciation time for milk production	5 lactations	4 lactations	6 lactations
4 Price			
Penalties on milk price for SCC	3, 9, 15 €/1000 l [7]	2, 6, 10 € /1000 l	4, 12, 20 € /1000 l
Annual average milk price	300 € / 1000 l	270 € / 1000 l	330 € / 1000 l
Feeding cost for extra milk yield	100 € / 1000 l	70 € / 1000 l	130 € / 1000 l
Price of the milk substitute for calves	200 € / 1000 l	170 € / 1000 l	230 € / 1000 l
Price of a freshening heifer	1200 €	900 €	1500 €
Price of a culled cow	700 €	500 €	900 €
Contagious clinical mastitis treatment cost	45 €	30 €	75 €
Environmental clinical mastitis treatment cost	60 €	30 €	150 €

[1]respectively for lactation number 1, 2-3, 4

[2]respectively for the intervals 0-60 days, 61-150 days, > 150 days

[3]respectively for 0-60 days, 61-90 days, > 90 days

[4]respectively for CMT scores of +, ++, +++

[5]respectively for S. aureus, streptococci, CNS

[6]respectively for each month ≤ 2 and for each month > 2

[7]respectively above BMSCC threshold of 250 000, 300 000, 400 000 cells/ml

and SCC; the monthly variations of basic milk price and feeding cost. The monthly BMSCC was considered to follow a lognormal distribution with a coefficient of variation of 25% throughout the year.

Parameters

At each step, the calculation of the effects was modulated by specific parameters The table 1 lists the 36 active parameters which was made to vary in the simulations: 7 referred to strategy of treatment, 14 to epidemiology, 7 to herd management and zootechnical performances, 8 to prices.

Considering the purpose of the study, other parameters linked to drug were fixed: administration by the local route; 8 days of treatment plus withholding time; 15 € of drug cost; apparent bacteriological cure rates of 45%, 80% and 60% respectively for *Staphylococcus aureus*, streptococci and CNS. It was also admitted that the quarter milk SCC linearly decreased for 20 days after the bacteriological cure to a minimum level of 200 000, 300 000 and 400 000 cells/ml for cows respectively in their 1^{st}, 2^{nd} or 3^{rd}, 4^{th} or more lactations and that the milk yield was reduced of 2% per 10^5 extra cells of BMSCC. The simulations were made for 20% of cows in the herd treated in 1.5 quarter in average and for a farm milk quota fixed at 275 000 liters.

Range of values and hierarchy of the parameters

On the basis of meta-analysis of the literature and of expert opinions, three levels of parameter values were defined: medium, extreme low and extreme high (table 1).

The hierarchy in influence of the parameters was established according to the amplitude of the variation of the economic result when each single parameter varied between the extreme values, the other parameters remaining fixed at medium.

Results

Effects of the treatment for medium values of the parameters

When the medium values were attributed to all the parameters, the treatment appeared economically justified with a benefit after one year of 178 € (table 2).

The decrease of 20 000 cells/ml in the annual average BMSCC generated a decrease of penalties which represented 54% of the zootechnical gains.

The extra milk yield, the reduction of clinical mastitis treatments, the less number of cows culled for incurable mastitis respectively represented about 15% of the total gain. The discarded milk exceeded the extra milk yield in amount and the cost of treatment product in money.

Hierarchy of the parameters

The three most influencing parameters were the initial CMT scores of the treated quarters, the initial bulk milk SCC and the penalty SCC scheme (table 3), stressing the crucial importance of SCC on the economic result of the treatment.

The initial BMSCC was the only parameter which determined a bell-shaped curve for the treatment benefit with a maximum for an intermediate value situated between the extreme penalty thresholds (figure 1). When it moved away below the lowest or above the highest threshold, the benefit decreased towards negative figures.

Table 2. Sanitary, zootechnical and economical effects of the treatment of subclinical mastitis in lactating cows for model parameters at medium values.

Treatment effects	Amounts	Unit values (€)	Value	
	€	%		
Improved health				
• Infected quarter x lactation (nb)	-3,59			
– *resulting from cure*	*- 3,13*			
– *resulting from less contagion*	*- 0,77*			
– *resulting from re-infection*	*+ 0,31*			
• Clinical mastitis (nb)	- 1,83			
– *including extra environmental mastitis*	*+ 0,29*			
• Incurable infections (nb)	- 1,24			
• Annual average BMSCC (10^3 cells/ml)	- 20			
Zootechnical gains			610	
• Penalties on milk price for SCC	275[a]	1,21[b]	332	54[c]
• Herd milk yield	967	0,10	97	16[c]
• Treatments of clinical mastitis	1,83	43	78	13[c]
• Culled cows	0,62	167	103	17[c]
Treatment costs			432	
• Product	12	15	180	42[d]
• Discarded milk	1681	0,15	252	58[d]
Economical result			178	

[a]annual amount of marketed milk in thousands of litres

[b]reduction of penalties per 1000 l of marketed milk

[c]relative to the total of zootechnical gains

[d]relative to the total of treatment costs

The other main influencing parameters were the parity of the treated cows (negative), the spontaneous cure rates (negative), the on-farm valorisation of unmarketable milk, the relative risk of new infection in the cured quarters (negative), the freshening heifer price, the treatment cost of clinical mastitis by contagious pathogens, the cow depreciation time for milk production. Unexpectedly, the proportion of treated infections due to *S. aureus* relative to streptococci and the shortage of milk to produce the farm quota had few influence on the economic result.

Discussion and conclusion

It appears that the interest to treat subclinical mastitis during lactation is mainly related to SCC through different factors: the selection of quarters to be treated exhibiting enough CMT ++ and +++, an initial BMSCC at intermediate values relative to the penalty thresholds, high levels of penalty for SCC. The inopportuneness of the treatment for initial BSCC beyond the upper SCC penalty threshold is nevertheless not true if the regulation limit for milk sale is exceeded.

As already stressed by Deluyker (2005), the parity of the treated cows appeared very influential because it modulates several other factors in a way unfavourable to the oldest

Table 3. Hierarchy of the parameters according to the variation of the economic result when each parameter varies from high to low value.

Parameters	variation %[1]
CMT scores of the treated quarters	365
Annual BMSCC of the marketed milk	177
Penalties on milk price for SCC	124
Parity of the treated cows	-122
Spontaneous cure rates	-119
% unmarketable milk given to calves	94
Relative infection risk in cured quarters	-73
Price of a freshening heifer	70
Contagious clinical mastitis treatment cost	53
Cow depreciation time for milk production	48
Price of a culled cow	-47
Stage of lactation at time of treatment	-45
% of quarters treated twice	-43
Prevalence of infected quarters	-43
Cure rates during the dry period	-42
Staphylococcal / other treated infections	-39
Contagious/environmental new infections	38
Marketable milk relative to the farm quota	38
Incurable quarters in mastitis culled cows	-37
Feeding cost for extra milk yield	-33
Annual milk yield per cow	29
Contagion rate during lactation	24
Interval infection beginning - clinical signs	24
Cure rates correction for infection age	23
Environmental mastitis treatment cost	-19
Age of infection at the time of treatment	-15
% of overlaying intercurrent treatments	15
Cure rates of clinical mastitis	-15
Calving period	10
Price of the milk substitute for calves	-10
Overlaid withholding time	7
% cure rates reduction if 2^{d} treatment	-5
Clinicity rates of subclinical infections	3
Environmental new infection clinicity rate	-3
Cure rates correction for parity	2
Annual average milk price	0

[1]relative to the economic result for parameter medium value

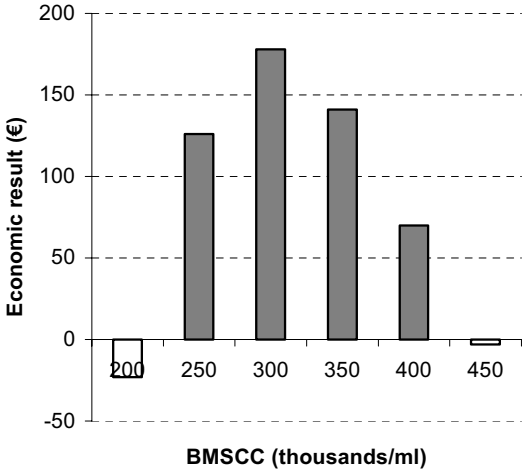

Figure 1. Economic result of the treatment of subclinical mastitis during lactation according to the initial bulk milk somatic cell count.

cows: lower cure rates, lower SCC when infected, higher SCC when cured, higher new infection rate, lower residual dairy value of the cow if culled.

The spontaneous cure rate influences as much as the apparent cure rate, the real cure rate of the treatment which is the pertinent measure of the drug efficacy. This factor appears all the more important to consider since the spontaneous cure rates of subclinical mastitis observed in different studies were very variable (Abric and Sérieys, 2001; Deluyker, 2005; Durel *et al.*, 2005).

The relatively small difference in the economic result according to the dominant pathogen, *S. aureus* or streptococci, can be explained by the short duration of the advantage due to the better cure rate of the streptococcal infections which, more often than the staphylococcal ones, are going to be eliminated some weeks later by treatment of clinical mastitis or at drying off.

The usual reduction of the marketable milk following treatment may explained the better economic result in case of over-realisation of the quota: the amount of actually marketed milk remains unchanged in this situation. For the same reason, the milk price influences the economic result only in case of under-realisation of the quota: then, higher the price, lower the economic result, excepted if the quota under-realisation was specifically due to the withdrawal of milk from cows with high SCC. Nevertheless, the variations of the economic result linked to the amount of marketable milk are relatively small because the increase of discarded milk following treatment is more or less compensated by an extra milk yield.

In conclusion, the treatment of subclinical mastitis during lactation appears to be economically justified as a transitory measure to accelerate the reduction of BMSCC in herds engaged in a control program. The treatment must be targeted to CMT positive quarters with at least 25% of CMT ++ or +++, preferably in young cows at the beginning of the lactation.

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Stochastic economic modeling of the use of penethamate hydriodide (Mamyzin®) in heifers around calving to control peri-parturient mastitis

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Abstract

In the spring of 2002 a prospective cohort study was carried out on the effect of the parenteral use of 10 g penethamate hydriodide (Mamyzin®) on heifers around calving to prevent mastitis within a commercial dairy herd in Central Southland, New Zealand.

Treated heifers showed a significant reduction in the incidence of mastitis (22.8% vs. 41.0%; RR = 0.52, p=0.029) after calving, and treatment was associated with an increased milk production (p=0.06 at first herd test after calving, corrected for days in milk). Cost benefit and sensitivity analysis using a deterministic model showed an overall return on investment (ROI) of NZ$186 per heifer, or 6.18 times the investment (based on NZ$30 treatment cost).

Using the key indicators derived from this model (daily production, expected mastitis incidence, milk and heifer value), a stochastic model was produced to determine the sensitivity of the ROI to different scenarios with varying herd size and production. The model showed that the ROI essentially varied between 4 and 10 and had a very close mean over a high number of iterations of around 7.2.

These data suggest that, presented with a group of heifers, with a high risk of peri-calving mastitis, therapy at or around calving with Mamyzin® would likely be of significant preventative and economic benefit.

Keywords: heifers, penethamate hydriodide, stochastic model, economics, return on investment (ROI)

Introduction

The peri-calving period is the commonest risk period for mastitis infection, with reported incidence varying worldwide. The most recent survey in New Zealand found that the overall incidence of clinical mastitis in heifers varied widely across farms from 0% up to > 61% (Pankey *et al*., 1995). In New Zealand, most cows and heifers calve outside on pasture, with large numbers of animals calving over a very short period of time.

In many herds, heifers are disproportionately represented in pericalving mastitis outbreaks, and this group of animals also presents the greatest challenge in treatment and management. With most pericalving infections detected between 24 and 72 hours post-calving, at a time when management is directed at settling newly calved heifers into a large

herd and into an alien milking routine, any further complications such as mastitis and its treatment present real challenges for both dairy management and animal welfare.

Recently, much success has been found with parenteral treatment of intramammary infections in heifers. The advantages are many: simplicity of administration; simultaneous treatment of multiquarter infections; no risk of teat canal damage in small heifer teats; cost-effectiveness; and efficacy.

Given the growing body of evidence that heifers detected with mastitis within 96 hours of calving may have been infected for a varying period pre-calving, attention has been drawn to pre-treatment of heifers at risk, prior to calving. The advantages of this in the welfare of the newly-calved heifer and the improvements in her management are significant.

Pre-treatment of heifers has been performed at various stages prior to calving. Treatment shortly before or around calving would be expected to be of greater benefit by shortening the period of risk prior to calving and any subsequent mammary flushing.

Materials and methods

96 heifers from one of the smaller dairy herds in Central Southland, New Zealand, had been synchronized to calve in early spring (August onwards). Of this group, 39 became untreated (controls); 57 were treated. Treatment was 10g of penethamate hydriodide (Mamyzin®, Boehringer Ingelheim Vetmedica GmbH) at 7 days before expected calving date.

Heifers calved within a mean time period of 3.47 days (SD 3.47) after treatment with a range of 1 - 10 days. Heifers that did not calve within 7 days received a second treatment (n = 3 heifers). All heifers calved outside on grass.

Both a cost benefit analysis and sensitivity analysis were performed on the data from this study, to assess key indicators that determined any potential economic benefits to the wider dairy farming community. The data from this study was used to develop a stochastic model to predict the economics of this approach to managing mastitis within a larger and/or differently producing herd.

Results

The study demonstrated that treated cows showed a significant reduction in the incidence of mastitis (22.8% vs 41.0%; RR = 0.52, p=0.029) after calving, and treatment was associated with an increased milk production (p=0.06 at first herd test after calving, corrected for days in milk).

The key economic indicators identified by the initial deterministic model were, in order of significance: daily production, expected mastitis incidence, milk value and heifer value. These factors were modeled in the stochastic model using random number generation (RNG) within predetermined limits to produce complex series of calculations with an infinite number of possible variations. This allowed us to determine the sensitivity of the ROI to the 4 key indicators.

The values of these factors were modified to reflect more realistic NZ values. In particular, the key variables had the following predetermined minimum-maximum values:

- value of heifers (NZ$600-1100)
- value of kgMS (milk solids) (NZ$3.5-5.0)
- expected daily MS production (1.5-2.4kg)
- expected mastitis incidence (10-90%)

In addition, the number of heifers in a group was in the range of 100-400.

The sensitivity analyses showed that the ROI essentially varied between 4 and 10 and had a very close mean over a significant number of iterations (each iteration = 200*n*) of around 7.2. The analyses showed that the number of heifers in the group had almost no effect on ROI.

However, ROI was reasonably sensitive to:

- Daily MS $R^2 \sim 0.4$
- Expected mastitis incidence $R^2 \sim 0.3$

Less sensitive to:

- MS $ value $R^2 \sim 0.2$
- Heifer $value $R^2 \sim 0.1$

And not at all sensitive to:

- n heifers $R^2 \sim 0.05$

Discussion and conclusions

Mastitis in heifers not only has a large economic impact, but also presents a significant risk to the welfare of heifers. Newly calved heifers are under extreme stress from adapting to a new environment, new herd situation, new human interactions, and facing new immunological challenges.

Pericalving mastitis in heifers varies in incidence from farm to farm and season to season. There is evidence that glands may become infected long before calving- possibly long before mating in young animals, however the risk factors for infection have yet to be satisfactorily identified. In the absence of robust data identifying risk factors that would allow management changes to minimize the risk of mastitis to heifers, it seems sensible to seek other means of either prevention or amelioration of effects of infection.

As discussed, first preliminary results from other workers indicate that parenteral treatment with penethamate hydriodide (Mamyzin®) shortly before or around calving seems to be efficacious (Kreiger *et al.*, 2005). Parenteral treatment is not only effective but has significant benefits over intramammary treatment. There is no risk of damage to teat canal integrity, and the risks to operators are far lower.

The benefits shown by this study, in both halving the risk of mastitis and also in the very significant cost benefits, suggest that this regime offers a viable option in the management of this disease. Furthermore, the comprehensive stochastic modeling has shown that the economic benefits are present under a wide range of conditions.

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Importance of uncertainties in the effects of pathogen specific mastitis on profit in dairy herds estimated by stochastic simulation

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Introduction

The method of stochastic simulation modeling has been suggested as a relevant approach to assess the economic value of mastitis control strategies (Allore and Erb, 1999; Seegers *et al.*, 2003). One important element in such models is the quantification of each of the various effects of mastitis. The direct effects of mastitis on health and production, include increased milk withdrawal, elevated somatic cell count, infection risk of herd-mates, increased risk of culling and death and reduced feed intake, body weight and milk yield. These effects may vary due to different mastitis pathogens and severity of the mastitis case. In the present literature, the estimated effects of mastitis vary substantially. The objective of this study was to develop a model simulating mastitis control in dairy herds and to investigate how sensitive the model is when varying the effect parameters according to the uncertainty.

Material and methods

Model structure

The model developed in this study was an extended and modified version of the mechanistic, dynamic and stochastic dairy herd model called SimHerd III (Østergaard *et al.*, 2003). Overall the extension includes modeling of SCC and mastitis.

The SCC of the individual cow is modeled by the natural logarithm of cells/ml of milk as a function of parity, lactation stage, mastitis occurrence and with a Gaussian random effect. Because differences have been found in effect of mastitis depending on the pathogen involved (Haas *et al.*, 2002; Gröhn *et al.*, 2004), the model simulates pathogen specific mastitis types. Nine mastitis types based on agents are represented:

1. *Staphylococcus aureus*;
2. *Streptococcus dysgalactiae*;
3. *Streptococcus uberis*;
4. *Eschericia coli*;
5. *Klebsiella* spp.;
6. *Acanobacterium pyogenes*;
7. Minor pathogens;
8. No pathogen isolated;
9. *Streptococcus agalactia*.

Subsequently, the numbers are used as reference for these mastitis types. The model structure and variables are the same for all mastitis types. Specific attributes of the individual mastitis types are represented through different parameterizations of the mastitis variables.

Mastitis occurrence was defined as the event, where a mastitis pathogen begins (week of occurrence) to cause subclinical or clinical mastitis symptoms in the affected cow. State variables of the individual cow include for each mastitis type: infectiousness, severity, parity, and days after occurrence of a previous mastitis case. The severity is represented by subclinical and four clinical severities: mild, moderate, severe and permanent effect. A new case of the same mastitis type is forced to have the same severity as a previous occurrence of the same mastitis type, if the severity of the new case otherwise would have caused less reduction in milk yield. The effects of mastitis are specific to each mastitis type and to each of the five severities within each mastitis type.

The risk factors for each clinical mastitis type includes lactation stage (base line) parity (1, 2 and 3+), yield level (continuous), presence of previous cases of the same mastitis type, presence of previous milk fever case, season (two seasons) and contagious spread of the infection from herd mates.

Occurrence of mastitis is modeled to have direct effects on feed intake, body weight, milk yield, SCC, subsequent mastitis cases in the cow herself and herd mates (described under risk factors), voluntary and involuntary culling, mortality and milk withdrawal. As the effects of the different mastitis types and severities are represented in the model no additional effect of SCC on milk yield is included. The effect of the subclinical, mild clinical and severe clinical occurrence of mastitis is modeled by scale variables, which scale proportionally the effects of the moderate cases on feed intake, body weight, milk yield and SCC. The effect of clinical cases with permanent effect is modeled by a proportional permanent reduction in the yield capacity of the affected cow.

The effect of non-permanent cases on daily feed intake, body weight gain and milk yield are specified by proportions of without-the-disease level according to the time since occurrence. These effects on milk yield, weight loss and feed intake are interrelated in the model. The effect of mastitis on SCC is modeled similarly as for the effect on milk yield, including the proportional scaled effect according to severity. However, the effects are specified as additive absolute effects on the number of SCC per ml. of milk. Milk withdrawal due to mastitis treatments is modeled for a specifiable number of days after clinical mastitis occurrence.

Default parameterization of the model

The default parameter values were intended to represent a typical loose housing system and management strategy for a 200-cow dairy herd with additional young stock. The default parameterization of mean Ln(SCC) in cows not affected by the modeled mastitis cases was based on Haas *et al*. (2002) and a standardized normal distribution of 0.875 was assumed.

The default values of the variables for the distribution among the mild, moderate, severe and permanent clinical cases were specified as 0.40, 0.50, 0.10 and 0.00, respectively, for mastitis types 3, 4, 5, 7 and 8. This distribution among the mild, moderate and severe was suggested by Seegers *et al*. (2003) based on a review on the effect of mastitis on milk yield. For mastitis type 1 and 2 the default distribution were 0.40, 0.50, 0.05 and 0.05, respectively, based on an assumption of 5% of severe clinical cases resulting in blinding

off the affected quarter. For mastitis type 9 we assumed all of the severe clinical cases result in a dry quarter, specified by the distribution: 0.40, 0.50, 0.0 and 0.10. For mastitis type 6 we assumed 50% of all clinical cases result in a dry quarter specified by the distribution: 0.20, 0.25, 0.05 and 0.50.

The default values for the effects of the mild and severe clinical cases compared to the effects of moderate cases on feed intake, body weight, milk yield and SCC, were specified to be 0.10 and 2.5, respectively. These values are also based on suggestions from Seegers *et al*. (2003). The default values for the effects of the subclinical cases, compared to the effects of moderate clinical cases on these responses, were specified to be 0.50. The default value for the permanent effect of permanent clinical cases on yield capacity was specified to be a 15% reduction.

The default values of the variables for the effect of clinical mastitis on mortality are specified as a 0.3% and a 2.0% risk of death at the time of occurrence of mastitis, where the high risk is applied for the gram negative pathogens (mastitis types 4 and 5). Furthermore, this default value was chosen to represent that no cow is culled for slaughter directly caused by mastitis at the time of mastitis occurrence and that the effect of mastitis on voluntary culling is represented by the indirect effect of milk yield.

The default values of the variables for the effect of moderate clinical mastitis on daily feed intake were specified as a 7% reduction in the first week of mastitis occurrence and linear decline of -0.33% per day thereafter based on Bareille *et al*. (2003) and Østergaard and Gröhn (2000). The default values for the variables for the effect of moderate clinical mastitis on milk yield were based on the estimates for multiparous cows of Gröhn *et al*. (2004). The default values of the variables for the effect of moderate clinical mastitis on daily weight gain were specified so that the body weight was unaffected due to the mastitis occurrence (Østergaard and Gröhn, 1999; Seegers *et al*., 2003).

The default values for the variables for the effect of clinical mastitis on milk withdrawal were based on 7 days withdrawal from the day of mastitis occurrence. The default values for the variables for the effect of moderate clinical mastitis on SCC were based on results of Haas *et al*. (2002).

Simulated scenarios

We simulated 17 scenarios to study model behavior and model sensitivity (Table 1). The scenarios for studying model behavior were very general reduction in mastitis incidence and mastitis effects. The scenarios for studying model sensitivity were selected to represent potential key uncertainties on mastitis effect parameters.

Analysis of scenarios

Each scenario was simulated over 10 years and replicated 500 times. From each replicate we used average annual results from the last 5 years of simulation.

The economic consequences of each scenario were studied by applying a set of assumed year 2005 Danish prices and costs for the different technical results. These include a milk price of € 0.282 per kg energy corrected milk (ECM), which is scaled according to bulk tank SCC (200.000 being the highest threshold). The value of slaughter cows was € 1.43 per kg body weight. The cost of veterinary treatment of a case of clinical mastitis was assumed to be € 90. Herd profit was calculated as sales income less variable costs (feed, AI's, veterinary assistance, medicine and other costs) for cows and heifers. Labor and management

Table 1. List of scenarios to study model behavior and model sensitivity.

Abbrev.	Description
Model behavior	
DEF	Default
NOMA	Elimination of all mastitis types
NOIN	Elimination of infectious mastitis types (type 1, 2, 7 and 9)
NOEN	Elimination of environmental mastitis type (type 3, 4, 5, 6 and 8)
MILD	All clinical cases being mild clinical cases
Model sensitivity	
SUB1	Half of subclinical cases assumed to be clinical cases
SUB2	Subclinical cases assumed to have effect on feed intake, milk yield, body weight and SCC like moderate clinical cases
PER	Moderate and severe clinical cases assumed to be permanent cases
NOPER	Permanent cases assumed to be non-permanent clinical cases
MOD	Non-permanent clinical cases assumed to be moderate cases
DRY	Effects of non-permanent mastitis cases assumed to stop at dry off regarding feed intake, body weight, milk yield and SCC
CUL1	Clinical cases assumed to reduce the insemination period by 28 days
CUL2	Clinical cases assumed to reduce the insemination period by 56 days
MOR1	Effect of clinical mastitis on cow mortality assumed to be zero
MOR2	Effect of clinical mastitis on cow mortality assumed to be doubled
WD1	4 days milk withdrawal after mastitis treatment assumed
WD2	10 days milk withdrawal after mastitis treatment assumed

costs were not included as variable expenses. To study how sensitive the model is when varying the mastitis effect parameters, we compared the contrast between scenarios DEF and NOMA with the contrasts between each of the sensitivity scenarios and the scenario NOMA.

Results

Technical and economic effects of the scenarios DEF, NOMA, NOIN, NOEV and MILD are presented in Table 2.

In the scenario DEF the incidence per cow-year of the mastitis types 1 through 9 were: 0.053, 0.055, 0.108, 0.065, 0.004, 0.018, 0.043, 0.071 and 0.003, respectively. The major items of the account resulting in the € 146 per cow-year, when eliminating all mastitis (NOMA), were € 119 from milk, € 4 from sale of pregnant heifers, € -16 from feed consumption and € 38 from less veterinary cost. The milk sale item is mainly increased milk yield, but also a € 0.001 increased milk price per kg ECM, which is a result of lower BTSCC. By dividing with the corresponding number of mastitis cases per cow-year in the scenario DEF the effect of scenario NOMA can be expressed as € 148 per case of mastitis (subclinical and clinical) case and € 347 per case of clinical mastitis. The latter figure assumes that subclinical cases are inherent parts of clinical cases.

Table 2. Technical and economic effect of the scenarios DEF, NOMA, NOIN, NOEV and MILD.

	DEF	NOMA	NOIN	NOEV	MILD
Output variable	Mean (sd.)	Avg. effect relative to default scenario			
Replacement %	33.7 (1.6)	-0.83	-0.37	0.35	-0.19
Avg. slaughter cow, kg	559 (4.2)	5.7	0.9	4.9	3.1
Dead cowsa	0.027 (0.051)	-0.002	0	-0.002	0
Sale of preg. Heifers[1]	0.16 (0.02)	0.005	0.003	0.003	0.001
Feed intake, SFU[1,2]	5905 (21)	77	42	34	44
Kg ECM[1]	9167 (47)	385	218	159	190
Kg withdrawal[1]	61 (5)	-61	-22	-37	8
Average 1000 SCC	169 (2)	-29	-17	-10	-23
Inc. of cli. mas.[1]	0.42 (0.02)	-42.0	-15.1	-26.5	-1.0
Inc. of subcli. mas.[1]	0.56 (0.03)	-56.3	-40.7	-15.2	2.4
Net return[1], €	1517 (13)	146	75	73	52
Net return / kg ECM, €	0.165 (0.001)	0.009	0.004	0.005	0.002
Vet. treatment cost[1], €	53.5 (2.4)	-37.5	-13.4	-23.8	-0.8

[1]Per cow-year

[2]SFU = Scandinavian feed unit

From the sensitivity scenarios the scenario PER showed 54% higher loss in net return due to mastitis, compared to the scenario DEF. This was provided by an even higher sensitivity in term of reduced income from milk sale, which was partly compensated by the largest reduction in feed cost due to mastitis. The scenario SUB2 and SUB1 showed 36 and 31% higher loss in net return due to mastitis compared to the scenario DEF. This was provided in scenario SUB2 by even higher reduction in income from milk sale (172 kg ECM per cow-year produced less), which was slightly compensated by saved feed cost for cows (32 SFU per cow-year). The milk withdrawal was not affected significantly but the SCC was increased by 21000, which caused a 0.4% reduction in milk price. In scenario SUB1 a 65% increase in veterinary cost contributed significantly. The scenario MOD showed 54% higher loss in net return due to mastitis compared to the scenario DEF, which was essentially caused by the effect on milk sale. In the remaining scenarios the sensitivities was less than 5%.

Discussion

The results of € 146 per cow-year increase in net return from eliminating mastitis do not include any control costs for labor, investments, etc. If these control costs were zero, the economic potential in mastitis control would be equivalent to the value of extra 508 kg (5.5%) ECM per cow-year. The scenarios NOEV and NOIF both increased the economic net return by half of the increase associated with eliminating all mastitis types. This indicates equal economic importance of the two types of mastitis. The scenario MILD resulted in an increased net return of € 52 per cow-year, which is equivalent to 36% of eliminating all mastitis.

Seegers *et al*. (2003) argued that it is not very relevant to compare the results from simulation studies obtained in different spatiotemporal contexts. Consequently, we will just

provide a few examples. Bennett *et al*. (1999) estimated total economic impact of clinical mastitis to be 119 £ per cow-case. In our study we did not estimate the specific effect of the clinical occurrences. However, our estimate for the cost per case (clinical and subclinical) was 14% lower than the estimate of Bennett *et al*. (1999). The average cumulative impact of mastitis (clinical and elevated SCC) was 78 EUR per cow year in a French study (Seegers *et al*., 2003). The estimate in our study was 86% higher.

Among existing simulation models, the presented extended SimHerd model bears the closest similarity to the SIMMAST (Allore *et al*., 1998) and ECOMAST (Seegers *et al*., 2000) models, but still some differences do exist. The SimHerd IV differs by simulating more pathogens, simulating with weekly time steps (as opposed to daily time steps), a cow can have more mastitis types simultaneously, effects of mastitis on feed intake and body weight are simulated, the effects of mastitis on milk yield and SCC can be simulated as independent or correlated responses, and long term interactions between mastitis control and feeding, reproduction and culling strategy can be simulated.

The sensitivity in terms of net return was highest when moderate and severe clinical cases were assumed to be permanent cases. As this is an extreme assumption we consider the sensitivity in the order of 36 and 31% provided by scenario SUB2 and SUB1 to be more important. These sensitivities emphasize the importance of the representation of the effect of subclinical mastitis when evaluating mastitis control strategies. The sensitivity of scenario MOD was 11%. The inclusion of different severities and not just one average effect of a certain mastitis type, was in itself a way to include directly the uncertainty on the biological effect of mastitis. Consequently, this sensitivity of 11% could be interpreted as a reduction in the effect of mastitis when uncertainty of the effect parameters is included in the model. From the remainder scenarios with sensitivities below 5%, the corresponding uncertainties of mastitis effect parameter is considered to be of minor importance.

Based on this sensitivity analysis the model appears to have a low sensitivity to uncertainty on most of the addressed parameters. Representation of the effect of subclinical mastitis and of variation in mastitis severity was concluded in this study to be important when modeling mastitis economics in dairy herd.

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Costs and benefits of improved milking practices in smallholder dairy farms in the 10th region of Chile

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Abstract

Chile is keen to expand exports of dairy products and so has introduced regulations to improve milk quality through financial incentives. Penalties for BMSCC start at 250,000 cells/ml and go up to minus 22% of the milk price at >750,000 cells/ml and penalties for TBC start at 20,000 cfu/ml and go up to minus 10% of the milk price at >400,000 cfu/ml.

A study was carried out to investigate the principal management factors that influenced bulk milk somatic cell count (BMSCC) and total bacterial count (TBC) of 150 smallholder dairy farms in the main dairy area of Chile (Van Schaik *et al.*, 2005). The predictions of the TBC and BMSCC models were used to estimate the TBC and BMSCC levels of herds with specific management measures. A division could be made in management practices that required low, moderate and high investment from the smallholder dairy farms and based on that three scenarios were considered. In the best case scenario all management practices were successfully applied, in the worst case scenario none of the beneficial practices were carried out, and in the cheap scenario only the low cost management practices were applied. The calculations were carried out for an average smallholder with 11 lactating cows that produce about 30,000 kg with an average milk price of 116 pesos (€0.16) with the standard penalties and bonuses. The extra net present value after 10 years was calculated for the three scenarios.

The study predicted that in the best management scenario a herd had a mean TBC of 1525 colony forming units (cfu)/ml and a BMSCC of approximately 46,166 cells/ml. Whereas a herd in the worst management scenario had a predicted TBC of $59x10^9$ cfu/ml and a mean BMSCC of $2x10^6$ cells/ml. In the cheap management scenario a herd would have a predicted TBC of 235x103 cfu/ml and a mean BMSCC of 215x103 cells/ml. The extra net present value that the worst, best and cheap management herd would gain after 10 years amounted -€4072, €980, and €333, respectively. The worst management herds would loose a considerable amount and the best and cheap management herds would have an extra increase in their yearly income from milk of 4.6% and 1.6% respectively.

Introduction

Eighty percent of the Chilean dairy herds and 66% of Chilean milk is produced in the 10^{th} Region (INE, 1997). About 84% of the 11,000 dairy herds in the 10^{th} Region are considered smallholder dairy farms. These are often subsistence farmers with 2 - 40 cattle that produce less than 100,000 kg of milk per year (Amtmann *et al.*, 1995). The farmers frequently have other enterprises (beef calves, poultry, and vegetables), use family labour

and live off their own produce. They sell milk to obtain a continuous flow of cash to support their family and the property can also serve as a guarantee to obtain credit from a bank (Amtmann *et al.*, 1995). The minimum monthly wage in Chile is about 130,000 pesos (€ 178).

Milk is usually collected by hand into buckets and poured into churns or collected by machine directly into churns. The churns are then left at the farm entrance and collected by a local co-operative milk collection centre (MCC) once or twice per day. At the MCC the quality of the bulk milk from each farm (bulk milk somatic cell count (BMSCC)) and total bacterial count (TBC) is checked before it is added to milk collected from other farms and cooled in a large refrigerated tank. There are 42 MCC in the 10th Region of Chile with 2,200 producers delivering their milk to these MCCs. The association is voluntary but restricted by the geographical location of the farm relative to the MCC. The number of producers for each MCC varies during the year, but on average an MCC has 56 producers delivering milk throughout the year (ACOLECHE, 2003, personal communication).

Good milk quality is important for a profitable dairy industry. The EU, New Zealand and Australia require that milk used for dairy products have BMSCC levels below 400,000 cells/ml, Canada below 500,000 cells/ml, and the USA below 750,000 cells/ml (Sargeant *et al.*, 1998, Norman *et al.*, 2000, Van Schaik *et al.*, 2002). In terms of bacterial standards, regulatory penalties are typically paid on milk with TBC>100,000 bacteria/ml. Chile is keen to expand both its liquid milk and processed milk markets to other countries and so has introduced penalties to improve milk quality, these penalties are also applied to smallholder dairy farmers. The basic milk price is about 115 Chilean pesos (€0.16) per kg and for milk with a bacterial count of less than 20,000 cfu/ml a bonus of 7.2 pesos per kg is paid. The penalty for milk with a bacterial count over 400,000 cfu/ml is 3.8 pesos per kg of milk. A smallholder dairy farmer that reduces the bacterial count from the highest to the lowest category can gain 11 pesos (€0.02) per kg of milk. The financial difference between low (<250,000) and high (>750,000) SCC milk is 25.5 pesos (€0.03). It is therefore important for the prosperity of these farmers that they produce milk of the highest quality.

Recently, a study was carried out to determine the factors that improved milk quality from smallholder dairy farms in the 10th Region of Chile (Van Schaik *et al.*, 2005). The purpose of this paper was to determine the financial returns when management was changed and milk quality improved.

Material and methods

Risk factors for high TBC and BMSCC

One hundred and fifty smallholder dairy cattle farmers were randomly selected from 42 MCCs in the 10th Region of Chile using sampling proportional to size of MCC. The herds had to produce milk all year round to be included in the study. The questionnaire used by Tadich *et al.* (2003) was changed to an interview format and adapted to include practices used by smallholder dairy farmers. In April and May of 2002 the two data collectors visited all 150 farms with the MCC veterinarian (when available). The fortnightly tests on BMSCC and TBC in the two months prior to the survey were obtained for each farm from the relevant MCC. The BMSCC was estimated using Fossomatic^{TM} FC (Foss A/S, Denmark). The MCCs tested bacterial count in one of two ways, Bactoscan^{TM} FC (Foss A/S, Denmark) or reduction time of methylene blue. The mean BMSCC and mean TBC of the four observations in the two

months prior to the visit were used as the dependent variables in the analyses. These variables were normalised by a natural logarithm transformation (LN).

The management factors were tested in two multiple linear regression models, one each for mean LNBMSCC and mean LNTBC as the dependent variables. Backward elimination was used to exclude non-significant (P>0.05) management factors from the saturated models. Variable were forced in the models to correct for herd size, yield and the effect of the two methods of bacterial counting (BactoScan™ and reduction time of methylene blue).

Multivariable mixed regression models were used to correct for the possible clustering of herds within MCC. For more detailed information see the paper of Van Schaik *et al*. (2005).

Economic calculations

The predictions of the LNTBC and LNBMSCC models were used to estimate the TBC and BMSCC levels of herds with specific management measures. A division could be made in management practices that required low, moderate and high investment from the smallholder dairy farms and based on that three scenarios were considered. In the best case scenario all management practices were successfully applied, in the worst case scenario none of the beneficial practices were carried out, and in the cheap scenario only the low cost management practices were applied (see Table 1). The calculations were carried out for an average smallholder with 11 lactating cows that produce about 30,000 kg with an average milk price of 116 pesos (€0.16) with the standard penalties and bonuses. It was assumed that the farmers will spend one hour extra when all management changes are applied. A 10-year period was assumed for writing-off costs of a cement waiting yard and the cooling tank. The interest rate was assumed 18%, a high figure because smallholders can not easily get funding from banks. The costs and benefits were assumed to be the same each year. The extra net present value after 10 years was calculated for the three scenarios based on the penalties or bonuses for the milk.

Results

Descriptive statistics

One to 10 farms from each of the 42 MCC, were included in the study. The herds had on average 28 (SD 21.6) ha. of land and 11 (SD 8) milking cows that produced 7.3 (SD 4.7) kg of milk per cow per day. The mean rate of mastitis was approximately 17 (SD 21) cases per 100 cows per year.

The TBC was not determined by 7 MCCs, which excluded 30 herds. The TBC of 82 herds was based on BactoScan™ and for the remaining 38 farms from 11 MCCs the reduction time of methylene blue was used. The mean TBC was 332,000 (SD 822,000) cfu/ml; 40% of the herds had a TBC below 100,000 in the four fortnightly observations. The mean TBC estimates were not significantly different by method of measurement. The geometric mean BMSCC was 408,000 (SD 309,000) cells/ml: 45% of the herds had a BMSCC < 400,000 cells/ml in the four fortnightly observations. The LNTBC did not follow a perfectly normal distribution. For BMSCC both the Kolmogorov-Smirnov test statistic (*P*>0.20) and the histogram indicated that the LNBMSCC was normally distributed.

Multivariable model for LNTBC without random effect for MCC

The final model for mean LNTBC explained 35% of the total variance. In the null model the random effect of MCCs was highly significant. It was explained by three covariates: milk collected once a day or less compared with collection twice a day, not cleaning the bucket after milking mastitic cows versus cleaning the bucket and cooling milk in a vat of water versus not cooling milk or using ice or a bulk tank to cool milk. Other factors that increased the LNTBC were a waiting yard with a soil or gravel floor versus concrete, use of plastic buckets for milking instead of metal buckets, not feeding California Mastitis Test (CMT) positive milk to the calves and cows of dual purpose breed. From the model it was predicted that a herd that did not comply with any of these management factors had a predicted TBC of $59x10^9$ colony forming units (cfu)/ml whereas a herd that complied with all the management practices had a mean predicted TBC of 105 cfu/ml.

Multivariable model for LNBMSCC

The model of mean LNBMSCC explained 18% of the variance; the random effect of MCC was not significant. Management factors that decreased the mean LNBMSCC were: using the CMT for one year versus using the test for more than one year or not at all, absence of a concrete waiting yard, not filtering the milk or using filters other than a plastic sieve to filter the milk, milking cows with mastitis last, and sometimes or always examining the udder before milking. A herd that complied with all of these management factors had a BMSCC of approximately 46,166 cells/ml whereas a herd that did not comply with all management practices above had a mean BMSCC of $2x10^6$ cells/ml.

Costs and benefits of improved milk quality

Table 1 contains the yearly costs and benefits of the three scenarios for the smallholder dairy farms and the extra net present value after 10 years.

In the worst management scenario a farm would have very high TBC and BMSCC levels and would loose €906 in yearly income as a result of the low quality of the milk. In the best management scenario a farmer would have very low TBC and BMSCC and a high milk price which generated a yearly extra milk income of €590. The extra net present value after 10 years would only be €980 as a result of the high costs of especially the milk cooling tank. Farmers that complied with the cheap management changes would still have a fairly high TBC but that penalty is overruled by the bonus for the low BMSCC and they would have a higher milk price and gain an extra €139 per year. With low costs for their management changes the extra net present value in 10 years would amount €333.

Discussion

It is unlikely that all herds would make such improvements in BMSCC and TBC since other, unmeasurables influence the outcome of change. However, for simplicity we assume that the predicted changes in TBC and BMSCC were accurate. The estimates give an indication about the potential gain in profitability of smallholder dairy farms.

Smallholder dairy farmers can increase their income by improving the quality of their milk. In a worst case scenario in which a farmer does not apply any of the recommended management practices a farmer would yearly loose an extra €906. A farmer that would apply

Table 1. The extra net present value for three management scenarios to improve milk quality of smallholder dairy farms.

	Costs	Worst management	Best management	Cheap changes
Metal buckets and sieves for milking	€ 40	No	Yes	Yes
Examine the udder before milking	Extra labour; €4/hr	No	Yes	Yes
Milk mastitic cows last	Extra labour; €4/hr	No	Yes	Yes
Wash the bucket after milking an mastitic cow	Extra labour; €4/hr	No	Yes	Yes
Using CMT fortnightly and not adding CMT positive milk to the bulk milk	CMT, labour and discarded milk; 7kg/day × €0.16/kg × 7days × 2 cows/yr =€16	No	Yes	No
Cooling the milk with ice-bottles	Electricity for freezing bottles; €5	No	No	Yes
Milk cooling tank	€2,200	No	Yes	No
A waiting yard with a cement floor	€400	No	Yes	No
Predicted TBC		58,820,442	1,525	235,626
Milk price differentiation for TBC (in pesos)		-3.8	+7.2	-3.8
Predicted BMSCC		1,943,498	46,166	215,346
Milk price differentiation for BMSCC (in pesos)		-18.3	+7.2	+7.2
Yearly benefits (in €); milk production 30,000 kg/yr.		- €906	+€590	€139
Yearly costs (in €) for management changes		€0	€372	€65
Extra net present value in 10 years (in €)		- €4.072	€980	€333

all recommended management practises would gain an extra €218 per year which is 4.6% of the total yearly milk returns (€4,750).

Not all farmers would be able to obtain a loan from a bank for a cement waiting yard or a milk cooling tank. However, when these farmers would apply only the cheap management changes they would make a yearly extra profit of €74, which is 1.6% of the total yearly milk returns.

A factor that was not included in the calculations was whether milk was collected once or twice a day. Van Schaik *et al*. (2005) predicted a decrease in TBC of 58,000 cfu/ml when milk was collected twice relative to once a day. MCC do not collect more often because of the long distances and high expenses but it would be worthwhile to determine the extra profit they may obtain when milk quality improves with more frequent milk collection.

This study has demonstrated that a considerable gain in milk quality and therefore profitability could be obtained if smallholder dairy farms in Chile complied with management practices reported in an earlier study of Van Schaik *et al*. (2005). Some of the recommendations such as use of metal buckets for milking, always examine the udder before milking, milk mastitic cows last, wash the bucket after milking a mastitic cow, use of CMT and discard CMT positive milk could be applied immediately and against low costs. The financial gain for a herd that decreases TBC and BMSCC can be considerable.

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Udder health, treatments and pathogens in organic dairy herds in the Netherlands

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Abstract

83 organic dairy farmers in the Netherlands were questioned about management and risk factors for poor udder health, treatment of mastitis and their strategies for good udder health. Milk recording data, including cow somatic cell count (SCC), and bulk tank milk cell count (BMSCC) from 2001 until 2003 were collected. All quarters of high cell count cows (> 250,000 cells/ml; heifers>150,000) were sampled for SCC and pathogens. The aim of the study was to determine the range of problems in udder health and to investigate management and treatment strategies practiced by farmers to maintain good udder health in organic dairy herds.

Sub clinical mastitis is a frequent occurring health problem in organic dairy herds in the Netherlands. The percentage of cows with high SCC ranged between 10 - 52. Eighty percent of the farmers used antibiotics against clinical mastitis as the only treatment or in combination with other treatments such as frequent milking, homeopathy and massage with peppermint oil. Homeopathy was used on more than 50% of the farms but only 10% of the farmers used it without antibiotics. The percentage of high cell count cows on farms using antibiotics to treat clinical mastitis ranged between 10 - 46; on farms not using antibiotics in treating clinical mastitis this figure ranged between 12 - 52%. Some farmers succeed in maintaining good udder health with preventive measures without the use of antibiotics. Ten percent of BMSCC were above the Dutch penalty limit of 400.000 cells/ml milk. *Streptococcus uberis* (19.6%), *Staphylococcus aureus* (16.1%) and *Staphylococcus not aureus* (45.1%) were the most common pathogens in the culture positive samples of high SCC cows. Types of pathogens in organic milk samples did not significantly differ from those in conventional milk samples.

Keywords: strategies, alternative, SCC

Introduction

In organic dairy farming udder health is one of the main topics: it causes milk losses by decreasing production and discarded milk from bulk tank, it attacks animal welfare, treatment takes labour and medicine costs and it is bad for farmers welfare to be confronted regularly with diseased cows. Information about udder health of organic cows in the Netherlands was scattered. In previous research (Eijk *et al.*, 2003, Groot *et al.*, 2003) the importance of mastitis was stressed. There was a lack of information about implementation of preventive measurements on practical dairy farms, frequency of different treatments, type and application of medicine, the effect of treatments and farmers attitude concerning udder

health. Simultaneously with the questionnaire for udder health, one questionnaire with topics about Johne's disease and one about parasites were performed.

Material and methods

All organic dairy farmers in the Netherlands were invited to join the research by filling in a form with some questions about date of conversion, size of the farm and the herd and the possibility to gather information about milk production and somatic cell count (SCC). After a selection based on this criteria, from September - December 2003 84 farms were visited with a questionnaire. Except some common questions about the farm, the questions focussed on udder health: frequency of clinical mastitis, type of treatment in subsequent cases, effect of different treatments, management practices and preventive measures on the farm.

All milk recording data from January 2001 till December 2003 were available from the *Nederlands Rundvee Syndicaat* (NRS) and also data about cows breed, date of birth, calving dates were collected.

All cows with a SCC above 250.000 cells/ml (heifers above 150.000 cells/ml) in one milk recordings in the period September - December 2003 were sampled at four quarters for quarter somatic cell count (QSCC) and pathogens. Samples were taken according to instructions of the Animal Health Service, stored at -20^{o} C and analysed for Somatic cell count and pathogens according IDF procedures.

Percentage of high SCC cows (%HCC) per period were accounted as the number of cows with a SCC above 250.0000 cells/ml (heifers above 150.000 cells/ml), divided by the total number of cows per milk recording date. The percentage of new cows with a high SCC is the number of cows with a high SCC and a low SCC in the previous milk recording, divided by the total number of cows.

All data were stored in a Microsoft Access databank.

Results

About 80% of the organic cows in the Netherlands are Holstein Frisian (HF). Meuse Rine Yssel (MRY) is about 7 percent and Jersey (J), Fries Hollands (FH), Brown Swiss (BS) and Montbeliarde (MB) breeds all score about 2 - 2.5 percent. In young stock the amount of other breeds but HF is growing. The number of calvings per year is on average 57 per farm with about 28% of the calving from heifers. The average age at calving is 4 years and 4 month and is about 6 month older than conventional cows in the Netherlands. Organic heifers calf for the first time at an age of 28 month, one month later than conventional ones. The average interval between calvings is 412 days.

The average 305 days milk yield is 7181 kg with 4.32% fat and 3.38% protein. Compared to the average yield of Dutch cows, organic cows have a 900 kg milk lower yield and have lower fat and protein content, 0.07 and 0.08% respectively. The lactation of organic cows lasts on average 346 days. Ninety-seven percent of the farms houses the cows in cubicle houses, 14 percent in deep litter barns and only 7 percent in tied bars. Somatic cell count in bulk milk (BMSCC) was on average 262.000 cells per ml milk. The amount of bulk milk tanks with a BMSCC above the penalty limit (> 400.000 cells/ml) decreases (Table 1).

Table 1. Percentage of bulk milk tanks per cell count class per year

	*Somatic cell count class (BMSCC *1000)*				
Year	<101	101-200	201-300	301-400	>400
2001	1.2	30.6	39.0	19.5	9.8
2002	2.4	31.4	39.9	16.8	9.4
2003	1.8	32.2	40.9	16.1	9.0

Somatic cell count

The average SCC for first and second calvers and older cows during the lactation are given in Figure 1. After a steep decline in the first two weeks of lactation, there is a gradually increase in SCC for all three parities. At the end of lactation, the heifers average just over 100.000 cells/ml milk, the second calvers reach 160.000 cells/ml and the older cows average just over 250.000 cells/ml at the end of lactation.

The percentage of HCC per month and year is given in Figure 2. The average HCC is 28% and well over the border set for conventional cows. In the summer period the SCC raises with about 10% and declines in autumn to basic level again. The amount of cows with a new high SCC varies around 10% and is comparable with conventional standards.

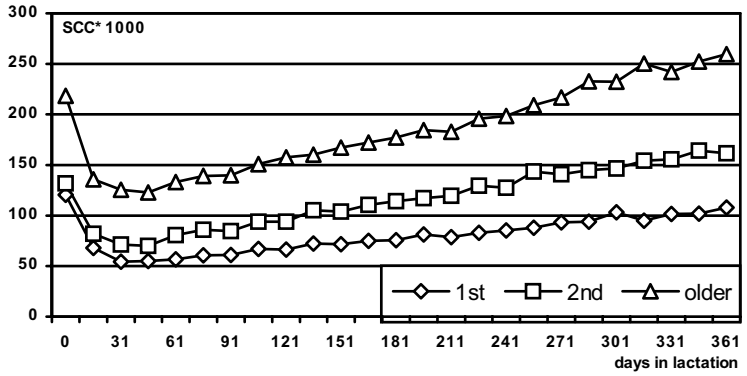

Figure 1. Somatic cell count in organic dairy cows during lactation.

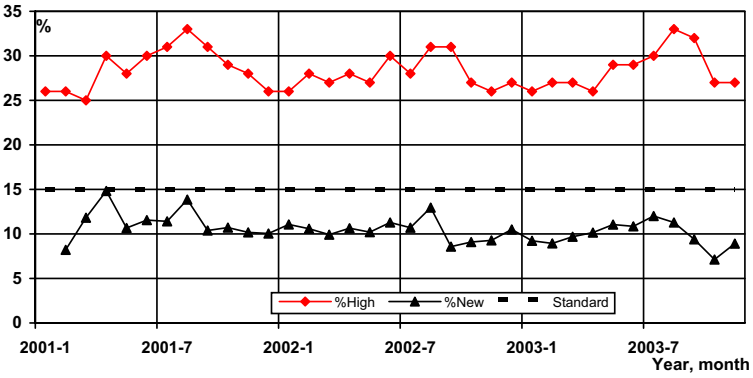

Figure 2. Average percentage of high somatic cell count cows per period.

Preventive measures

Figure 3 shows that in three different housing systems, the range in percentage HCC is about the same. Farms with deep litter stables are longer organic and a larger part is biodynamic in stead of ecological. In Table 2 an overview of preventive measures is given divided by barn type. The average incidence of (sub)clinical mastitis in cubicle housing seems lower than in the other housing systems. At farms with deep litter housing, more farms had their one drinking water supply and hygienic status were not optimal. At deep litter barns with a high hygienic standard for both milking parlour and milking equipment, percentage of sub clinical mastitis was 24.5 compared to 34.5% in deep litter barns with insufficient hygiene at milking. Compared to farms with cubicle barns, at deep litter barns farmers used more washable towels instead of disposable towels for pre milking udder cleaning and teat cubs were not cleaned with hot water after milking a cow with (sub)clinical mastitis. With washable towels on deep litter barns up to 8 cows per towel were cleaned compared to maximum 5 cows per disposable towels. At 50% of the deep litter barns farmers did not use any medication for dry cow therapy (90 percent of the cows or more). Another difference in management between housing systems was the treatment of clinical mastitis. A division was made between the treatment of the first mastitis cases and the repeated cases. The use of antibiotics in deep litter barns was lowest for both types of mastitis. At deep litter barns, farmers used more peppermint oil especially for first cases instead of antibiotics. With regard to sub clinical mastitis, 70% of the deep litter barn farmers did not act at all, while about 40% of the farmers with cubicles acted by frequent milking, massage with peppermint oil or use of homeopathy. Seventy percent of the farms sprays or dips the cows post milking (mostly a Iodine dip or spray) and on two third of the farms no complete check of the milking equipment was carried out in the last 3 years.

Type of treatment

Table 3 shows the percentage of the farms acting in case of (sub) clinical mastitis. In case of clinical mastitis, farmers take more actions than in case of sub clinical mastitis. Separation of cows with mastitis is not a popular action: on farms practising separation and practicing frequent milking %HCC are remarkably below the average %HCC. Application of antibiotics increases dramatically in recidivist cows instead of alternative treatment

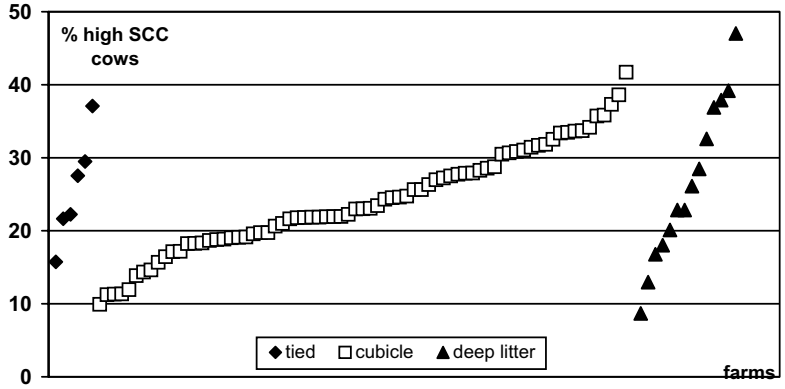

Figure 3. Percentage high SCC cows and housing system.

Table 2. Factors affecting SCC in relation to barn type presented as the percentage of positive cows or positive farmers.

Barn type	Tied	Cubicles	Deep litter
Percentage of cows			
Clinical mastitis cows (incidence)	25	20	24
Sub clinical mastitis cows (incidence)	26	24	28
Percentage of farms			
Drinking water from tap	71	43	30
Hygienic insufficient	14	11	20
Extra minerals for dairy cows and/or for dry cows	33	75	55
Cows may lay down after milking immediately	100	72	20
Separate milking mastitis cows	70	20	20
Washable towels for cleaning udder pre milking	71	43	60
Cleaning teat cubs with hot water after mastitis cow	71	57	30
No medication for dry cow therapy	29	34	50
Treatment: first mastitis with antibiotics	57	53	10
Repeated mastitis with antibiotics	86	65	50
First mastitis with mint oil massage	57	57	70
Repeated mastitis with mint oil massage	29	35	40

Table 3. Percentage of farms per action in (sub) clinical mastitis cows.

Cause of action	# actions	no action	separate group	other action	antibiotics	homeopathy	mint oil	freq. milking	culling
Recidive subclinical mastitis	116	30	5	6	13	29	19	10	27
1st clinical mastitis	169	4	4	6	46	48	59	41	
Recidive clinical mastitis	177	5	5	10	64	22	33	26	52

(massage, frequent milking) or alternative medicines. The range in percentage of HCC on farms using antibiotics or no antibiotics as a treatment of clinical mastitis is about the same (Figure 4). In both groups farmers are able to achieve low percentage of HCC.

Pathogens

In organic cows with sub clinical mastitis about 60% of quarters are not infected with any pathogen. That is comparable with conventional cows with sub clinical mastitis. In organic cows Staphylococcus not aureus is the most common pathogen, followed by Staphylococcus aureus and Streptococcus uberis (Table 4). That is very similar as the importance in conventional cows.

Discussion

The longer farmers practice organic farming, the more they deviate from their less experienced organic and their conventional colleagues. More variety in breeds, more variety

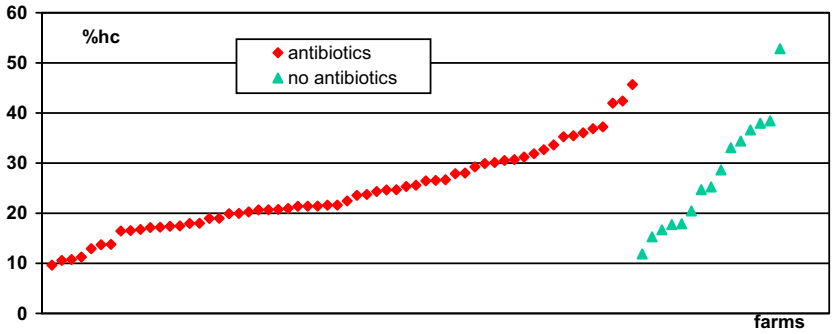

Figure 4. Percentage high SCC cows on farms with and without antibiotics.

Table 4. Precentage of samples and type of pathogens in positive samples of cows with sub clinical mastitis.

Farm type	Organic	Conventional
Staphylococcus not aureus	46.0	40.9
Staphylococcus aureus	15.8	18.6
Streptococcus uberis	17.5	16.4
Bacillus	8.3	10.9
Streptococcus dysgalactiae	7.8	5.7
Escherichia coli	1.0	2.7

in housing systems and more alternative treatments and medicine. Organic dairy farms have higher percentage of HCC compared to conventional farms, but equal numbers of cows with clinical mastitis. Also most common pathogens seems similar on both types of farms. There are big differences in preventive measurements between farms, especially in dry cow management, hygiene at milking and milking procedures (pre milking teat cleaning, rinsing teat cubs with hot water or separate milking of infected cows). Although some farmers prove to be able to work well without antibiotics, most farmers are not to confident in the effect of alternative medicine or find themselves not experienced enough to choose the proper treatment for a diseased cow. Preventive measures depend on farmers attitude. Successful treatments on one farm were said to be unsuccessful on other farms under different management. The combination of measures and treatments fitting to the farmer leads to success. In alternative treatment it is important that each individual farmer finds the proper set of measures for the specific circumstances on his farm.

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High milk yields and the risk of mastitis in different herd environments

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Abstract

It is well known that a high milk production increases the risk of mastitis. In this study the association between milk yield, milk composition and Somatic Cell Counts (SCC) were analysed in 4006 Dutch herds. As expected there was a close association between milk yield at start of lactation and the incidence of SCC-peaks later in lactation. The higher the milk yield the more likely that SCC-peaks occurred. Fat-protein ratios were non-linearly related to SCC-peak rates. Both a lower and a higher fat/protein ratio at the start of lactation increased the risks of SCC-peaks. Interestingly, there was considerable variation across herds, both in SCC-peak rates and in the association of SCC-peaks and milk yield. Unexpectedly the incidence of SCC peaks was lower in herds with a high average milk yield per cow. The highest producers in these herds, however, did have the highest risk of elevated SCC. In herds with both a low average production and a low fertility of the cows there was no association between milk yield and SCC-peaks. Thus a higher production does not always increase the incidence of mastitis. We hypothesize that in herds with an intensive management both a higher production and a lower mastitis incidence is achieved, but that mastitis cannot be prevented in these herds for the highest producing cows.

Keywords: SCC-peak rates, herd environment, milk yield, fat/protein ratio, genetic correlations

Introduction

In dairy cattle both high milk yields and high fat/protein ratios have been indicated as risk factors for mastitis (Heuer *et al.*, 1999; Ingvartsen *et al.*, 2003). The concern has even lead to suggestions milk production above a certain limit should not be allowed. Mastitis and milk yield are genetically positively correlated (De Haas *et al.*, 2002a; Simianer *et al.*, 1991; Van Dorp *et al.*, 1998). On a phenotypic scale mastitis is not or negatively correlated to milk yield, since infections lead to a decrease in production (e.g. Schepers and Dijkhuizen, 1991; Sloth *et al.*, 2003). This cannot be taken as an indication that high producing cows are at a lower risk, since a high production prior to infection may still be a risk factor. One way to solve this problem is to look at milk yields before infection either in the same lactation (Bartlett *et al.*, 1991) or in previous lactations without infection (Houben *et al.*, 1993).

Somatic cell counts (SCC) are in use as a routine measurement to detect mastitis cases. Although not all cases of clinical mastitis lead to an observable increase in SCC for monthly test day records, it has been proven as a useful tool to monitor mastitis incidence. Recently different patterns of increase and decrease of SCC have been linked to different pathogens

of mastitis (De Haas *et al.*, 2002b), especially when inferences are made for herds or large sire-daughter groups.

This research focuses on the relation between milk production and mastitis as defined by SCC patterns. It particularly addresses the question whether high producing cows show more SCC peaks. The analysis is performed at different levels, from the national level down to the herd and genetic level. By using SCC patterns inferences can be drawn from larger data sets than ever possible in experiments with more detailed measurements (e.g. bacteriological counts).

Material and methods

The data set contains data of 5000 herds with cows calving in the period 1st of September 1994 till 1st of March 2000. The cows had at least 75 percent Holstein Friesian genes. The herd had at least 50 heifers calving in this period, and at least 5 years of data in the data set. Furthermore the herds had to participate during this period in the national milk recording scheme, the herd classification program for conformation and use artificial insemination. This resulted in a data set of 4006 herds with for each lactation test days every 3 or 4 weeks. Lactations with 2 or less test days were ignored leaving a total of 1 962 752 lactations.

Somatic Cell Counts and production data were corrected for lactation stage (see Windig *et al.*, 2005a for details). Lactations were split into periods with low SCC and periods where SCC peaked, and production data were split into three periods: before during and after SCC-peaks. Four SCC peak patterns were defined following de Haas *et al.* (2002b) (Figure 1). Pattern 1 is a short and intense peak. The peak period lasts only one test day. This pattern is significantly more associated with environmental pathogens, in particular *Escherichia coli*. Pattern 3 is defined by at least two consecutive test days with a SCC>400000, preceded by a test day with a SCC < 150000. Such a pattern is often associated with infections of contagious pathogens in particular *Staphylococcus aureus*. Pattern 2 is in between pattern 1 and 3. There is as in pattern 1 only one test day with a SCC > 400000, but the previous and next test day both have a SCC between 150000 and 400000, and the peak does not last longer than 3 test days. Pattern 4 is defined as all peaks that do not fit in one of the other patterns.

At the national level SCC peak rates were compared with production levels before the start of the first SCC-peak in a lactation. For kilogram milk and fat/protein ratio test days were split into test days before, during and after the SCC peak period. For each lactation values for the production in these periods were calculated by taking the average over test days. Since production was corrected for test day averages can be compared directly. Lactations were grouped according to average production level before SCC-peak, and SCC-peak rates calculated per group. Statistical tests for comparing production levels always yielded highly significant ($P < 0.0001$) differences, due to the high number of lactations in the data set, and are not specified further.

To quantify variation between herds, correlations between production and SCC-peak (scored as 0 = no peak and 1 = peak) were calculated within the 53 largest herds (all herds in the dataset with > 100 lactations for heifers and cows). All herds were grouped according to average production levels within the herds, and the correlation within and across groups calculated (details in Windig *et al.* 2005b). Variation in the genetic correlation between

milk production and SCC level was estimated with a reaction norm model (following Calus *et al*. 2005).

Results

Overall rate of SCC-peaks was 27.8%. More peaks occurred in multiparous cows than in heifers (Table 1). In heifers pattern 1 (fast) was the most common pattern of infection while in multiparous cows pattern 4 (other) was the most common. Rates of pattern 3 and 4, thus the longer lasting infections, were each about 4-6% higher in multiparous cows, while rates for pattern 1 and 2 was only about 1-2% higher.

The frequency of SCC-peaks of animals grouped according to their average milk yield before the SCC peak or, if no SCC peak occurred in the entire lactation, the average milk yield of the entire lactation shows that high producing cows suffer more SCC-peaks later in lactation (Figure 1). At pre-peak milk yields below average daily production around 20% of the animals show SCC-peaks, whereas at a daily milk yield prior to infection of more than 20kg above average around 80% of the animals have SCC-peaks. Average pre-peak milk yield was highest for pattern 3 and 4. Milk yield decreased sharply during SCC-peaks. During SCC-peaks animals with patterns 1 and 2 suffered the highest drop in milk yield (up to 32 kg daily milk yield). There is also a clear relationship between f/p ratio and SCC-peak rate. This relationship is, however, not linear (Figure 1). Peak rates increase both for f/p ratios lower and f/p ratios higher than average. The ratios during SCC-peaks depend on the pattern. For pattern 1 and 2 the ratios increase (=relatively more fat), while for pattern 3 and 4 the ratios decrease (=relatively more protein).

The correlation between milk yield and SCC-peak *within* herds ranged from -0.375 to +0.597 for heifers and from -0.071 to + 0.708 for cows, while correlations with f/p ratio varied from -0.395 to +0.548 (heifers) and from -0.272 to +0.230 (cows). Across herds SCC-peak rates decreased with increasing average production levels, while within herds the correlation of SCC-peaks with milk production was higher for herds with high average production levels (Figure 2). Thus in herds with low average production all cows had similar risks for SCC-peaks, while in high production herds only the highest producing cows had high risks. The genetic correlation between milk yield and SCC decreased, however, from 0.315 (low production) to 0.163 (high production).

Table 1. Number of analysed lactations and rate of lactations with SCC-peak patterns (in brackets pattern of consecutive test days characterising pattern).

SCC Peak Pattern	Parity			
	1		2	
no infection	537 757		879 690	
1 (LHL)	52 267	7.9%	125 950	9.7%
2 (LIHIL)	7 981	1.2%	31 130	2.4%
3 (LHH..)	18 415	2.8%	91 688	7.0%
4 (other)	44 851	6.8%	173 043	13.3%
total infection	123 514	18.7%	421 791	32.4%

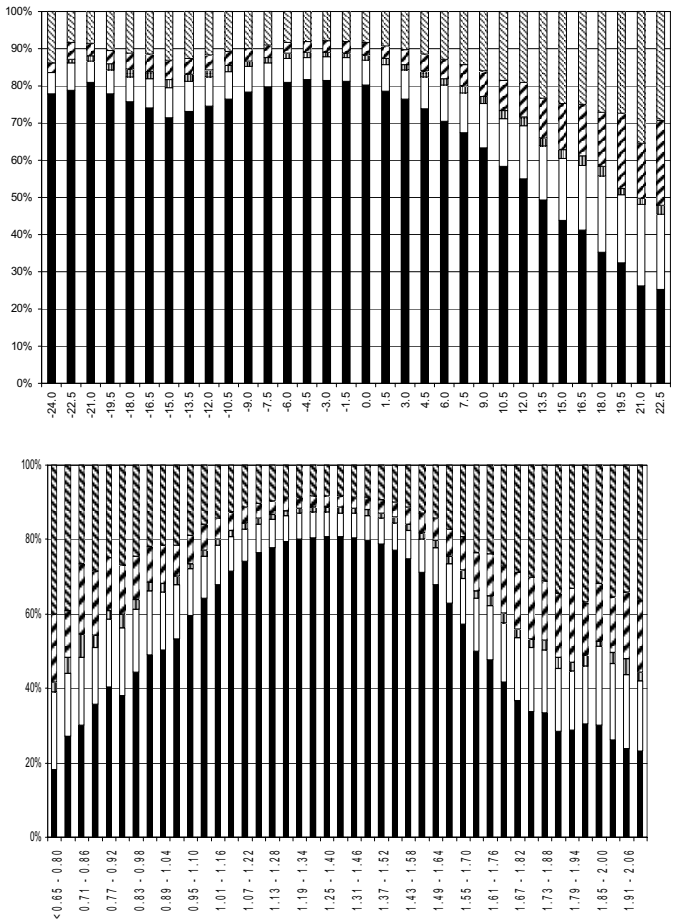

Figure 1. SCC peak incidence rates at different levels of milk production (left) and fat/protein ratio (right). Daily milk yield is based on test days prior to peak and expressed as deviation of daily milk yield in kg from standard lactation curve. Fat/protein ratio is calculated as deviation of ratio from standard lactation curve. Each deviation class is given as the range of fat/protein ratios (for 200 days in milk to 14 days in milk) of a lactation curve with that deviation.

Discussion

There is a clear relation between milk yield, fat-protein ratios and SCC-peaks. In this study we showed that animals with a SCC-peak have a higher milk yield and either a higher or lower fat/protein ratio before the peak than animals for which SCC levels remain low throughout the lactation. Although at the individual level (absence of) a SCC peak is not the same as (absence of) mastitis, at the herd or higher level elevated SCC levels indicate more mastitis. Thus we can conclude that a high milk yield increases the risk of mastitis. This is in accordance with the review of Ingvartsen *et al.* (2003) who concluded that "mastitis is the only disease with a clear relationship with milk yield". By using test-days and SCC patterns we were able to quantify this relationship on a nation wide scale.

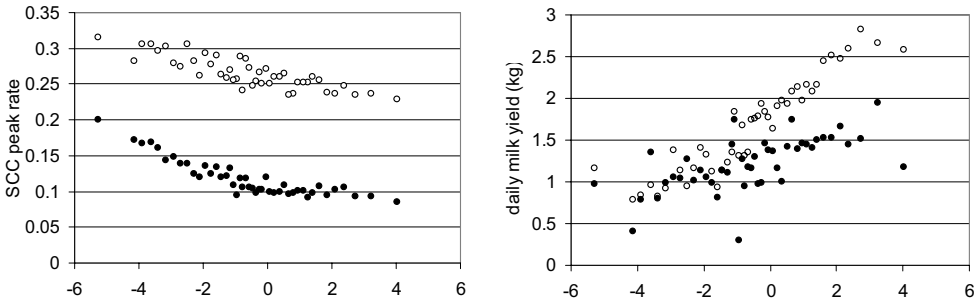

Figure 2. Relationship herd environment, production and SCC-peak rates. Herds are grouped according to production levels. X-axis is score of first Principal Component summarising 45 herd characteristics into average production levels per cow. Left: SCC peak rate per herd group, right: difference in milk yield between lactations with and without SCC-peaks. Solid circles heifers, open circles cows.

Milk yield was not the only factor identified as a risk factor. The fat protein ratio was the second factor in this study with a clear relationship with SCC peaks. Surprisingly both an increased and a decreased ratio increased the risk of mastitis. Higher infection rates with a higher fat/protein ratio is in agreement with Heuer *et al*. (Heuer *et al*., 1999) who reported a higher mastitis risk at fat/protein ratios above 1.5. They, however, did not report higher risks with decreasing ratios. De Vries and Veerkamp (2000) reported that a high initial fat percentage at the start of lactation and a lower fat percentage later in lactation is related to a negative energy balance. Thus a negative energy balance might play a role in the relationship between fat/protein ration and mastitis, but the exact relationship is not clear. It is noticeable that the lowest SCC-peak rates occur at average milk yields and f/p ratios, which are the most frequent milk yields and f/p ratios in the population.

Although clearcut patterns between milk production and SCC-peaks were found at the national level, this does not mean that a high milk production automatically leads to a higher risk of mastitis infections. Indeed, there is considerable variation across herds in the correlations between production and SCC peaks. Interestingly less SCC-peaks are found in high production herds. Probably, it is not only the production level that changes with increasing production levels, but also other aspects of management such as overall hygiene and medication. Although in these high production levels less SCC-peaks occur, and thus less mastitis, this is not the case for the highest producing cows in these herds. Thus it seems that the management is effective in reducing mastitis only up to a certain level of production.

Acknowledgements

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Association between somatic cell count in early lactation and culling of dairy heifers using Cox frailty models

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Abstract

The association between the somatic cell count (SCC) of dairy heifers in early lactation (SCCel) [measured between 5 and 14 days in milk (DIM)] and the culling hazard during the first lactation was studied using Cox frailty models. Udder health problems were the culling reason for 10% of the culled heifers. For each unit increase in the natural log-transformed SCCel (LnSCCel), the culling hazard increased by 11% (Hazard ratio (HR) = 1.11). The strength of the association depended on five factors. Firstly, the association was stronger when SCCel was recorded after 10 DIM than at an earlier DIM. Secondly, the association was stronger if only culling events for udder disorders were considered (HR = 1.32) instead of all culling events (HR = 1.11). Furthermore, for each unit increase of test-day LnSCC after 14 DIM, modelled as a time-varying covariate, the culling hazard in the first lactation increased by 26% (HR = 1.26). Including LnSCC in the model already containing LnSCCel, reduced the estimate of LnSCCel slightly. Fourth, a higher test-day milk yield, modelled as a time-varying covariate, protected against culling and reduced the magnitude of the effect of LnSCCel as well when taken into account. Finally, the association between LnSCCel and culling was still present, although smaller in size, in the group of heifers with a second test-day SCC ≤50,000 cells/mL.

Keywords: Cox frailty model, culling, dairy heifer, early lactation somatic cell count

Introduction

Mastitis is an important culling reason in cows (Beaudeau *et al.*, 1995; Barkema *et al.*, 1998; Gröhn *et al.*, 1998; Rajala-Schultz and Gröhn, 1999a, 1999b). Nearly 11% of heifers that were treated for clinical mastitis before calving or within the first 14 DIM were culled within one month after treatment (Waage *et al.*, 2000). The main culling reason for 96% of these heifers was mastitis. Elevated SCCel in heifers, suggesting presence of an intramammary infection around calving, is associated with elevated test-day SCC and higher probabilities of test-day SCC >200,000 cells/mL (De Vliegher *et al.*, 2004a), with an increased probability of clinical mastitis during the first lactation (Rupp and Boichard, 2000), and with lower milk production (Coffey *et al.*, 1986; De Vliegher *et al.*, 2005). Moreover, elevated SCCel in heifers could be associated with an increased culling hazard during the first

lactation, possibly to some extent because of the aforementioned effects. The indirect effects of disease (including mastitis) may be reflected by, for instance, milk yield (MY) (Gröhn *et al.*, 1997) because most diseases cause a decline in MY, either temporarily or longer lasting (Gröhn *et al.*, 1998). Comparing models with and without MY helps to estimate the direct and indirect effects of mastitis in general on culling (Rajala-Schultz and Gröhn, 1999b).

The objectives of the study were twofold:

1. to examine the association between SCCel of heifers and culling during the first lactation while accounting for the day of assessment of SCCel and for the variability between herds, and

2. to determine what part of the effect of SCCel on culling acts indirectly through increased test-day SCC and decreased MY, by adjusting the models for test-day SCC and MY, modelled as time-varying covariates.

Materials and methods

Data set and data handling

Dairy Herd Improvement (DHI) data (Flemish Cattle Breeding Association, Oosterzele, Belgium) were used in the present study. In short, 14,234 heifers belonging to 3264 herds (enrolled in the Belgian DHI program) that calved between January 1, 2000 and December 31, 2000, and of which the first test-day took place between 5 and 14 DIM, were followed up until 365 DIM, drying off or culling. The primary culling reason was recorded by the farmer (low milk production, reproductive disorders, udder disorders, foot/leg problems, behavioural problems, death, and non-specified reasons). Heifers for which it was unclear whether they were culled or not and heifers belonging to herds that stopped activities during the study period were omitted for further analyses, resulting in data from 13,835 heifers belonging to 3192 herds. In total, the dataset contained 114,906 test-day records measured after 14 DIM. A heifer was considered to be culled or censored at its last available test-day. Somatic cell count was measured in composite milk samples collected from two successive milkings (Fossomatic 5000, Foss Electric, Hillerød, Denmark).

Two datasets were created based on the full dataset. An event was defined as culling for all reasons combined in the first dataset (CULLall; 3204 events), and as culling for udder disorders only in the second dataset (CULLudd; 325 events).

Statistical analysis

The association between LnSCCel and the culling hazard was studied by a semi-parametric Cox model (Cox, 1972). The Cox model was extended to a frailty model by introducing herd as a random effect to account for the clustering of heifers within herds (Duchateau and Janssens, 2004). The time to culling information for the k^{th} heifer from herd j that was assessed at DIM equal to i was given by (t_{ijk}, δ_{ijk}), where t_{ijk} stands for the time of culling, and δ_{ijk} was equal to 0 if the heifer was censored and to 1 in the case of culling.

Seven different models were fitted using CULLall and CULLudd respectively, with breed incorporated as a fixed effect in all models. Both natural log-transformed test-day SCC (LnSCC) and test-day MY (measured between 15 and 365 DIM) were considered time-varying covariates when included in the models. In order to adjust for the fact that (Ln)SCCel was not assessed at the same day after calving for each heifer, the Cox models were stratified according to DIM (5 to 14) on which (Ln)SCCel was measured in the period called "early

lactation". This means that for each DIM value (each stratum) another baseline hazard function was assumed.

In the first model, LnSCCel was introduced assuming a constant effect over the different DIM in early lactation (Model 1). In the second model, the same relationship between LnSCCel and the culling hazard was studied, but a different relationship between LnSCCel and the culling hazard was allowed according to DIM on which LnSCCel was recorded (Model 2). These two models were compared with each other based on the likelihood-ratio test. Models 3 and 4 contained LnSCC and MY, respectively. Models 5 and 6 contained both LnSCCel (as in Model 2) and LnSCC, and LnSCCel (as in Model 2) and MY, respectively, to evaluate the changes in LnSCCel when the time-varying covariates were included separately. Model 7 was the full model containing LnSCCel (as in Model 2), LnSCC, and MY.

Hazard ratios (HR) with 95% confidence intervals were obtained for all covariates. All models were fitted with S-Plus 6.0 for Windows (Insightful Corp, Seattle, US).

Results

Descriptive analysis

Geometric mean SCCel of the 13,835 heifers was 111,000 cells/mL, ranging from 5,000 to 25,000,000 cells/mL. In total, 3204 heifers (23.2%) were culled during their first lactation. The two main specified reasons of culling were reproductive disorders and low MY. In total, 325 heifers (2.3% of all heifers and 10.1% of the cows culled in first lactation) were culled for udder health disorders as the primary reason. No reason was specified in 40.5% of all cullings.

Heifers with a higher SCCel were more at risk of being culled during lactation (Figure 1). For instance, at 100 DIM, 3% of the heifers with a SCCel ≤50,000 cell/mL were culled,

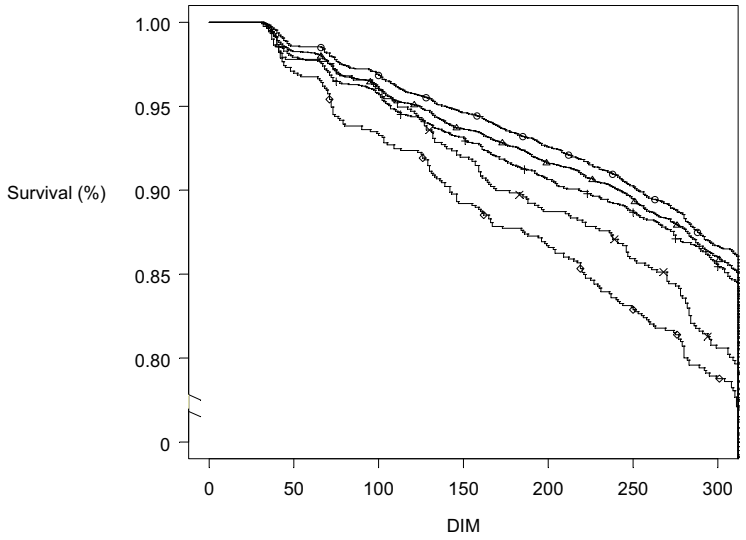

Figure 1. Kaplan-Meier graph of culling of heifers for all reasons (until 305 DIM) with a SCC in early lactation (SCCel, measured between 5 and 14 DIM, x 1000 cells/mL) of 0 to 50 (○), 51 to 200 (Δ), 201 to 500 (+), 501 to 1000 (×), and > 1000 cells/mL (◇).

whereas 7% of heifers with a SCCel >1,000,000 cells/mL were culled. At 200 DIM, this was 7 and 13%, respectively. The same trends were present in the heifers that were culled for udder disorders, but the differences between the SCCel-levels were smaller. Heifers with a SCCel >500,000 cells/mL were culled earlier in lactation compared with the other heifers.

Cox models

For each unit increase in the LnSCCel, the culling hazard of 13,835 dairy heifers in the first lactation increased by 11% (HR = 1.11; 95% CI: 1.08-1.14) (CULLall, Model 1). Black Holstein-Friesian heifers were culled less frequently compared with Belgian White-Blue double-purpose heifers and heifers of unknown breed. The association between LnSCCel and culling was stronger (HR = 1.32; 95% CI: 1.22-1.44) if only culling for udder disorders (CULLudd, Model 1) was considered.

The model allowing for a different association between LnSCCel and the culling hazard per DIM in early lactation (CULLall, Model 2) was significantly better (likelihood ratio test; Chi-square = 41.2, df = 9, P <0.001) than Model 1 (CULLall): an increase in LnSCCel was associated with an increased culling hazard, except for DIM 5. The association was significant from DIM 10 and onwards (except for DIM 11).

Log-transformed test-day SCC was significantly related to the culling hazard of dairy heifers with a HR of 1.26 (95% CI: 1.22-1.30) for each unit increase in LnSCC (CULLall, Model 3). The HR increased to 1.80 (95% CI: 1.66-1.95) when only culling for udder disorders specifically (CULLudd, Model 3) was considered. Introducing LnSCC into the models that already comprised LnSCCel, reduced the estimate of LnSCCel at every DIM in early lactation (Models 5). The reduction was larger when studying the association in heifers that were culled for udder problems only.

Higher MY protected heifers against culling [CULLall: HR = 0.90 (95% CI: 0.89-0.91) and CULLudd: HR = 0.92 (95% CI: 0.89-0.94), Model 4]. The magnitude of LnSCCel at the different DIM in early lactation was slightly reduced when MY was taken into account (Models 6). The changes were smaller compared with the changes due to introducing LnSCC. In addition, incorporating MY into the models took away the breed effect: Black Holstein-Friesian heifers were no longer protected from culling.

Studying the association between LnSCCel and culling in heifers with a second test-day SCC (measured between 15 and 75 DIM) ≤50,000 cells/mL revealed that, although the association was smaller, an elevated LnSCCel was, in general, still associated with a higher culling hazard, especially for SCCel measured in the second part of the period called “early lactation”.

Discussion

In this study, Cox frailty models were used to study the association between SCCel and the culling of heifers belonging to different herds. The influence of test-day SCC and MY on the magnitude of SCCel was assessed.

Reproduction was the primary culling reason in our study, whereas production was second and mastitis third, which corresponds with the findings from Bascom and Young (1998). Some of the factors that needed to be evaluated or adjusted for (e.g. test-day SCC and test-day MY) change over the course of lactation. In order to assess their immediate effect, they were introduced as time-varying covariates, because summary measures (e.g. lactation

average SCC or MY) are not able to determine the effect of a covariate throughout lactation (Gröhn *et al.*, 1997). Furthermore, the clustering effects due to the fact that heifers belong to different herds had to be taken into account by introducing a random herd effect in the Cox model, rather than including herd as a fixed effect because the individual farm is not of interest by itself (Duchateau and Janssen, 2004). Because SCCel decreases substantially in the first 2 weeks after calving (Laevens *et al.*, 1997; De Vliegher *et al.*, 2001; De Vliegher *et al.*, 2004a; De Vliegher *et al.*, 2004b), models that stratified for day of assessment of SCCel were fitted.

Considering only the heifers that were culled for udder health reasons (CULLudd) versus all culled heifers (CULLall) increased the magnitude of association between SCCel and SCC, and the culling hazard. This is comprehensible as the effect is not diluted by the other reasons why heifers are culled. We wanted to present both approaches because the DHI program only allows farmers to identify one single culling reason per heifer, whereas farmers usually consider many factors when deciding to cull an animal (Bascom and Young, 1998). In addition, for 40.5% of the culled heifers no specific reason was available, although some of them were probably culled because of udder health problems. Still, even when considering all culling reasons, an elevated SCCel predicted a higher culling hazard, confirming the negative economic consequences of heifer mastitis at freshening, even though the HR was only significantly >1 when recorded after 9 DIM.

High yielding cows, even if they are diseased, are more likely to be kept in the herd (Gröhn *et al.*, 1998). Comparing models with and without MY, can therefore help to estimate the direct and indirect effects of mastitis or disease in general on culling (Rajala-Schultz and Gröhn, 1999b). Hence, MY was included in the models to find out whether this changed the magnitude of the effect of SCCel on culling. Acute mastitis in the first month of lactation had a significant effect on culling throughout lactation (Rajala-Schultz and Gröhn, 1999a), but adding MY to the model, in general, reduced the effect (Rajala-Schultz and Gröhn, 1999b). The same was true in our study, indicating that a small part of the effect of an elevated SCCel on test-day SCC was mediated through MY. Fitting a model with both LnSCCel and LnSCC showed that part of the LnSCCel effect acted through the associated test-day SCC. This was not unexpected as an elevated SCCel increases the odds on elevated test-day SCC (De Vliegher *et al.*, 2004a).

Heifers with an excellent udder health at the second test-day but with an elevated SCCel were still more at risk of being culled in their first lactation compared with heifers with an equally low second-test-day SCC but a lower SCCel. Most probably this finding is related to the fact that the latter heifers will have fewer test-day SCC >200,000 cells/mL (De Vliegher *et al.*, 2004a) and will out-produce the heifers with the higher SCCel (De Vliegher *et al.*, 2005). This suggests that prevention against elevated SCCel, especially in the second part of the early lactation period as we defined it, should be preferred over treating an elevated SCCel. Additionally, some of the heifers with (sub)clinical mastitis in early lactation will lose milk production in the affected quarter(s) and will consequently have a low second test-day SCC.

Conclusion

Heifers with elevated SCC were at an increased risk of being culled during first lactation. Part of the effect was associated with the consequential elevation of test-day SCC and

suppression of test-day MY. High yielding heifers were, on average, protected against culling, even if their SCCel was elevated. The association between LnSCCel and culling was still present, although smaller in size, in heifers with a second test-day SCC ≤50,000 cells/mL, suggesting that prevention rather than cure of an elevated SCCel is needed.

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Therapy and immunization

Reducing subclinical and clinical mastitis in dairy heifers by precalving infusion of a teat sealant and/or parenteral antibiotic therapy

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Abstract

Heifers (n=1067 from 30 herds) were randomly allocated within herd to treatment in a 2 by 2 factorial arrangement approximately one month prepartum. The treatments were bismuth subnitrate teatsealent (Teat seal, Pfizer Animal Health, Auckland New Zealand) infused into all 4 glands (n=236), injection of 5 g of tylosin (Tylan 200, Elanco Animal Health, Manukau City, New Zealand) i.m. daily for 3 days (n=234), both teatsealent and tylosin (n=232), or no treatment (n=236). Milk samples were collected at enrolment, again at 0 to 4 d postpartum, and from glands with clinical mastitis. Data were analysed at the heifer level using logistic regression models.

Bacteria were isolated from one or more glands of 37% and 36% of the heifers, pre and postpartum, respectively. Treatment with teatsealent (RR = 0.38 (95% CI = 0.29-0.49), p < 0.001), but not tylosin (RR = 1.09 (95% CI = 0.88-1.32), p = 0.40) reduced the prevalence of infected heifers postpartum. Infection prepartum increased the risk of infection postpartum (RR = 1.66 (95% CI = 1.37-1.95), P<0.001).

The cumulative incidence of clinical mastitis over the first 2 weeks postpartum was 168/938 (17.9%). The cumulative incidence was reduced by teatsealent (RR=0.71 (95% CI=0.51-0.95), P=0.02), but not by tylosin (R=0.97 (95% CI=0.72-1.30), P=0.86). There were no tylosin by teatsealent interactions (P>0.2).

It is concluded that infusion of a teatsealent into all four glands of heifers precalving reduced the postpartum prevalence of infection and the incidence of clinical mastitis.

Keywords: heifers, teatsealent, antibiotic, prepartum

Introduction

Current mastitis control programmes do not specifically address mastitis in heifers. However, the prevalence of intramammary infection (IMI) in heifers pre-calving is high (20% to 97%; Oliver and Mitchell, 1983; Trinidad *et al.*, 1990) with resultant long term production losses (Woolford *et al.*, 1983), an increased risk of clinical mastitis in the subsequent lactation and of premature removal from the herd (Rupp *et al.*, 2000).

Intramammary infusion of antibiotics pre-calving in heifers has been shown to reduce the prevalence of IMI and to reduce the incidence of clinical mastitis post-calving (Oliver *et al.*, 2003). Parenteral antibiotic treatment of heifers prepartum has not been previously reported. Infusion of a teatsealent at the end of lactation has been shown to reduce the

new infection rate over the non-lactation period in adult cattle (Woolford *et al.*, 1998; Berry and Hillerton, 2002; Huxley *et al.*, 2002). It was hypothesised that infusion of a teat sealant and/or parenteral antibiotic treatment prepartum would reduce the prevalence of IMI around calving and reduce the incidence of clinical mastitis postpartum in heifers.

Materials and methods

Heifers (n=1067) from herds (n=30) from seasonally calving, pasture-fed dairy herds located in the Wiakato region of New Zealand were enrolled.

Heifers in each herd were enrolled on one calendar day, on average 40.2 (SEM = 0.5 range = 2 to 116 days) days pre-calving. At enrolment, a sample of secretion was collected from every gland of every heifer. Treatment was assigned randomly within herd in a 2 by 2 factorial arrangement. The treatments were bismuth subnitrate teatsealent (Teat seal, Pfizer Animal Health, Auckland New Zealand) infused into all 4 glands (n=268), 5 g of tylosin (Tylan 200, Elanco Animal Health, Manukau City, New Zealand) i.m. daily for 3 days (n=268), both teatsealent and tylosin (n=266) or untreated controls (n=265).

The body condition score (on a 1-10 scale; Roche *et al.*2004), the udder and escutcheon hygiene score (Schreiner and Ruegg, 2003), tail length (cut short, cut long, switch trimmed but the tail not cut and not trimmed or cut) and the presence of udder oedema was recorded for each animal at enrolment and again at the postpartum sampling. The minimum height of the lowest teat end from the ground (in cm) was recorded for each animal post-calving.

Duplicate milk samples (~20 ml each) were collected from every gland within 4 days of calving and from every gland diagnosed with clinical mastitis before treatment for bacterial culture.

Animal data including birth date, calving date, calving type (e.g. assisted vs. not assisted) and breed were retrieved from the national database (Livestock Improvement Corporation, Hamilton, New Zealand).

Bacteriology

Fresh milk (10 µl) was streaked onto a 5% sheep blood agar plate containing 0.1% esculin (Fort Richard, Auckland, New Zealand), and incubated at 37^{o}C for 48 h. The genus of bacteria was provisionally determined on the basis of colony morphology, Gram stain, hemolysis pattern, catalase test and esculin reaction following NMC recommendations.

Data analysis

The heifer was the unit of interest. A gland was defined as infected where 3 or more of single colony morphology types were found and a heifer defined as infected where one or more glands were infected. A new infection was defined as occurring where bacteria were cultured from a gland post partum that had not been cultured pre partum. At heifer level, cure was defined as occurring where each gland within a heifer from which a bacteria was cultured pre partum either did not culture any bacteria or cultured a different bacterial species from that cultured pre partum. Again at heifer level, new infection was defined as occurring where any gland acquired a new infection.

The outcome variables of interest were cumulative incidence of new infection from pre to post partum, cure of existing infection from pre to post partum, prevalence of infection postpartum and incidence of clinical mastitis within 14 days post partum. Independent

variables included treatment, herd, age at calving (days), breed (coded as >11/16th Friesian = Friesian, >11/16th Jersey = Jersey and the rest as crossbreed), interval between treatment and calving, interval between calving and post partum sampling, breeding worth (i.e. the New Zealand defined genetic potential), height of the lowest teat from the ground (cm), body condition score and body condition score change, udder oedema score, hygiene score and tail length.

The independent variables were initially tested using univariate analysis (χ^2 for categorical variables and logistic regression for continuous variables) and variables associated i.e. P<0.2) were then offered to a reverse stepwise regression model using likelihood ratio as the exclusion/inclusion criteria. Biological meaningful first order interactions were then tested among the remaining variables. Herd and treatment were always included in the model. The model fit was assessed via the Hosmer-Lemeshow test and by checking that there were <5% of the cases >2 SD from the expected.

Results

A total of 37% (344/938) and 35% (325/938) heifers were infected pre and post partum respectively.

Teatsealent reduced the risk that a heifer would acquire a new IMI between treatment and postpartum sampling (RR=0.39 (95% CI=0.29-0.53), P<0.001). Tylosin had no effect on new IMI incidence (RR=1.08 (95% CI=0.83-1.36), P=0.56). Risk of new IMI infection increased with soiling of the escutcheon (P=0.04), with increasing body condition score at post partum sampling (P=0.04), with increasing age at first calving (OR=1.01 (95% CI=1.00-1.02; P=0.04) and decreased with increasing interval between calving and sampling (P<0.001; Table 1).

Teatsealent increased the risk cure of an existing IMI would occur (RR=1.50 (95% CI=1.37-1.59), P<0.001). Tylosin had no effect on cure proportion of existing IMI (RR=1.11 (95% CI=0.93-1.25), P=0.21). Risk of cure of IMI increased with time postpartum at sampling (P=0.004) and varied with calving date (P=0.05) and height of teat above the ground (P=0.011).

Teatsealent reduced the risk of IMI post partum (RR=0.38 (95% CI=0.29-0.49), P<0.001). However, treatment with tylosin did not affect the risk of IMI post partum (RR=1.09 (95% CI=0.88-1.32), P=0.40). Infection pre partum increased the risk of IMI post partum (RR=1.66 (95% CI=1.37-1.95), P<0.001). The risk of IMI declined with increasing distance from the lowest teat-end to the ground (OR=0.947 (95% CI=0.906-0.989), P=0.015). The risk of IMI declined with days post partum at sampling (P<0.001) and increased with increasing body condition score at postpartum sampling (P=0.005).

The cumulative incidence of clinical mastitis over the first 2 weeks post partum was 168/938 (17.9%). The cumulative incidence was reduced by teatsealent (RR=0.71 (95% CI=0.51-0.95), P=0.02), but not by tylosin (R=0.97 (95% CI=0.72-1.30), P=0.86). There was no tylosin by teatsealent interaction (P>0.2). Heifers calving at a later calendar date were at increased risk of clinical mastitis (P=0.001). Cows which lost >1.5 units of body condition score were at less risk of clinical mastitis than cows losing less body condition score (P=0.06). Herds differed in risk of clinical mastitis (P=0.004).

A major pathogen was isolated from clinical mastitis cases from one or more glands of 92 (10%) of 938 heifers. Teatsealent reduced incidence of clinical mastitis cases from which

major mastitis pathogens were grown (RR=0.26 (95% CI=0.16-0.43), P<0.001), but incidence of major pathogen clinical mastitis was not effected by tylosin (RR=1.07 (95% CI=0.71-1.60), P=0.74).

Heifers treated with teatsealent were more likely have cases of clinical mastitis from which no pathogen was isolated (RR=2.23 (95% CI=1.90-2.44), P<0.001). Additionally, where diagnosis occurred before Day 4 post partum the incidence of heifers with clinical mastitis from which no pathogen was isolated was higher than where diagnosis occurred ≥4 days postpartum in teatsealent-treated heifers (P=0.002). In control heifers, time post partum at diagnosis had no effect on the incidence of clinical mastitis cases from which no pathogen was grown (P=0.47).

Discussion

Teatsealent reduced the prevalence of heifers with IMI post partum and reduced the incidence of clinical mastitis within 14 days of calving. This was associated with a reduced risk of new infection between enrolment (~40 days before first calving) and sampling within 4 days of calving. The hypothesis that the teatsealent treatment would reduce prevalence of IMI and reduce clinical mastitis incidence postpartum was supported. The postpartum IMI was reduced both because the new infection incidence was reduced and because there was a higher proportion of cure of existing infections. New IMI were occurring approaching calving. The presence of the physical barrier provided by the teatsealent would likely reduce the risk that bacteria would enter the teat canal or cistern. However, it is not clear why the teatsealent treatment resulted in a greater proportion of previously infected glands and heifers being uninfected around calving. This may be due to the teatsealent having some antimicrobial activity. Alternatively, it is possible that in the non-teatsealent groups that cure was occurring, but that new infections were occurring with the same species as prepartum and hence the gland or heifer was defined as having ongoing infection. Use of genotypic methods may help clarify this question.

Usage of the teatsealent was associated with an increased risk of clinical mastitis from which no pathogens was isolated. Appearance of 'flecks' or 'fragments' of the teastsealent may have led herdowners to misdiagnose clinical mastitis.

Treatment with a parenteral antibiotic (Tylosin) did not reduce the incidence of new infections pre calving, the prevalence of infection postpartum nor the incidence of clinical mastitis. It had been hypothesised that treatment would reduce the prevalence of infection prepartum which had previously been shown to be a risk factor for infection postpartum and clinical mastitis (Parker *et al.*2004). It is not clear whether the treatment had failed to produce bacterial cure of existing infections or whether cure was followed by re-infection.

It is concluded that infusion of a teatsealent into all four glands of heifers precalving reduced the postpartum prevalence of infection and the incidence of clinical mastitis.

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Chemotherapeutic approaches to bovine mastitis caused by *Staphylococcus aureus* through strain identification using random amplified polymorphic DNA analysis

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Abstract

Bovine mastitis caused by multi drug resistant *Staphylococcus aureus* with an increase in global prevalence poses potential threat to the dairy industry in developing tropics. Strain mediated drug resistance of *S.aureus* necessitates accurate and reliable methods of strain identification as a prerequisite for the design of effective chemotherapeutic regimens. *S.aureus* (63 isolates) obtained after screening 1053 quarters from animals brought to the University veterinary teaching hospital and University livestock farms were subjected to detailed bacteriological analysis and antibiotic sensitivity. DNA from 24 *S.aureus* isolates were genotyped using a Random amplified polymorphic DNA assay. RAPD protocols for genotyping *S aureus* strains were standardized using a 21 mer arbitrarily primer 5'AGTAAGTGACTGGGGTGAGCG 3'. DNA bands of molecular weight ranging from 234 bp to 4261 bp were recognized from these bacterial isolates. On the basis of RAPD assay the 24 S.aureus isolates could be grouped in to seven distinct strains designated as A,B,C,D,E,F and G respectively. Fifteen (62 per cent) of the isolated belonged to strain A which had 80 per cent clinical recovery on appropriate antibiotic regimen. The arbitrary primed PCR was found to be more reliable in differentiating *S aureus* strains from bovine mastitis cases. This protocol, which does not require prior knowledge of genome, appears to be a promising tool to verify the antimicrobial efficacy in determining the chemotherapeutic approaches to bovine mastitis caused by *Staphylococcus aureus*.

Keywords: *S aureus,* chemotherapy, RAPD

Introduction

Mastitis continues to be the most important infectious disease among dairy cattle, in spite of continuing research and control programmes. Surveys on dairy cattle indicate that the prevalence of mastitis in cows is about 50 per cent and a quarter infection rate of about 25 per cent. *Staphylococcus aureus* is a major pathogen of the mammary gland and is a common cause of contagious mastitis in dairy cattle worldwide (Radostits *et al.*, 2000). The existence of many genotypes may attribute to the variations in antibiotic sensitivity.

Randomly amplified polymorphic DNA (RAPD) typing, also known as arbitrarily primed polymerase chain reaction (AP-PCR) is a technique suited for rapid detection of genomic

polymorphisms. RAPD analysis has been used successfully to characterize *S.aureus* strains from bovine mastitis (Mathews *et al.*, 1994; Fitzgerald *et al.*, 1997). More over it is a rapid, simple and reproducible characterization technique. In this study RAPD assay was employed to differentiate *S aureus* isolates obtained from clinical cases of bovine mastitis and it is used to correlate the antimicrobial efficacy, which helps in determining the chemotherapeutic approaches to bovine mastitis.

Materials and methods

Mastitis cases brought to the University Veterinary Teaching Hospital. Mannuthy and University Livestock Farm were subjected to detailed bacteriological analysis and antibiotic sensitivity. Milk samples were collected aseptically for bacteriological isolation on the first day of illness and 72 hours after the completion of the antibiotic therapy. Cultural examination was carried out as per the method described by Cowan (1974). Three commonly used broad spectrum chemotherapeutic agents viz. amoxicillin-cloxacillin combination, enrofloxacin and oxytetracycline were tested for their efficacy in the treatment of clinical cases.

Twenty four isolates of *S. aureus* were subjected to RAPD assay. The bacterial DNA isolation was carried out by high salt method described by Lahiri and Nurnberger (1991). The extracted DNA was checked by agarose gel electrophoresis. G3 primer having the sequence 5′ AGTAAGTGACTGGGGTGA GCG 3′ (Van den berg *et al.,* 1999) was used in RAPD assay. DNA dissolved in 20 micro litre of LTE buffer was boiled at 100 degree celcius for 5 minutes. After boiling the samples were immediately cooled in ice for maintaining the DNA template strands in single strands. From this sample, one micro litre was used for RAPD reactions. Thermal cycler programme for Staphylococcus spp was one cycle of 5 minutes at 94° c followed by 34 cycles of 5 seconds at 94 ° c, 30 seconds at 34 ° c and I minute at 72 ° c. Then it was kept at 4 ° c over night. The RAPD products were examined by electrophoresis in 1.5 per cent agarose gel in TAE buffer containing ethidium bromide. Then the gel was visualized under UV transilluminator and photographed using Polaroid camera with wratten gelatin filter.

DNA from twenty four samples of *S aureus* was typed using RAPD finger printing. By comparing the banding patterns of different isolates they were classified in to seven groups arbitrarily designated as A,B,C,D,E,F AND G. among the 24 isolates 15 belong to the same group, genotype A. All the samples showed bands with the molecular weight ranging from 234 bp to 4261 bp. Table one shows the genotype of different bacterial isolates with the treatment adopted and the response of the treatment indicating clinical and bacteriological cure.

In the present study seven different genotypes of *Staphylococcus aureus* were identified among 24 isolates from different cows. However *Staphylococcus aureus* genotype A predominated and accounted for more than 62 per cent of isolates. The seven different genotypes identified had variation with regard to the response to treatment. Among the fifteen *Staphylococcus aureus* genotype A identified, 80 per cent showed clinical cure (Table 1). The clinical cure assessed by the remission of clinical symptoms of mastitis exhibited by the animal and a bacteriological cure was ascertained by a negative bacterial culture of milk sample, collected 72 hours after the completion of the antibiotic therapy, which varied

Table 1. Bacterial genotype with treatment adopted and the response.

Sl. No.	*Bacterial isolate*	*Treatment adopted*	*Clinical cure*	*Bacteriological cure*
1	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin combination	Yes	Yes
2	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin combination	Yes	Yes
3	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin Combination	Yes	Yes
4	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin Combination	Yes	Yes
5	*Staphylococcus aureus* Genotype B	Amoxycillin-Cloxacillin Combination	Yes	Yes
6	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin Combination	No	Yes
7	*Staphylococcus aureus* Genotype E	Amoxycillin-Cloxacillin Combination	No	No
8	*Staphylococcus aureus* Genotype F	Amoxycillin-Cloxacillin Combination	No	No
9	*Staphylococcus aureus* Genotype A	Amoxycillin-Cloxacillin combination	Treatment changed	-
10	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	Yes
11	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	Yes
12	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	Yes
13	*Staphylococcus aureus* Genotype C	Enrofloxacin	No	No
14	*Staphylococcus aureus* Genotype A	Enrofloxacin	No	Yes
15	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	Yes
16	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	Yes
17	*Staphylococcus aureus* Genotype A	Enrofloxacin	Yes	No
18	*Staphylococcus aureus* Genotype A	Oxytetracycline	Yes	Yes
19	*Staphylococcus aureus* Genotype D	Oxytetracycline	Yes	Yes
20	*Staphylococcus aureus* Genotype A	Oxytetracycline	Yes	Yes
21	*Staphylococcus aureus* Genotype C	Oxytetracycline	Yes	Yes
22	*Staphylococcus aureus* Genotype E	Oxytetracycline	No	No
23	*Staphylococcus aureus* Genotype G	Oxytetracycline	Yes	Yes
24	*Staphylococcus aureus* Genotype E	Oxytetracycline	Yes	No

with different genotypes. Hence the molecular genotyping can be used as an effective tool in predicting the outcome of the chemotherapeutic regimen employed.

Fitzgerald *et al.* (1997) used RAPD PCR to elucidate molecular epidemiology of *Staphylococcus aureus* isolated from cows and concluded that only a few specialized clones of staphylococcus were responsible for majority of cases of bovine mastitis and these clones have a broad geographic distribution. The different DNA fragment pattern observed in the RAPD assay clearly indicates the suitability of this assay to genotypically differentiate the bacterial isolates. More detailed investigations using more number of isolates will help to identify the genotype that is predominant in the locality and it can be utilized for the production of a candidate vaccine.

The number and size of amplification products depend on the complimentarity of the sequences of the particular primer and the template DNA. The RAPD primer gave significant DNA polymorphism changes, since it produces different DNA bands. RAPD analysis is thought to be the most specific and best reproducible differentiation method for *Staphylococcus aureus* strains (Wang *et al.,* 1993). Many workers have attempted extraction of DNA, directly

from milk samples for RAPD, but the results may be unreliable due to mixed infections possible in case of mastitis. Hence DNA extraction from pure culture of organism was done in this study to enhance the reliability of the RAPD study.

The different DNA fragment patterns were observed using RAPD assay clearly indicate the suitability of this assay to differentiate *Staphylococcus aureus* isolates in mastitis cases. This methodology is rapid and preferable over biotyping, gives good level of discrimination and it does not require prior knowledge of genome (Wang *et al.,* 1993: Lipman *et al.,* 1995). RAPD is a less laborious procedure and it requires only smaller quantities of DNA and does not depend on the use of selective PCR primers when compared with other DNA based procedures.

In conclusion, RAPD assay can be considered as a simple and convenient method to study DNA polymorphism and can be regarded as a valuable molecular tool in the epidemiological analysis of bacterial organisms of milk origin as well as to assess the efficacy of the antibiotic regimen employed.

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Efficacy of a cloxacillin dry cow product for treatment of heifers on farms with a low and a high prevalence of heifer mastitis in The Netherlands

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Abstract

A high percentage of heifers have an intramammary infection (IMI) at calving. Treatment with antibiotics before calving has been shown to reduce the number of IMI's at calving and to increase milk-production.

To quantify this effect a trial was carried out on 13 Dutch dairy farms where 196 heifers were treated with 600 mg dynomilled cloxacillin (Orbenin Extra Dry Cow, Pfizer®) 8-10 weeks before the expected calving date. Another 196 heifers served as untreated controls. An employee of the Animal health Service performed the random selection and treatment. Bacteriological culturing and determination of the somatic cell count (SCC) was carried out at calving and repeated 10 days later. All farms recorded milk-production and cow SCC.

There was a difference between farms. Farms with less than 15% of heifers with a cow SCC above 150.000 cells/ml at the start of the trial were considered to have a low prevalence of heifer mastitis (LPF group) while farms with over 15% were considered as high prevalence farms (HPF group). The expected 305-day milk-production, in the treated heifers, was significantly higher (496L) in the HPF group in comparison with the untreated animals but this difference was only 77L (not significant) in the LPF group. In both groups of farms the cow SCC was significantly lower in the treated heifers compared to the untreated controls.

It is concluded that treatment of heifers is beneficial on HPF but not on LPF farms. Determination of the causes of heifer mastitis is recommended on HPF farms.

Keywords: heifers, treatment, milk-production, somatic cell count

Introduction

Up to 97% of heifers are infected with mastitis pathogens, often months before calving. The most common pathogens are *Staphylococcus chromogenes*, *Staphylococcus hyicus* and *S. aureus* (Trinidad *et al.*, 1990, Pankey *et al.*, 1991, Fox *et al.*, 1995) and in the studies from Oliver (Oliver *et al.*, 1992; Oliver *et al.*, 1997; Oliver *et al.*, 2003) *Streptococcus uberis* and *Streptococcus dysgalactiae*. The presence of mastitis pathogens is not usually noticed by most farmers until freshening. An IMI may already have existed for a year or more before calving (Nickerson *et al.*, 1995). Farmers lack of awareness that heifers could be infected with contagious mastitis pathogens before calving is mainly a result of the fact heifers are not milked twice a day.

Several therapy studies have been performed to reduce the IMI after calving. A dry cow product containing 300 mg cephapirin benzathine had a 95% cure rate for *S. aureus* on the day of calving (Owens *et al.*, 1994). A dry cow product with Penicillin and streptomycin was used in another study (Trinidad *et al.*, 1990) where the prevalence of *S. aureus* decreased from 17.1% to 2.9% after treatment. In the control group, prevalence of *S. aureus* decreased from 26.3% to 15.8%. The third trimester of pregnancy was, according to Owens *et al.*, 2001, the optimum period to commence treatment with a dry cow product. An intramammary infusion of 200 mg of sodium cloxacillin in comparison to an intramammary infusion of 200 mg of cephapirin sodium 7 days before expected parturition was effective in eliminating many IMI in heifers, especially those caused by coagulase negative staphylococci (Oliver *et al.*, 1992).

Information about treatment efficacy, of a dry cow product, on cow SCC, milk-production and clinical mastitis after calving in heifers, on farms with a different level of heifer mastitis prevalence is unknown.

The purpose of this study was to determine the efficacy of 600 mg dynomilled cloxacillin (Orbenin Extra Dry Cow, Pfizer®) administered 8-10 weeks before the expected calving date in heifers. Evaluations were made on IMI after calving, clinical mastitis after calving, SCC and milk-production on farms with a low and a high prevalence of heifer mastitis.

Materials and methods

Herds

13 farms in the province of Overijssel were included in the trial. The farms had a good animal identification system, milk-recording was carried out every four weeks and there was a good record of treatments. The herd size varied between 38 and 118 milking cows with an average milk-production of 8500 litres per cow per lactation. The BMSCC on the farms ranged from 95.000 to 463.000 cells per ml.

During the winter period, the heifers were housed in free stalls with cubicles and slatted floors. In spring, summer and autumn the heifers were kept on pasture. Eleven farms milked the heifers after parturition in a herringbone milking parlour and 2 farms used an automatic milking system. Milking machines were checked once a year, which is compulsory in the Dutch Milk Quality System (KKM). After milking 12 farms used a post-milking teat dip and 1 farm did not spray or dip.

A farm was defined as a low prevalence farm (LPF) when at the start of the trial less than 15% of the heifers had a CSCC above 150.000 cells per ml. A farm was defined as high prevalence farm (HPF) when at the start of the trial more than 15% of the heifers had a CSCC above 150.000 cells per ml.

Animals and treatment

Three hundred ninety two primigravid heifers were included in the trial. An employee of the Animal Health Service randomly assigned these, to treated and untreated control groups. The 196 treated heifers received 600 mg of dynomilled cloxacillin (Orbenin Extra Dry Cow, Pfizer®) intramammary, between eight and ten weeks before the expected calving date; the other 196 untreated controls did not receive any intramammary infusion. After calving CSCC, milk-production and the expected 305 day milk-production of the trial heifers were collected from the milk recording data of the first 4 months of lactation. The study started in September 2001 and ended in November 2003.

Sampling

The farmer sampled all clinical mastitis cases before treatment. Immediately after calving the farmer took milk-samples from all 4 quarters. Employees of the Animal Health Service took duplicate samples 10 to 12 days after calving. The teat end was sanitized with cotton balls moistened with 85% ethanol. Milk samples were collected aseptically into sterile tubes. The udders were examined for abnormalities on the days of sampling.

Bacteriology and somatic cell count

Milk samples were incubated according to the standard procedures of the International Dairy Federation (1981) within 24h of collection. An inoculum of 0.01 ml was spread on a sheep blood agar plate and on Edward's medium. Edward's medium was used for selective isolation of streptococci (groups A, B, C, G, E, and L), and crystal violet in the medium inhibited growth of *S. aureus*. Incubation temperature was 37°C, and plates were examined after 24 and 48h of incubation. Strains producing α, β or α and β haemolysis were usually presumptively identified as *S. aureus*. A sample was confirmed as positive for *S. aureus* using a tube coagulase test.

Sensitivity to β-lactam antimicrobials like penicillin was tested on all isolated strains of *S. aureus* using nitrocephin (Oxoid, SR0112C, Haarlem, The Netherlands). Strains that were positive for β-lactamase were considered resistant to penicillin. Sensitivity for other antibiotics was tested with the agar diffusion method using Isosensitest agar (Oxoid, CM0471, Haarlem, The Netherlands), and tablets loaded with amoxicillin-clavulic acid, oxacillin, cephalotin, neomycin, erythromycin, lincomycin, and trimethroprim-sulphonamid (Rosco, Taarstrup, Denmark) according to the procedure described by the Dutch Working group on the standardisation of antibiotic sensitivity testing (Mouton and Van Klingeren, 1981). Strains that were negative for β-lactamase were considered penicillin sensitive. Somatic cell count was determined on milk samples using an electronic cell counter (Foss, Hillerod, Denmark).

Statistical analysis

Differences between the treatment group and the untreated controls were analyzed with the Student's *t*-test (Trinidad *et al.*, 1990). Quarter and cow SCC were first transformed by use of natural logarithm and then analyzed with the two-sample *t*-test. Chi-square analysis (Rothman, 1986) was used to evaluate the differences between the two treatment groups for clinical mastitis.

Results

Thirteen farms were included in this trial, 5 LPF with 72 heifers in the treatment group and 73 heifers in the untreated control group and 8 HPF with 124 heifers in the treatment group and 123 heifers in the untreated control group. In table 1 the remarks around calving are given for the heifers.

A number of heifers were excluded from the trial (Table 1); 4 animals were not pregnant, 3 were sold shortly before calving for export, no samples were collected by the farmer at calving from 3 heifers, 1 aborted, data were incomplete from 30 heifers and 1 heifer was treated intramuscularly for several days for a metritis.

Table 1. Remarks around calving for heifers in the different treatment and prevalence groups.

	Treatment group			Control group		
Reason	LPF	HPF	Total	LPF	HPF	Total
Not in calf	3		3		1	1
Sold export	2	3	5			
No samples taken				3		3
Abortion	1		1			
Metritis		1	1			
Data incomplete	4	12	16	3	11	14
Total	10	16	26	6	12	18

All the other treated and control heifers were included in the trial.

Clinical mastitis occurred in 46 heifers, 17 in the treated and 29 in the control group (P=0.06), mainly around and in the first 14 days after calving.

Intramammary infections at parturation and 10 till 12 days after calving are summarised in Table 2. Staphylococcus non aureus or Coagulase Negative Staphylococci was most isolated. At D10 the number of infected quarters was much lower in all groups compared with D0.

The average daily milk-production in the first 100 days after calving (Figure 1) was higher (P<0.05) in the treated heifers compared to the untreated controls in the HPF group but not in the LPF group.

Table 2. Intramammary infections in heifers at parturition (D0) and 10 till 12 days (D10) after calving for the different treatment and prevalence groups.

	Treatment group		Control group	
Pathogen	D0	D10	D0	D10
Negative	381[1]	562[3]	304	506
Staphylococcus aureus	29	11[3]	35	20
Staphylococcus non aureus	240[1]	110[3]	301	135
Streptococcus uberis	5	1[3]	5	5
Streptococcus dysgalactiae	8[2]	1[3]	16	6
Escherichia coli	17	6	12	3
Bacillus	15[2]	13[4]	7	28
Corynebacterium bovis	13	14	11	14
Strep. green	10	8	9	10
Other	4	5	12	3
Contaminated	11	15[4]	14	5
No sample	49	38	57	49
Total	784	784	784	784

[1]Different (P<0.05) from D0 control group

[2]Tendency (0.05≤P≤0.10) for difference from D0 control group

[3]Tendency (0.05≤P≤0.10) for difference from D10 control group

[4]Different (P<0.05) from D10 control group

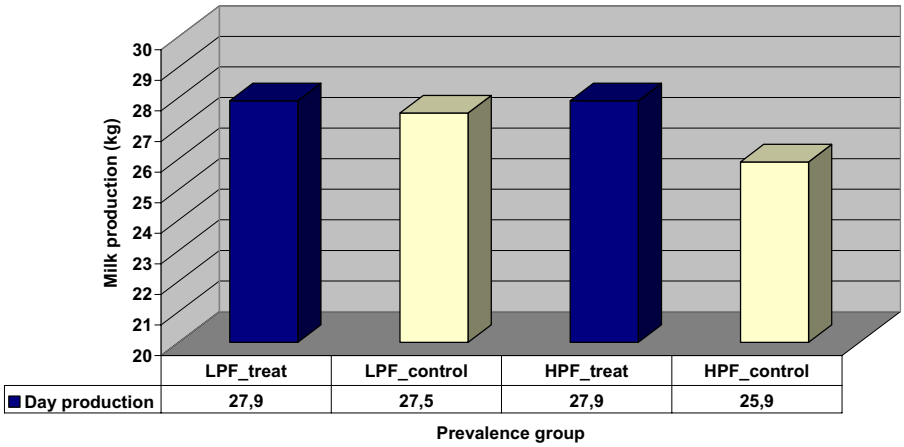

Figure 1. Average milk-production in the first 100 days of lactation for the low (LPF) and high prevalence (HPF) group.

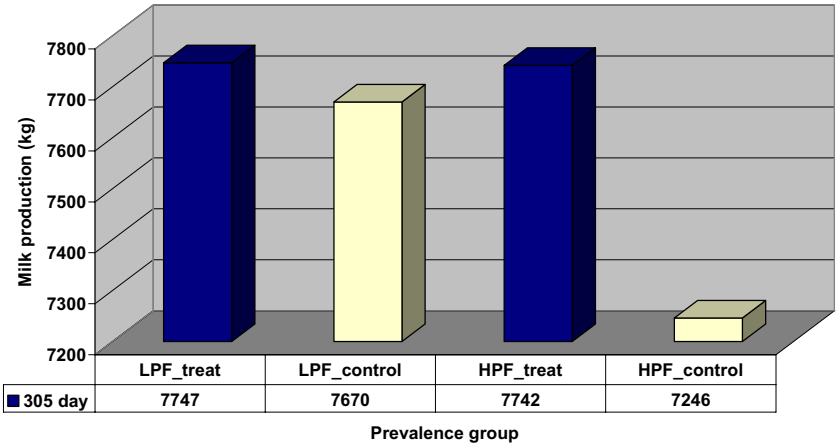

Figure 2. Expected 305 day milk-production for the low (LPF) and high prevalence (HPF) group.

The expected 305-day milk production (Figure 2) was 496L higher ($P<0.05$) in the treated heifers in the HPF group in comparison with the untreated animals but this difference was only 77L (not significant) in the LPF group.

In both groups of farms (Figure 3) the cow SCC was lower ($P<0.05$) in the treated heifers compared to the untreated controls.

Discussion

Treatment of heifers with 100 mg dynomilled cloxacillin (Orbenin Extra Dry Cow, Pfizer®) administered 8-10 weeks before the expected calving date is beneficial on farms with a high prevalence of heifer mastitis but not much on farms with a low prevalence.

Therefore the reasons for the high prevalence of heifer mastitis must be analysed. Infection of quarters is already possible shortly after calves are born and suck each other.

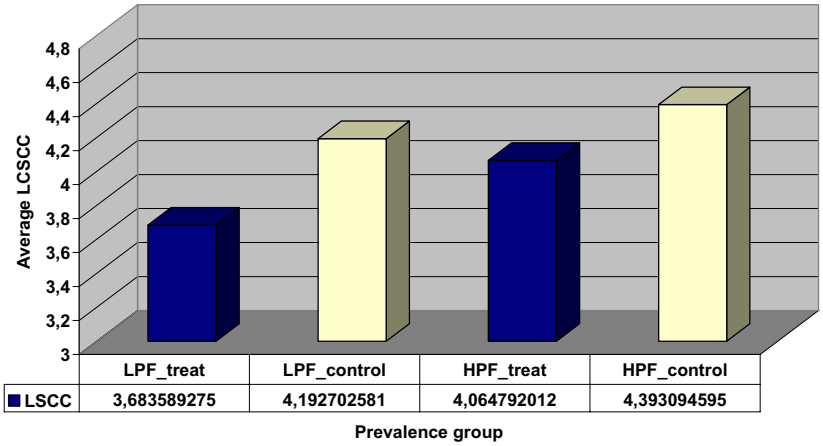

Figure 3. Log somatic cell count in the first 100 days of lactation for the low (LPF) and high prevalence (HPF) group.

Also suckling in older age groups is a threat. Less is known about feeding calves milk from infected quarters but that is also a possible cause of an early infection. For this reason milk from cows with mastitis should be discarded and not fed to calves.

Flies are able to transmit *S. aureus*. Therefore a good fly control is necessary. Mineral deficiencies may cause immunodeficiency and make quarters more sensitive for infection. Therefore in parrticular the blood selenium level must be sufficient.

Also housing conditions, especially around calving are important. Too much energy in a dirty environment may cause more udder infections.

After analysing possible risk factors appropriate measures should be taken. Preventive treatment with antibiotics should be discontinued as soon as the prevalence of heifer mastitis is again at a low level.

Conclusions

It is concluded that treatment of heifers with 600 mg dynomilled cloxacillin (Orbenin Extra Dry Cow, Pfizer®) administered 8-10 weeks before the expected calving date is beneficial on HPF but not on LPF farms. Determination of the causes of heifer mastitis is recommended on HPF farms.

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Efficacy of a J-5 Escherichia coli bacterin in clinical coliform mastitis of dairy cattle

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Abstract

The study objective was to determine the efficacy of Enviracor™ (Pfizer), a J-5 *Escherichia coli* bacterin, as an aid in preventing of clinical coliform mastitis under field conditions. Cows and heifers with seven month pregnancies in thirteen herds were randomly assigned to Enviracor™ or placebo (saline) treatment groups. Enviracor™ animals received three doses subcutaneously with the first dose targeted at 56 days pre-calving, the second dose 28±7 days later, and the third within 16 days after calving for a study period of 150 days of lactation. Of 1314 animals enrolled, 1118 animals (556 in placebo and 562 in Enviracor™ groups, respectively) were qualified for final analysis. The frequency of visual injection site swelling in vaccinates peaked at 48 hours post injection (60-65%) and was reduced by 72 hours. The incidence of toxic coliform mastitis was significantly higher for the placebo group than the Enviracor™ group ($p=0.04$) with a relative risk of 1.89. The likelihood of occurrence of one or more risk factors for mortality due to coliform mastitis was significantly lower for Enviracor™ treated cows compared to placebo ($p<0.05$) with an odds ratio of 3. This study shows that cows vaccinated with Enviracor™ were better able to react to severe coliform infections. As a result, they experienced a lower incidence of toxic mastitis and were at a lower risk of mortality following an incident of coliform mastitis.

Introduction

It is estimated that 30% to 40% of all cases of clinical mastitis in some dairy herds are caused by coliform bacteria, particularly in herds where contagious pathogens are well controlled. The most common organisms are *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *K. oxytoca,* collectively they are known as coliforms. Since most of these organisms are either normal gut inhabitants or are found in bedding materials, there is almost constant exposure of the mammary gland to these environmental bacteria.

The clinical manifestation of infection is acute, sometimes per-acute disease with high fevers and toxemia. Chronic infections characterized with quiescent periods punctuated with periodic acute flare-ups may also occur.

Cell wall components, particularly, endotoxins, can cause release of pre-formed inflammatory compounds resulting in mediator shock, which may be fatal. The effects of these mediators include contraction of smooth muscle, increased vascular permeability resulting in oedema, and an early increase in vascular resistance, which may be followed by vascular collapse if sufficient concentrations of mediators are present. Uncorrected, the

clinical signs of mediator shock are followed by metabolic acidosis, cyanosis, altered vascular resistance and cardiac output, coma and death.

Treatment of acute and per-acute clinical coliform mastitis is difficult and not always curative, and is consequently costly to the producer. Prevention of the disease is considered to be the best form of control. Methods of prevention include decreasing the exposure of the teat end to the coliforms and increasing the animal's resistance to infection. Maintaining a good sanitation program and practicing good milking procedures should reduce the amount of exposure to coliform organisms.

However, dairy cattle that do become exposed to the organisms may not possess the appropriate immune status to resist clinical infection. Peri-parturient cows, cows in high production and cows under nutritional stress are especially susceptible to coliform mastitis due to a decrease in the effectiveness of nonspecific resistance mechanisms. The severity of the disease may be reduced by the use of an effective immunogen. Immunization of dairy cattle with J5 *E. coli* bacterins has been shown to reduce the occurrence of clinical coliform mastitis under field conditions (Gonzales *et al.*, 1989; Lucas *et al.*, unpublished 1992). The use of this a J-5 vaccination program is just one part of an overall preventive program, which included appropriate management.

The purpose of this study was to evaluate the efficacy of a new bacterin formulation made with a J-5 mutant strain of *E. coli* (Enviracor™) as an aid in the prevention of clinical mastitis due to coliforms under field use conditions. Study data were generated in 13 dairy herds throughout the EU, following a standardized protocol.

Materials and methods

This study was conducted in thirteen herds, comprising a total of 1,314 multiparous cows and heifers. Animals that were confirmed approximately seven months pregnant were assigned to either of two treatment groups: Enviracor™ or placebo. The Enviracor™ vaccine contained 7.5×10^9 colony forming units of J-5 *E. coli* mutant per 2 mL dose, mixed with a proprietary adjuvant. The placebo consisted of saline only.

Animals entered the study in the order of their predicted calving date; every two animals formed a randomization block. Within each randomization block, the animals were randomly assigned to one of the two treatment groups.

The animals received three 2 mL doses: the first dose was administered at 56 days pre-calving, the second dose 28 days ± 7 days later and the third dose within 16 days after calving. The vaccine and saline were injected subcutaneously in the neck region or behind the shoulder. The first injection was made on the left side, the second on the right, and the third on the left again.

The safety of the vaccine was evaluated by daily visual observation of the vaccinated cows both at the site of injection and for any systemic clinical signs related to the injection, for 72 hours post injection. The time, date, diagnosis, and final outcome of any health event were recorded during the entire study. The date of calving and the outcome thereof for the calf were recorded as well.

All animals enrolled in the study were monitored for the presence of clinical mastitis at each milking until 150 days of lactation. When a diagnosis of clinical mastitis had been made, a single pre-milking quarter milk sample was taken for microbiological culture. For each clinical mastitis event, the following was recorded: animal identification, date of

diagnosis, rectal temperature, systemic clinical signs, affected quarter(s), clinical appearance of the milk and quarter, and the date the animal's milk and quarter first returned to normal.

The variables of interest included measures of coliform mastitis incidence and severity. Incidence measures pertained to incidence of first occurrence of clinical coliform mastitis, and incidence of severe clinical coliform mastitis. Severity measures were rectal temperature at time of diagnosis, proportion of animals removed due to severe coliform mastitis and incidence of risk factors for mortality, clinical status of milk and quarter at diagnosis, and time to return to normal milk and normal quarter. All these variables were evaluated for differences between treatment groups.

Results

There were 656 enrolled animals that received the first of the three injections of placebo and 658 that received the first of three injections of Enviracor™. From these 1314 animals, 1118 animals (556 in the placebo group and 562 animals in the Enviracor™ group) met the vaccination schedule and calved at least 7 days after second vaccination, and therefore qualified for final enrollment.

Safety-There were two cows with systemic clinical signs within 72 hours post-injection. One was a case of lameness in the Enviracor™ group at 72 hours following first injection, and another concerned a dull and depressed animal at 48 hours following first injection of saline in the placebo group. These two observations were noted at a single time point and were not accompanied by other clinical signs. These results show that injection with Enviracor™ does not lead to systemic reactions.

Injection site reactions were observed much more frequently following injection of Enviracor™ than placebo. The frequency of visually observable swelling following Enviracor™ peaked at 60-65% after 48 hours and by 72 hours the proportion of cows with visually observable swelling was already reduced. There were no reports of open or draining lesions.

Efficacy-There was little difference between treatment groups in the overall frequencies of first occurrence of clinical mastitis due to coliforms. However, the statistical analysis showed that the incidence of clinical coliform mastitis cases which match the characterization of toxic mastitis (i.e. two or more systemic clinical signs at time of diagnosis) was significantly higher for the placebo group than for the Enviracor™ group ($p=0.04$). The relative risk for such a case of toxic mastitis was 1.89 times higher in the placebo group than in the Enviracor™ group.

In cows diagnosed with coliform mastitis, rectal temperature was higher ($p=0.055$) in the Enviracor™ group than in the placebo group (Table 1). This was due to a lower number of animals with abnormally low temperatures (<38.3° C) in the Enviracor™ group compared to the controls. Thus, cows severely challenged with coliform mastitis were more likely to mount a strong inflammatory response following vaccination with Enviracor™ than cows in the placebo group, which were at a higher risk of not being able to mount such a vigorous response.

Mortality is the result of disease severity and the degree of care that a severely ill animal receives following diagnosis. In order to compare the risk of mortality at the time of diagnosis between the two treatment groups, the frequency of occurrence of risk factors for mortality measured at the time of diagnosis (subnormal temperature, recumbency, and higher packed cell volume) were compared in cases of clinical coliform mastitis. For this

Table 1. The results of an Enviracor™ study in cows with naturally occurring coliform mastitis.

Variable	Placebo	Enviracor™
Number of cows	556	562
Rectal temperature (C)	39.0[a]	39.2[b]
Abnormal milk appearance (%)	28.6	34.4
Abnormal quarter appearance (%)	87.3	87.5
Likelihood of mortality (odds ratio)*	3[c]	1[d]

[ab](p=0.055)

[cd]Differ significantly (p<0.5)

*Based on the presence of at least two of the following risk factors: sub-normal temperature, recumbency, and higher packed cell volume (measure of dehydration).

purpose, packed cell volume was considered a sign of dehydration. Each of the risk factors occurred more frequently in the placebo group than in the Enviracor™ group. The difference in occurrence of one or several of the risk factors for mortality in cases of coliform mastitis was significantly different between the two treatment groups ($p<0.05$) and when expressed as an odds ratio was about 3 fold higher for the placebo group than the Enviracor™ group.

Neither the milk appearance nor the quarter appearance at time of diagnosis differed significantly between the placebo and the Enviracor™ group. Following coliform mastitis eight and five cows did not return to normal milk and normal quarter in the placebo group and the Enviracor™ group, respectively. This was almost always a consequence of the coliform mastitis event, except for one Enviracor™ group which was removed due to a disease condition other than coliform mastitis. The time to return to normal milk and normal quarter was not significantly different between the two treatment groups.

Conclusions

The results from this study show that cows vaccinated with Enviracor™ were better able to react to severe coliform infections. As a result, they experienced a lower incidence of toxic mastitis and were at a lower risk of mortality to be removed from the herd following coliform mastitis.

Toxic coliform mastitis is an important disease condition in modern dairies (Tadich *et al.*, 1998; Green *et al.*, 1996). Systemic treatment with antibiotics (above and beyond the common intramammary antibiotic therapy) has been recommended to combat this condition (Cebra *et al.*, 1996; Shpigel and Schmid, 1997) and is widely used in veterinary practice. The current study indicates that Enviracor™ vaccination lowers the incidence of toxic mastitis with its impact on animal health and animal welfare.

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Effect of carprofen following experimentally induced *E. coli* mastitis in primiparous cows

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Abstract

Acute *Escherichia coli* mastitis is one of the major sources of economic loss in the dairy industry, due to reduced milk production, treatment costs, discarded milk and occasional fatal disease. Non-steroidal anti-inflammatory drugs (NSAID's) are frequently used as adjunctive therapy to antibiotics. The objective of the current study was to evaluate the effect of carprofen treatment following infusion of *E. coli* into the mammary glands of primiparous cows during the periparturient period. Carprofen is a powerful inhibitor of inducible enzymes of the cyclo-oxygenase pathway. The study was conducted to GCP standard under veterinary supervision and complied with applicable animal welfare and regulatory requirements. Severity of mastitis was scored based on the average milk production in the uninfected quarters on d+2 post-inoculation. All heifers reacted as moderate responders. Carprofen was administered at 9 h post-challenge, when clinical signs of mastitis appeared. In previous work, efficacy of NSAID's was mainly evaluated using clinical symptoms. In the present study, the effect of carprofen on innate immune response was also assessed by quantification of inflammatory mediators. Primiparous cows were intramammarily inoculated with 1×10^4 CFU *E. coli* P4:O32 in two left quarters. Analysis of blood and milk parameters, including IL-8, C5a, LBP, sCD14, PGE_2 and TXB_2, was performed from d0 to d+6 relative to intramammary inoculation. Rectal temperature in carprofen-treated animals was lower than that of control animals 3 and 6 h post-treatment ($P < 0.05$). Treatment also restored the decreased reticulorumen motility that occurs during *E. coli* mastitis to pre-infection levels faster than in control animals. Eicosanoid (PGE_2 and TXB_2) production in milk tended to be inhibited by carprofen. No significant differences in the kinetic patterns of SCC, IL-8, C5a, LBP and sCD14 were observed. In conclusion, in this study carprofen improved general clinical condition by effective antipyrexia and restoration of reticulorumen motility.

Keywords: primiparous cow, carprofen, *Escherichia coli* mastitis, moderate inflammation

Introduction

Production costs attributed to mastitis represent the largest economic loss to the dairy industry. Mastitis caused by coliform bacteria is responsible for a major portion of these

losses, predominantly as the result of acute clinical disease, and is associated with animal welfare problems related to significant clinical severity, toxaemia, pain and occasional mortality. Coliform mastitis in cattle may be associated with systemic clinical disease, which occurs predominantly in the period immediately after calving (Hill, 1981). Systemic signs include general depression, fever, tachycardia, inhibition of reticulorumen motility, and many non-specific responses, such as neutropenia, followed by leucocytosis (Verheijden *et al.*, 1983). The pathophysiology of *E. coli* mastitis is characterized by the presence of endotoxin or lipopolysaccharide (LPS) in the outer membrane of the etiological bacteria. Binding of LPS to cell membranes activates the membrane-bound enzyme, phospholipase A2, liberating arachidonic acid which can be metabolized by two major enzyme systems, cyclo-oxygenase (COX) and lipoxygenase. The COX antagonists currently used in veterinary medicine are all regarded as non-specific inhibitors of COX-1 and 2, with similar potencies against both enzymes, or even some selectivity for COX-1 inhibition (Lees *et al.*, 2000). Exceptions are nimesulide, carprofen and meloxicam, which have a partial selectivity for COX-2 inhibition and may be described as preferential COX-2 inhibitors (Lees *et al.*, 2000).

Carprofen ((±)-6-chloro-α-methylcarbazole-2-acetic acid) is a NSAID that is well tolerated in the bovine (Ludwig *et al.*, 1989). In healthy cows, carprofen is pharmacokinetically characterized by a small distribution volume (0.09 l/kg), a relatively low systemic clearance (2.4 mL/h/kg) and a long terminal half-life (30.7 h) (Lohuis *et al.*, 1991). During an endotoxin-induced mastitic episode, systemic clearance decreased, whereas terminal half-life significantly increased (43.0 h). Following carprofen treatment at 2 h post-challenge, a significant reduction in severity of clinical parameters was observed.

A role for soluble CD14 (sCD14) and LPS-binding protein (LBP) in mediating bovine host responses to intramammary LPS or *E. coli* challenge has recently been demonstrated (Wang *et al.*, 2002; Bannerman *et al.*, 2003; Lee *et al.*, 2003a; 2003b). Following intramammary LPS infusion, sCD14 increases in milk (Bannerman *et al.*, 2003; Lee *et al.*, 2003a) paralleled by an increase in LBP (Bannerman *et al.*, 2003). Moreover, sCD14 has been shown to sensitize the mammary gland to LPS (Wang *et al.*, 2002) and to reduce the severity of experimental *E. coli* mastitis in mice (Lee *et al.*, 2003c) and cows (Lee *et al.*, 2003b).

Until now, the few reports on the treatment of experimentally induced *E. coli* mastitis with *NSAID's* have focused on the effects of these drugs on clinical symptoms rather than biochemical parameters and eicosanoids. The objective of the present study was to examine the modulatory effect of carprofen treatment on different clinical, blood and milk parameters following moderate inflammation in primiparous cows.

Materials and methods

Experimental animals and study facilities

Experimental infections were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, Merelbeke, Belgium). The study was conducted to GCP standard under veterinary care, provision for withdrawal of animals based on pre-defined severity criteria and blinding. Animals treated within 10 d before the intramammary inoculation were not included in the trial. Therefore, only healthy animals, free of major mastitis pathogens through 3 consecutive bacteriologically negative examinations were included for the intramammary *E. coli* challenge. Primiparous cows accepted for the

intramammary challenge were inoculated between 12 and 28 d post-parturition. Milking was performed daily using a quarter milking device.

Experimental design

Inoculation was performed as described previously (Vangroenweghe *et al.*, 2004a; 2004b).

The inducible COX-2 enzyme was inhibited using a NSAID, carprofen, administered at PIH 9, when acute clinical symptoms were already present. Carprofen (1.4 mg/kg, Rimadyl™; kindly provided by Pfizer Animal Health) was administered according to body weight (2.9 mL/100 kg). Control animals received saline solution (0.9% NaCl; Baxter N.V.) according to body weight (2.9 mL/100 kg).

Blood and milk samples were collected at d-4, d-1, d0, d+1, d+2, d+3 and d+6 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21. Classical clinical parameters were examined. Rectal temperature (RT), heart rate (HR), respiration rate (RR), reticulorumen motility, skin turgor, fecal appearance, appetite, general attitude and aspects of the mammary gland were recorded at each blood and milk sampling time.

Severity determination and clinical severity score

The severity of *E. coli* mastitis was determined based on quarter milk production in the uninfected quarters at d+2 post-infusion (Vandeputte-Van Messom *et al.*, 1993; Dosogne *et al.*, 1997; 1999; Vangroenweghe *et al.*, 2004a; 2004b). Clinical severity scoring was performed based on Wenz *et al.* (2001) with some slight modifications (Vangroenweghe *et al.*, 2004a; 2004b).

Milk composition and determination of IL-8, C5a, sCD14, LBP, PGE2, TXB2

Serum albumin and ion concentration were quantified as previously described (Vangroenweghe *et al.*, 2004a; 2004b). Chemotactic factors (IL-8, C5a) and innate immune molecules (sCD14, LBP) were determined using ELISA as previously described (Vangroenweghe *et al.*, 2004b). For analysis of milk whey and plasma PGE_2 and TXB_2 concentrations, quarter milk samples were prepared as previously described (Boutet *et al.*, 2003). Subsequently, eicosanoids were determined using commercially available competitive ELISA kits (Neogen, Lexington, USA).

Statistical analysis

In order to compare the two treatment groups with respect to the various parameters analysed in blood and milk, a mixed model was used with cow as random effect and treatment, time and their interaction as categorical fixed effects. The effect of treatment on the local aspects of the mammary gland and clinical severity score was tested by the Wilcoxon rank sum test.

Results

Local and systemic inflammatory response

Following intramammary *E. coli* inoculation, RT rapidly increased from PIH 9 onward, to reach its maximum at PIH 12 in the saline-treated group. Carprofen administration at PIH

9 immediately reduced RT ($P < 0.0001$) at 3 (PIH 12) and 6 h (PIH 15) post-treatment, and RT normalized at PIH 15 (6 h post-treatment). Heart rate followed almost identical kinetics as described for RT. In the carprofen-treated group, HR was ($P < 0.05$) lower at PIH 12 and 15. Carprofen treatment following intramammary *E. coli* inoculation has a beneficial effect on the duration of reticulorumen motility depression. Although reticulorumen motility was equally depressed in both treatment groups at PIH 9 (time of treatment), the depression disappeared following carprofen treatment ($P < 0.01$), whereas in the saline-treated group, depression of reticulorumen motility reached its maximum at PIH 12.

Local swelling appeared at PIH 6 and reached its maximum at PIH 12 in both treatment groups. In the carprofen-treated animals, quarter swelling decreased from PIH 18 and normal quarter consistency was present at PIH 144. Quarter swelling decreased more slowly in control animals, although normal quarter consistency at palpation was also reached by PIH 144. Maximal quarter swelling score was lower ($P < 0.05$) in the carprofen-treated animals.

Clinical severity scoring and quarter milk production

Based on the clinical severity scoring system by Vangroenweghe *et al.* (2004a), clinical scores increased to a maximum in both treatment groups at PIH 12. The clinical severity score in the carprofen-treated group decreased more rapidly from 6 h post-treatment onwards. At PIH 15, clinical severity score was lower ($P < 0.01$) in the carprofen-treated group compared to the saline-treated group.

Milk production in the infected quarters decreased equally on d0, the day of intramammary *E. coli* challenge, in both treatment groups. Carprofen treatment did not significantly affect the recovery of milk production in the infected quarters compared with saline treatment. In the uninfected control quarters, no significant differences in milk production were observed throughout the entire study period. As expected, none of the animals in either treatment group reacted as a severe responder, based on the quarter milk production of the uninfected quarters at d+2 compared to the quarter milk production in these quarters at d-1 (Vandeputte-Van Messom *et al.*, 1993).

Milk composition

Maximal concentrations of serum albumin in milk were reached at PIH 15. In the interval PIH 12-48, serum albumin concentration showed an interaction between time and treatment ($P = 0.013$), meaning that serum albumin kinetics were different between groups. At PIH 21, carprofen-treated animals had lower concentrations of serum albumin in the milk of the affected quarters ($P = 0.0099$).

There was a treatment by time interaction for milk Na^+ and K^+ concentrations. Na^+ concentration was lower in the carprofen-treated animals at PIH 21 ($P = 0.005$) and PIH 24 ($P = 0.007$), whereas the K^+ concentration was higher in the carprofen-treated animals at PIH 21 ($P = 0.002$) and PIH 24 ($P = 0.005$).

Milk IL-8, C5a, sCD14 and LBP

No significant differences in IL-8, C5a and sCD14 could be observed between treatments throughout the study period. Milk LBP was lower in the carprofen-treated than saline-treated animals at PIH 12 ($P = 0.0001$).

Plasma and milk PGE_2 and TXB_2

Plasma PGE_2 and TXB_2 concentrations did not significantly differ between carprofen and saline-treated animals. Milk PGE_2 and TXB_2 concentrations tended to be lower (NS) in carprofen-treated than saline-treated animals.

Discussion

The aim of the present study was to evaluate the potential modulatory effects of carprofen, a PG synthetase inhibitor through COX-2 inhibition, treatment on a moderate inflammatory reaction following *E. coli* challenge. Therefore, the study design included carprofen administration following the appearance of initial clinical symptoms (Shpigel *et al.*, 1994) and inflammatory dynamics of the moderate inflammatory model used (Vangroenweghe *et al.*, 2004a; 2004b).

Carprofen-treated animals demonstrated an immediate and significant decrease in RT at 3 h post-treatment, whereas pyrexia continued in the control animals with a peak fever at PIH 12. Single dose carprofen administration in the present study resulted in a more pronounced and prolonged antipyretic effect compared to meloxicam treatment in an *E. coli* endotoxin model (Banting *et al.*, 2000), where peak fever was reached 2 h post-treatment in both groups. Reticulorumen motility was equally depressed in both treatment groups at the time of carprofen administration. However, 3 h post-treatment, reticulorumen motility in the carprofen-treated animals returned to normal, whereas a maximal depression was reached in the control group at PIH 12. A similar effect on reticulorumen motility was observed with meloxicam treatment in the *E. coli* endotoxin model (Banting *et al.*, 2000). Improvement of local clinical signs at the level of the affected mammary quarters by carprofen treatment was limited to swelling and milk appearance. These observations are in accordance with Anderson *et al.* (1986), who reported significant improvement of quarter temperature, edema, pain and size following flunixin meglumine treatment of cows suffering from endotoxin-induced mastitis. However, the administration of flunixin meglumine in that study was performed much earlier (PIH 2) as compared to our study, where carprofen was only administered at appearance of the first clinical symptoms.

Clinical scores, combining several clinical parameters, have been described (Wenz *et al.*, 2001; Friton *et al.*, 2002; Vangroenweghe *et al.*, 2004a). Using the clinical severity score described by Vangroenweghe *et al.* (2004a), carprofen-treated animals had a significant lower clinical score at PIH 15 compared to the saline-treated group. Quarter inflammation was associated with a temporary loss of milk production, combined with secretion of abnormal milk from the infected glands. Maximal depression in milk production in the infected and uninfected quarters occurred on the day of challenge (d0), and was followed by a rapid recovery during subsequent days. Carprofen treatment exerted no significant beneficial effect on milk yield in the infected quarters following intramammary *E. coli* challenge.

Serum albumin and all ions (Na^+ and K^+) showed similar kinetics, with a significant interaction between time and treatment from 12 h post-treatment (PIH 21) onward in the carprofen-treated animals. These results indicate that carprofen induces a more rapid recovery of normal milk composition in animals intramammarily challenged with *E. coli*.

Although the effect of NSAID's on eicosanoids production following inflammation is well documented in the literature (Anderson *et al.*, 1986), no reports are available on the possible

effect on other immunological inflammatory parameters, such as the chemotactic agents IL-8 and C5a or the early innate immune molecules sCD14 and LBP. Carprofen treatment at PIH 9 did not significantly affect either parameter.

Conclusions

The inflammatory model using primiparous cows during the periparturient period is a minimal and moderate state of inflammation, necessary to eliminate the invading pathogens from the affected mammary quarters. Carprofen treatment was administered late during the acute-phase reaction, when first clinical signs appeared, and had modulatory effects on clinical, production and immunological parameters. The main modulatory potential occurred at the level of improved clinical condition, mainly due to the antipyretic effects and the ability of carprofen to improve reticulorumen motility. Milk composition was significantly affected by carprofen treatment at PIH 21 and 24, but no further effects on milk composition occurred and milk production was not significantly affected. Carprofen treatment did not significantly affect PGE_2 and TXB_2 in plasma but tended to reduce concentrations in milk. Carprofen treatment did not result in a significant decrease of chemotactic inflammatory mediators, IL-8 and C5a, and early innate immune molecules, such as sCD14 and LBP. Major immunomodulatory effects from NSAID administration were therefore not observed in this model, although a larger study might confirm some apparent trends.

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Iron uptake and growth responses by Escherichia coli cultured with antibodies from cows immunized with high affinity ferric receptors

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Abstract

A series of experiments examined the ability of bovine immunoglobulin to bind high affinity iron receptors of *Escherichia coli*. *Escherichia coli* isolated from bovine intramammary infections each expressed the ferric enterobacterin receptor (FepA) when cultured in iron-deplete conditions and the ferric citrate receptor (FecA) when cultured in iron-deplete media containing citrate. Both FepA and FecA were immunogenic in cows. *Escherichia coli* incubated in iron-deplete media containing anti-FepA IgG or anti-FecA IgG had reduced iron uptake and extended generation intervals compared with growth in iron-deplete media with IgG from unvaccinated cows. Culturing *E. coli* in iron-deplete media containing both anti-FepA IgG and anti-FecA IgG had a synergistic effect on reducing iron uptake. However, the accumulative results of trials on blocking iron uptake and growth of *E. coli* by bovine antibody specific for high affinity ferric receptors indicates that this approach may not be feasible in lessening the incidence or severity of coliform mastitis. The concentrations of purified antibody used for in vitro iron uptake and growth inhibition trials far exceeded the concentrations of antibody in mammary secretions. Cows immunized with FecA had increased antibody titers to FecA, but vaccination showed minimal effect on clinical severity of mastitis compared with unimmunized control cows. The myriad of high affinity and low affinity iron acquisition systems of *E. coli* appear to allow for the pathogen to acquire iron and replicate despite the presence of IgG specific for one or more iron regulated outer membrane proteins.

Introduction

Iron is an essential element for survival and multiplication of coliform bacteria that commonly cause bovine intramammary infections (IMI). All Gram-negative bacteria have an absolute requirement for iron in the range 0.4 to 4.0 μ*M* (Weinberg, 1978). Higher organisms have evolved mechanisms for lowering the levels of free iron to well below those required for the growth of Gram-negative bacteria (Briat, 1992), therefore the concentration of bioavailable iron (10^{-18} *M*) is too low for bacterial growth (Bullen *et al.*, 1978). Most iron is bound intracellularly to proteins such as ferritin, hemoglobin, and myoglobin and extracellularly to high-affinity iron-binding proteins, such as transferrin and lactoferrin in serum and mucosal secretions (Briat, 1992). Coliform bacteria may utilize one or more iron acquisition systems depending on the environmental conditions.

High affinity iron uptake systems involve the synthesis of low molecular weight siderophore, the expression of iron-regulated outer membrane proteins (IROMP) and

enzymes to utilize the chelated iron. The enterobactin system is common in coliforms isolated from a variety of sources. Ferric enterobactin is specifically recognized by ferric enterobactin receptor FepA, a surface-exposed IROMP. Molecular weight and antigenic properties of FepA were highly conserved in different genera of coliform bacteria (Rutz *et al.*, 1991). Our hypothesis is FepA is critically involved in the pathogenesis of coliform mastitis during the nonlactating period when most iron is bound to lactoferrin in bovine mammary secretions. However, the role of ferric enterobactin in iron acquisition appears to diminish in the lactating gland as the concentration of lactoferrin decreases and the concentration of citrate increases.

Utilization of the ferric citrate iron transport system appears pivotal for coliform bacteria to acquire iron in milk. Coliform bacteria have developed the ability to acquire iron directly from citrate. Although citric acid is not a siderophore, the uptake system has all the properties of siderophore-mediated high-affinity systems (Braun, 1995). Interaction of ferric citrate with the IROMP FecA induces transcription of the *fec* transport gene operon (Pressler *et al.*, 1988). Rather high concentrations of citrate (>0.1 m*M*) are required to induce the system. Milk appears to provide an ideal environment for induction of ferric citrate iron transport system. Free iron in bovine milk is limited because most iron is primarily bound to citrate. Therefore, the bovine mammary gland is an iron-restricted environment for coliform bacteria with the average concentration of citrate in bovine milk adequate for the induction of ferric citrate iron-uptake system. In addition, the citrate-to-iron molar ratio in milk is in excess of 1000 (Jenness, 1974), which can easily result in ferric dicitrate.

The purpose of the trials outlined below was to examine the ability of bovine immunoglobulin to bind high affinity iron receptors FepA and FecA, block iron uptake, and alter growth responses of coliform isolates.

FepA

Monoclonal antibody specific for the enterobactin ligand-binding site of FepA inhibited the growth of *E. coli* that was isolated from bovine IMI (Lin *et al.*, 1998a). The ability of a murine monoclonal antibody that blocks the enterobactin ligand-binding site of the ferric enterobactin receptor FepA to inhibit the growth of coliform bacteria derived from a bovine IMI was determined in an iron-restricted medium. Bacterial isolates from bovine IMI in five herds were tested by the chrome azurol sulfonate assay to detect siderophore production. Each of the isolates of *Escherichia coli* (n = 25) and Klebsiella pneumoniae (n = 25) were positive for siderophore production. Each isolate expressed iron-regulated outer membrane proteins when grown in trypticase soy broth plus the iron chelator alpha-alpha'-dipyridyl. Immunoblots revealed that the monoclonal antibody recognized FepA that was expressed by each of the *E. coli* isolates (n = 25). Only 4 of 25 K. pneumoniae isolates produced FepA that reacted with the monoclonal antibody. This result coincided with the results of an in vitro growth assay. Growth of all *E. coli* isolates was significantly inhibited by the addition of monoclonal antibody to synthetic medium containing apolactoferrin. Antigenic variation in the enterobactin-binding site resulted in a low percentage of *K. pneumoniae* isolates that were inhibited by the monoclonal antibody. Inhibition of bacterial growth by the monoclonal antibody was dose-dependent. As little as 50 µg/ml of purified antibody had an inhibitory effect on bacterial growth in the synthetic iron-restricted medium.

The next step in delineating the ability of IgG to bind and block FepA was to immunize cows to determine if the protein was immunogenic in dairy cows. Immunization with FepA elicited an IgG response in lactating dairy cows (Lin *et al.*,1998b). Primary immunization was at approximately 200 d in milk, and booster immunizations were given 14 and 28 d later. Serum and whey IgG titers to FepA in cows vaccinated with FepA were significantly higher than those from cows vaccinated with control vaccines. Serum and whey IgG titers to FepA were elevated by 14 d in cows vaccinated with FepA. The degree of cross-reactivity of purified IgG from cows vaccinated with FepA to *E. coli* and *K. pneumoniae* isolates was significantly higher than that to a control isolate that lacked FepA production.

The ability of purified bovine IgG from cows immunized with FepA to inhibit the growth of coliform bacteria derived from bovine IMI was investigated in iron-restricted media (Lin *et al.*, 1999a). All isolates of Escherichia coli (n = 21) and Klebsiella pneumoniae (n = 21) were tested for growth in a chemically defined medium containing 0.5 mg/ml of apolactoferrin and in a pooled source of dry cow secretion. The addition of 4 mg/ml of purified bovine IgG directed against FepA in the synthetic medium resulted in significant growth inhibition for both *E. coli* and *K. pneumoniae* isolates. Growth reduction of *E. coli* was greater than that of *K. pneumoniae*. In dry cow secretions, the growth of each *E. coli* isolate was inhibited by IgG from cows immunized with FepA. However, growth of less than half of *K. pneumoniae* isolates (43%) in dry cow secretion was inhibited by IgG from cows immunized with FepA. Supplementation with 50 µM of ferric chloride to the medium completely reversed the inhibitory effects of the antibodies and lactoferrin. Bovine IgG directed against FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of the ferric enterobactin complex to the cell surface receptor FepA.

FecA

Expression of ferric citrate receptor FecA by *E. coli* and *K. pneumoniae* isolated from bovine mastitis was investigated (Lin *et al.*, 1999b). Transformant *E. coli* UT5600/pSV66, which produces large quantities of FecA in the presence of citrate, was constructed. The FecA of *E. coli* UT5600/pSV66 was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and used to prepare polyclonal antiserum in rabbits. All coliform isolates of *E. coli* (n = 18) and *K. pneumoniae* (n = 17) from naturally occurring bovine IMI in five herds induced iron-regulated outer membrane proteins when grown in Trypticase soy broth containing 200 µM alpha -alpha '-dipyridyl and 1 mM citrate. Rabbit polyclonal antiserum against FecA was used in conjunction with an immunoblot technique to determine the degree of antigenic homology of FecA among isolates. In the presence of citrate, each isolate expressed FecA that reacted with the anti-FecA polyclonal antiserum. The molecular mass of FecA (~80.5 kDa) was also highly conserved among isolates. Therefore, the conclusions were ferric citrate iron transport may be induced in coliform bacteria and utilized to acquire iron in milk for survival and growth.

The effects of IgG from cows immunized with the ferric citrate receptor (FecA) on iron uptake by *E. coli* were investigated (Takemura *et al.*, 2003). FecA was purified from *E. coli* UT5600/pSV66. Cows were immunized with FecA during late lactation and the nonlactating period. Immunoglobulin G was purified from colostral whey. The purified IgG from FecA immunized cows had higher IgG titers against FecA compared with control IgG. Fifteen *E. coli* isolated from IMI and *E. coli* UT5600/pSV66 were grown in an iron-depleted medium

containing 1 mM citrate to induce FecA. The bacterial cells were mixed with 0, 2, and 4 mg/ml purified IgG, and ^{55}Fe was added to the assay. The radioactivity of ^{55}Fe taken up by the bacterial cells was measured by a liquid scintillation counter. The presence of IgG decreased ^{55}Fe uptake by *E. coli* mastitis isolates and *E. coli* UT5600/pSV66. Anti-FecA IgG reduced ^{55}Fe uptake by *E. coli* greater than IgG from unimmunized cows. However, vaccinating cows with FecA had little effect on the growth inhibitory properties of IgG toward *E. coli* mastitis isolates cultured in Fe-deplete media (Takemura *et al.*, 2004). Iron depletion decreased the growth of E. coli compared with growth in Fe-replete medium. The presence of IgG further decreased the growth compared with the growth under iron restriction alone. Bacterial growth did not differ between IgG from FecA vaccinated cows and IgG from control cows. Replenishing media with exogenous iron overrode the inhibitory effects of the Fe-depletion and IgG.

Synergism between FepA and FecA

The synergistic effects of IgG from cows vaccinated with FecA and IgG from cows vaccinated with FepA were measured in the in vitro iron uptake assay (Wolf *et al.*, 2004). Serum was isolated and pooled within treatment from five cows each vaccinated with FepA or FecA or not vaccinated. Six *E. coli* isolates from bovine IMI were cultured in an iron-depleted media to specifically induce FecA and/or FepA. The bacterial cells were mixed with either 3 or 6 mg/mL of purified IgG and ^{55}Fe. The combination of anti-FecA IgG and anti-FepA IgG reduced ^{55}Fe uptake compared with either anti-FecA or anti-FepA alone. Iron uptake was reduced more by anti-FecA IgG than by anti-FepA IgG when the ferric citrate system was induced. Reduction of iron uptake did not differ between anti-FepA alone and anti-FecA alone when citrate was absent from the medium.

Conclusions

Bovine antibodies directed against IROMP produced by *E. coli* altered iron uptake and reduced growth rate of bacterial isolates from bovine IMI. In addition, antibodies specific for FecA and FepA synergistically reduced iron transport compared with either antibody source alone. A series of experiments (Lin *et al.*, 1998a; 1999a; Takemura *et al.*, 2003) have shown that the FepA and FecA proteins are antigenic to dairy cows and IgG from vaccinated cows reduce iron uptake and replication in vitro. Despite the presence of antibody specific to FecA and FepA outer membrane receptors on *E. coli*, these antibody sources did not completely inhibited the uptake of iron into the bacterial cell or bacterial replication. This may be attributed to nonspecific transport of iron complexes through the porin channels, the induction of other high affinity transport systems, or the antibodies binding to areas of the IROMP not directly involved in recognition of their specific iron complexes.

The accumulative results of trials on blocking iron uptake and growth of *E. coli* by bovine antibody specific for high affinity ferric receptors indicates that this approach may not be feasible in lessening the incidence or severity of coliform mastitis. The concentrations of purified antibody used for in vitro iron uptake and growth inhibition trials conducted in minimal, defined media have exceed the concentrations of antibody in complex mammary secretions. Likewise, experimental IMI data implied that vaccination with IROMP lacks efficacy. Although FecA immunized cows had increased antibody titers to FecA, vaccination

showed minimal effect on clinical severity of mastitis compared with unimmunized control cows (Takemura *et al.*, 2002). The myriad of high affinity and low affinity iron acquisition systems of *E. coli* appear to allow for the pathogen to acquire iron and replicate despite the presence of IgG specific for one or more IROMP.

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In vitro susceptibility of biofilm growing *Staphylococcus aureus* isolates for 10 antibiotics

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Introduction

Epidemiological studies reveal that following treatment with antimicrobial agents bacteriological cure rate of *Staphylococcus aureus* infections can range between 0% and 70% and seem to depend on age, parity, stage of lactation, position of the infected quarter and somatic cell counts (SCC) (Owens *et al.*, 2001). In contrast, in lactating cows, bacteriological cure rates tend to range from 0% to 52%. Treatment of older cows or cows with high SCC is generally less successful, and treatment of chronically infected high SCC cows may be considered ineffective irrespective of the *in vitro* susceptibility (Wilson *et al.*, 1999; Sol *et al.*, 2000).

The general therapeutic approach towards mastitis is the use of antibiotics to combat the underlying infection. Various reports on *in vitro* antimicrobial susceptibility testing [performed in accordance with National Committee on Clinical Laboratory Standards (NCCLS) standards], confirmed that over the past decade mastitis pathogens have remained susceptible towards all common antibacterial agents (Erskine *et al.*, 2002). The success of mastitis therapy, however, often remains disappointing as in many cases udder health status does not improve to an appreciable degree, and somatic cell counts are not reduced, or start to increase again after a short lag period.

Previously various pharmacodynamic and pharmacokinetic aspects of the antimicrobial agents used in mastitis therapy have been discussed with the aim to explain the apparent resistance to therapy. Among other factors, the transfer of parentally applied antibiotics from blood to milk, depending on serum protein binding, lipid solubility and pK_a values of the antimicrobial agent, and the tissue localization of the pathogen (extracellular *vs*. intracellular) have been discussed. Moreover, binding to milk proteins and inactivation by chelating ions (Ca and Mg) in milk influence the efficacy of applied antimicrobial agents (du Preez, 2000).

One of the common approaches to try to improve cure rates is to extend the duration of therapy (Sol *et al.*, 2000). Treatment of *S. aureus* infections for 3 - 4 days resulted in a 29% bacteriological cure, whereas treatment for 5 days resulted in a 42 % cure (Pyorala and Pyorala, 1998).

Taken together, these data indicate with respect to bovine *S. aureus* mastitis that:

- antibiotic susceptibility (measured *in vitro*) is only partly related to bacteriological cure rates;
- acute infections respond better to therapy than persistent chronic infections;
- extended treatment over longer time periods may improve cure rates.

Assuming also that the lack of efficacy of current therapeutic regimes is related neither to intrinsic nor acquired resistance of common pathogens, nor to any basic pharmacokinetic shortcomings of the antimicrobial agent, nor the formulation, other factors have to be identified which protect pathogens form being affected by antimicrobials.

One of the most convincing hypotheses to explain therapy resistance, is the ability of many staphylococci, to grow in biofilms in infected tissues, thus developing an innate resistance to almost all therapeutic agents.

Biofilms

Biofilms are *a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface* (Costerton *et al.*, 1999). Biofilm-related infections by gram-positive bacteria have been recognized in human medicine for, inter alia, *Staphylococcus epidermidis* and *S. aureus* (Donlan and Costerton, 2002). As these bacterial species are also major pathogens involved in bovine mastitis, the difficulties of treating these infections might be related to the ability of pathogens to form biofilms.

In most natural environments, microorganisms try to adhere to available surfaces. Hence, the free-swimming (or planktonic) phase can be viewed as a mechanism of dispersal form one surface to another. Following initial attachment of cells to a surface, surface motility and binary division result in an aggregation of attached cells (Stoodley *et al.*, 2002).These primary cell aggregates produce exopolysaccharides to facilitate clumping. A secondary stage is characterized by cell multiplication and formation of a mature structure consisting of many layers of cells, connected to each by extracellular polysaccharides (Yarwood and Schlievert, 2003). Finally, in the process of maturation, many staphylococci generate a glycocalyx, a slime layer that further protects the biofilm bacteria.

Biofilm resistance to antimicrobial agents

Several in vitro studies have shown that bacteria growing in biofilm can become 10-1000 times more resistant to the effects of antimicrobial agents as compared to planktonic growing bacteria of the same strain (Olson *et al.*, 2002). Several mechanisms are known to be responsible for resistance of biofilms to antimicrobial agents, including:

- delayed penetration of the antimicrobial agents through the bioiflm matrix;
- altered growth rate of biofilm organisms;
- physiological changes due to the biofilm mode of growth, including “persister” cells.

The exopolysaccharide matrix impairs the access of antibiotics to the bacterial cells (Stewart, 2002). Either a reaction of the compound with, or its adsorption to the components of the biofilm matrix, can limit the transport of an antimicrobial agent within the biofilm. A linear correlation between the thickness of biofilms and their resistance to antibiotics has been observed (Monzon *et al.*, 2001; 2002).

Slow growth of the bacteria has been observed in mature biofilms (Costerton *et al.*, 1999). This phenomenon accounts for the decreased susceptibility of bacteria in biofilms to antimicrobial agents requiring growing organisms for their bactericidal effects. For example, penicillins and cephalosporins are virtually ineffective on non-growing cells, and the rate of bacterial killing is proportional to the growth rate. Various classes of antibiotics, including aminoglycosides and fluoroquinolones, however can kill non-diving cells, although being distinctly more effective in rapidly dividing cells.

Antibiotic penetration and slow rate of bacterial replication cannot explain entirely the resistance of biofilms to one important group of antimicrobial agents namely the fluorquinolones. A dose dependent killing study showed that a small fraction of "persisters" may remain even after administration of increasing concentrations of fluorquinolones and it has been suggested that these "persisters" are cells in which the programmed cell death is disabled (Lewis, 2000).

In general, bacteria that are removed from the biofilm are as sensitive as the "primary" planktonic cells of that species (Lewis, 2001).

The dynamic features of biofilm formation and shedding of cells from one biofilm to form a new biofilm may also explain the relapsing nature of biofilm infections and the need for extending antibiotica therapy to interrupt the dynamics of biofilm formation.

Biofilms and mastitis

Microscopic examinations of *S. aureus* in mammary tissue in acute and chronic infections showed that the bacteria are mainly located in clusters within the alveoli and lactiferous ducts in association with the epithelial cells and invaded in the interstitial tissue (Hensen *et al.*, 2000). Moreover the hypothesis, that mammary infections are associated with biofilm formation, is also supported by the observed shift in antimicrobial sensitivity (Amorena *et al.*, 1999).

Hence the aim of the presented study was to measure the antibiotic susceptibility of biofilm growing *S. aureus* isolates for a broad range of antibiotics and antibiotic combinations, which are used in today in the Dutch dairy practice.

Materials and methods

Standard reference strain *Staphylococcus aureus* Newbould 305 (ATCC29740) and 3 bovine mastitis isolates from the Intervet strain collection with high (Newbould 305, BMA/GE/032/0385) and low (BMA/UK/032/0106, BMA/GE/032/0412) slime production are stored in -70˚C.

MIC's were determined by the NCCLS microbroth dilution method according to NCCLS guideline M31-A2.

Antimicrobial susceptibility testing of bacterial biofilms were performed on the MBEC™ assay according to manufacturer's instruction with slight modifications for biofilms grown in milk. Biofilm susceptibility was measures in two ways, first susceptibility of detached bacteria during overnight growth in challenge plates, second susceptibility of biofilm bacteria through dislodging and recovery in CAMHB (Cation Adjusted Mueller Hinton Broth). Presence of viable bacteria both in the antibiotic challenge plates after 24 h 37˚C [BMIC (Biofilm Minimal Inhibitory Concentration)] and in the CAMHB recovery plates [MBEC (Minimal Biofilm Eradication Concentration)] was determined with turbidity at 655nm in a 96-well plate reader.

Assays were performed with the following antibiotics: penicillin and trimethoprim, sulfamethoxazole as well as various other antibiotics including amoxycillin, cloxacillin, tylosin, neomycin cefoperazon, pirlimycin cefquinome and two combinations of antibiotics

Results and discussion

As an example, the results of two antibiotics, penicillin and TMPS are presented in Figure 1. In this figure - a comparison of MIC (according to NCCLS guidelines) MBIC and MBEC (MBEC™ assay) values is made. Data obtained with penicillin show a clear difference between MIC and BMIC values with the penicillin resistant strains 0385 and 0106. MBEC values of penicillin susceptible strains (N305 and 0412) are 256µg/mL and 128 µg/mL for penicillin in CAMHB and milk respectively, however for the penicillin strains MBEC values are beyond the limit of detection (> 1024 µg/mL). Trimethoprimsulfamethoxazole (TMPS) shows low BMIC values (< 1) values with moderately high MBEC values.

Biofilm bacteria from the milk assay have a higher susceptibility for penicillin and for TMPS and they are even more effective in the low slime producing strains 0412 and 0106.

Susceptibility for the other 8 antibiotics (amoxycillin, cloxacillin, cefquinome, cefoperazon, penicillin with neomycin, penicllin with cloxacillin, pirlimycin and tylosin) that has been measured as well (data not shown) show also clear differences in susceptibility and efficacy in high and low slime producing strains, warranting further research with this assay. Although conclusions are difficult to draw from these preliminary results, susceptibility to antimicrobial agents seems higher for bacteria grown in milk, as compared to bacteria grown in CAMHB which is in concordance to previous results (Amorena *et al*., 1999).

The correlation between penicillin susceptibility and the prognosis of cure rates, as observed in previous studies, (Sol *et al*., 2000) might be explainable considering that these *in vitro* assays, indicating a very low susceptibility for bacteria shedding from biofilms (BMIC), and for bacteria actually in a biofilm (MBEC) from penicillin resistant strains to penicillin and several other antibiotics.

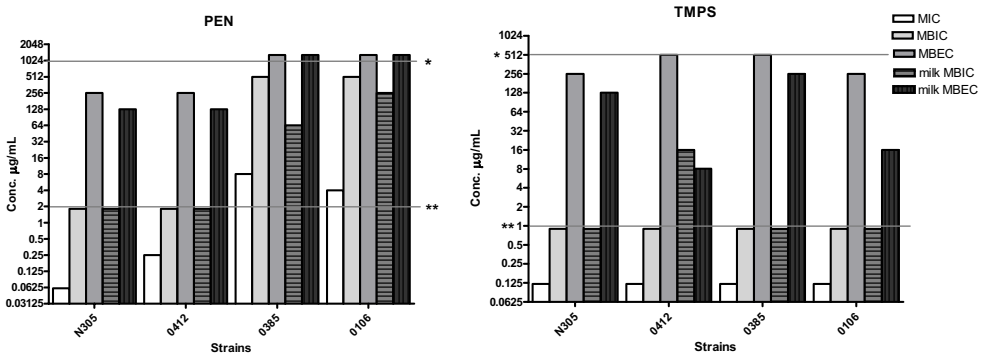

Figure 1. Concentration of Penicilline and Trimethoprimsulfa (µg/mL), determined with NCCLS and MBEC™ assay with inhibitory (MIC, BMIC) or eradicative (MBEC) effect on S. aureus mastitis isolates N305, 0412, 0385 and 0106.

**Upper limit of detection of MBEC™ assay.*

***Lower limit of detection of MBEC™ assay.*

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Better management of mastitis treatment: Contribution of consensus conference methodology

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Abstract

A consensus conference is a procedure to establish medical standards or clinical practice guidelines in all cases were there is uncertainty, controversy or logical inconsistencies. In terms of curative and preventive therapies for mastitis, veterinary practitioners repeatedly ask themselves the same questions: to treat or not to treat, with what, how should the different problems brought on by mastitis therapy be addressed - animal health, public health, economic issue? The French SNGTV (National Society of Veterinary Technical Groupings) have adopted a methodology to answer these questions, review the current state of the profession and knowledge as well as provide help in deciding on to implement mastitis therapy. A panel of European experts in the field of mastitis was queried to provide answers to questions raised by veterinarians on the diagnosis (epidemiological, bacteriological, etc.), the routes of administration, the treatment of subclinical and peracute mastitis in addition to the overall treatment strategy. After having drafted a critical bibliography on the aforementioned themes, the experts explained the outcomes of their research in a report. During the conference meeting, a debate on the questions raised and the answers provided took place among the experts and a group of vet practitioners representative of their French colleagues. After reviewing the consensus conference methodology, this article reports on the primary contributions that the conference made to help veterinarian practitioners manage mastitis therapy better.

Keywords: consensus conference, treatment, clinical practice guidelines, mastitis

Introduction

"A consensus conference is both a review of the current state of the profession and knowledge as well as a decision-making tool." (ANDEM, 1992)

Introduction: In terms of curative and preventive therapies for mastitis, veterinary practitioners repeatedly ask themselves the same questions: to treat or not to treat, with what, how should the different problems brought on by mastitis therapy be addressed - animal health, public health, economic issue? How to move concretely from theoretical pharmacological data to the practice of using pharmaceuticals, what are the decision making factors and their respective weight in making a treatment decision: this process is difficult for the practitioner. The French SNGTV (National Society of Veterinary Technical Groupings) have decided that consensus conference methodology is a consistent and structuring method to review knowledge on curative and preventive therapy for mastitis and develop guidelines

for veterinarians. This article explains the consensus conference methodology and the contributions of this conference in putting together practical guidelines for veterinarians.

Methodology (ANAES /Service Recommandations Professionnelles, 1999) and implementation

What is a consensus conference? What is its objective?

Methods for a consensus conference or practical clinical guidelines (PCG) have been developed in human medicine for nearly twenty years. Their goal is to establish, within a given community, relative points of agreement and divergence for a diagnostic procedure or a therapeutic strategy. The ultimate objective is to develop clinical practice guidelines in all cases where there are uncertainties, controversies, or inconsistencies.

The method process

"It entails writing guidelines by a Jury at the conclusion of the public presentation of expert reporting reviewing knowledge on a given topic. The critical analysis of the literature, carried out in this fashion, enables to find answers to pre-established questions." A public debate among practitioners, for example, enables the jury to define what the most relevant practices are by determining what results from scientific proof, presumption, and standard practice.

Consensus conference organization

The SNGTV took the initiative to sponsor a conference, select a theme, and then assign conference organization to a committee made up of members from the SNGTV milk quality commission and individuals from the Pfizer corporation.

The organization committee came up with questions to which the Conference was to provide substantive answers; chose the experts and a panel; and then asked the experts to come up with a critical bibliography on the suggested topics. This work was formalized in reports.

The conference took place over the course for a day and a half. The expert reports were submitted prior to the meeting both to the public and the panel. During the event, the experts presented the data from the literature and their personal experience on the selected themes. Additionally, they discussed them openly with the jury and the public. The results of a previously conducted survey on veterinarians' practices for prophylactic and curative mastitis treatments were brought to the public's, the experts' and the panel's knowledge. The audience, here limited to practitioner veterinarians and members of a specialized group in the area of milk quality from the SNGTV, debated along with the experts the findings taken from the reports. Whenever necessary, the panel had answers restated.

At the conclusion of the public conference, the jury met behind closed doors. They developed a summary that contains the points of consensus reached between the public and experts. Practical clinical guidelines were developed from them.

At the heart of the debate: themes, questions and experts tapped to contribute

The following themes and questions were addressed during the consensus conference:

- Efficacy and limitations of mastitis infection therapy. How can treatment failures be explained? What is the relative importance of bacteria location? Anti-infectious agent efficacy?
- Epidemiology of intramammary infections: what diagnostic help can knowledge of epidemiological data provide?
- Bacteriology of mastitis: what contribution does bacteriology make in the treatment of mastitis?
- How to make an early diagnosis? How to determine the infected quarter?
- Treatment of clinical mastitis: what are the choice criteria for local therapy and systemic therapy?
- Anti-inflammatory therapy: what is the real benefit of their use?
- The treatment of peracute mastitis: how to adapt therapy to the clinical state?
- The treatment of subclinical mastitis: should subclinical mastitis during lactation be treated and how?
- Treatment management: how to organize and implement a herd treatment strategy?

Contributions of the consensus conference for practitioners (SNGTV, 2005)

This conference addressed myriad issues and questions and as such it is a combination of a consensus conference and clinical practice guideline conference. At the conclusion of the conference, the jury submitted a report as provided for by the method: this report is a summary of "take aways" or "learnings" from the discussion and points of consensus. It was the subject of a publication.

The only points reviewed in this article are those that reinforce or improve concretely veterinarian practice in the area of treatment strategy or in areas of difficulty that require a conclusion.

Contributions to diagnostic aid provided by epidemiological data

The knowledge of the analytical epidemiology of individual pathogens has become much clearer over these past years with notably the detection:

- for enterobacteria: of multiclonal type isolates in the same herd and the existence of pathotypes causing latent mastitis which can become clinical after a few weeks;
- for staphylococci: of more oligoclonal isolates with a dominant strain;
- for *Streptococcus uberis* : oligoclonal and multiclonal strains depending on the herds, which has consequences notably in terms of the transmission of these strains.

These data, associated with the individual criteria of the herd (tank cell count - TCC— incidence of mastitis, types of mastitis) enable to define individual epidemiological models: a contagious model and an environmental one. Different germs come into play in these models: contagious staphylococci, enterobacteria, staphylococcus dominant submodel.

The information on these epidemiological models can help practitioners, at the herd level, to characterize the infectious type in each dairy herd and adapt the treatment strategy.

The contagious model is associated with a low level of strains (oligoclonal model); in this case, the findings of the antibiotic sensitivity testing performed on a few isolates can be generalized to the herd. The environmental mode is associated with a large number of

strains (multiclonal model) and the findings drawn from the antibiotic sensitivity testing a few isolates cannot subsequently be extrapolated.

Contributions on the means to make an early diagnosis and determination of the infected quarter

The purpose of the question is to determine if the treatments are more effective when administered early. If the answer is yes, then the next questions are: what are the most relevant means for making an early diagnosis and what criteria are used to decide on therapy?

It has been hypothesized that it stems from a scientific presumption to think that very early initiation of an anti-infectious for an udder infection limits the risks of a long-term infection of the udder.

The value of the individual means available for diagnosis in terms of earliness and identification of a quarter have been considered. Observation of clinical signs remains the method of choice; since it is specific but its sensitivity depends on the observer's acumen. Any clinical sign should result in treatment initiation; confirmation with the CMT (California Mastitis Test) is not necessary.

The CMT (California Mastitis Test) is scientifically and ordinarily recognized as the method of choice to detect an infected quarter. Its sensitivity can be enhanced through the operator's technical expertise; however, the CMT cannot guarantee early detection of new infections.

Scientific proof shows that electrical conductivity has inadequate sensitivity and specificity values to milk for it to be used alone. It is checked through standard practice. Only a measurement method based on the compilation of results taken from previous milkings, preferably quarter by quarter, seems to send up the appropriate warning signals allowing for an early diagnosis of udder infection.

Conductivity measurement is an important factor in early and meaningful screening provided that this measurement is considered only as a warning. This measurement must be associated with the CMT and the clinical examination in order to be used as decision-making tools for early treatment. A change in conductivity cannot be the only factor to come into play in the decision to treat.

Contribution on the value of bacteriology and antibiotic sensitivity testing

The discussions with the experts have led to the following practical guidelines. The bacteriologic analysis provides in the near term food for thought when the incidence of mastitis rises in a dairy herd, in the event of relapse or therapeutic failure. It is a complement to the epidemiological analysis but cannot replace the latter.

The objective of bacteriologic testing is to provide semi-quantitative data for prognostic and therapeutic purposes (regardless of the performance of antibiotic sensitivity testing). This requires collecting an adequate number of specimens while keeping in the mind the possibility of a bacteriologic under-diagnosis (false negatives). Standard practice ordinarily requires testing of a minimum of five samples. This threshold is, however, only indicative; since the number of samples depends on the desired outcomes. For example: it has been shown that with five samples, only 60 % of the herds infected with *Staphylococcus aureus* were diagnosed.

Bacteriology can also be used in the long term in an approach to set up an overall treatment strategy. The prior diagnosis of the herd relies on the recording of all cases of clinical mastitis and systematic sampling of these cases. The milk samples are stored frozen. As part of a follow up, a quarter of the samples (a minimum of five to ten) are analyzed *a posteriori* for bacterial identification.

To limit the effect of diluting potential outcomes in some bacterial populations due to freezing, it is preferable to store on glycerol type cryoprotectors.

The reliability of so-called "fast" methods is contested by scientists. They use selective media. Overall, the use of selective media is unadvised since this technique can cause false negatives. Additionally, the expediency of these methods compared to the times required for standard analysis is not improved.

Antibiotic sensitivity testing is of limited value in treatment decision making for udder infections. It provides purely theoretical and pharmacological data: these data observed *in vitro* do not allow for extrapolation into *in vivo* conditions.

A consensus has subsequently been reached on the non-systematization of antibiotic sensitivity testing. When it is performed, it can be used as a method of excluding antibiotics for which a resistance has been recorded. In the absence of setting a critical concentration threshold, the inhibitory diameter has no predictive value on the efficacy of an antibiotic family.

Contribution of the consensus conference on the treatment of mastitis

The efficacy of a treatment is assessed based on pharmacological and clinical trial data. Pharmacological data enable to justify *a priori* the use of such or such a drug or such or such route of administration. There are still no clinical trials available to confirm the efficacy that can be assumed from pharmacological data.

While many clinical trials on intramammary therapy are available, trials on the simultaneous use of a systemically administered antibiotic are still limited. The antibiotics tested in recent clinical trials on the use of the systemic route target rather specifically certain bacterial species.

In the absence of sufficient data to establish a decision-making tree, practitioners need to continue using the systemic route, in the event of observed or predictable failure, or if there is a risk of septicemia. Simultaneous use of both routes to treat mastitis with systemic signs in order to optimize clinical and bacteriologic cure is a common practice in France: this practice is not contested by the experts, even if rigorous proof of the added benefit is often still lacking.

The bibliographic review on the value of treating subclinical mastitis only provides partial data. Recent trials show an improvement in the bacteriological cure rate of subclinical mastitis when treatment is prolonged (example of pirlimycin). As for the benefit of the systemic route for treatment of these subclinical forms, the only trials available involve penethamate.

It should be noted that clinical trials are performed on animals with non cure or relapse risk factors, and this explains that the cure rates observed in "all types" of animals seems to be more disappointing than the rate obtained in these trials.

As for anti-inflammatory drugs, they are advised for the treatment of peracute mastitis; for other cases, their interest relies in their ability to improve animal welfare and pain management.

Contribution on the treatment of peracute mastitis

It seems to be standard practice to give priority to fighting the state of shock by using fluid therapy and anti-inflammatory drugs. The consensus focuses on the benefit of the analgesic value of NSAIDs enabling to provide for the pain management of the animal. Scientific presumption precludes from recommending calcium therapy as first line treatment with hypertonic energetic solutions. As an alternative to the use of an isotonic solution, a consensus on safety and efficacy has been reached: it prefers the use of hypertonic saline solution: a 7.5% solution. (5 ml/kg at 200 ml/min, i.e. 1 liter per 5 minutes). In the event of significant and early diarrhea, the injection of bicarbonates should only be done in the event of objectively confirmed severe acidosis. The efficacy of the administration of oral solutions is debatable, as it largely depends on gastro-intestinal tract function.

The objectives of systemic antibiotic therapy are not to limit the systemic clinical signs in their magnitude but more in their duration by controlling or avoiding early bacteremia and by controlling quickly udder infection. These objectives are justified and based on scientific rationale in the absence of formal evidence. Only a few clinical trials have been conducted to confirm this rationale. Some scientific experiments direct the choice towards the most recent generation of cephalosporins and quinolones.

In the event of peracute mastitis, the benefit of intramammary administration of antibiotics seems limited by the effects of the inflammation. Systemic therapies are preferred over local ones.

There is neither clinical proof, nor any standard practice on which to base a standardized treatment decision-making tree based on a clinical score.

A decision making tool that can be used in the event of endotoxinic shock can be based on a clinical score, capillary filling time, pulse perceived in the facial artery and the presence of diarrhea.

The prognosis becomes dim based on the clinical score and after serum assaying of ASAT, uremia, and creatineamia.

Contribution of the consensus conference for the implementation of a treatment strategy

Management must be “à la carte”: it is reminded that the strategy must be consistent with the overall objective set by the farmer: reduce tank counts or reduce the number of clinical mastitis or both: based on these objectives, the thresholds will be set for the different indicators: TCC, ICC (individual cell count), characteristics of mastitis (incidence, severity, time of development etc.) and the follow-up will be carried out relative to these thresholds.

Ongoing assessment of herd status is of help in the therapeutic decision. This assessment relies on the sorting of health animals and infected ones. To carry out the sorting, it utilizes individual cell counts.

One of the big debates for the French is to determine what discriminatory threshold is necessary to use for ICC. Based on the outcome of discussions, the threshold to use would depend on the desired outcomes for the herd and decision making elements. The threshold is only a compromise between sensitivity and specificity. For example, the threshold of 200,000 cells/ml is meaningful to select uninfected cows after calving. This threshold can be used for data analysis to assess and recommend a drying treatment strategy. In contrast,

a threshold of 300,000 cells/ml is preferable to target animals scheduled to undergo a bacteriologic analysis designed to collect epidemiological data on the herd.

Another frequent debate is to determine how many successive ICC are required to declare an animal healthy at a given point in time: based on discussions, a minimum of 3 successive counts below the set threshold are required. Regardless, anything over the 200,000 threshold during lactation is considered as the reflection of a new infection and the number of animals in this situation will be taken into account to determine the cause of the infections. The debate on the use of ICC to assess cure remains open.

Conclusions

The consensus conference addressed the many questions that French veterinarian practitioners raise on the treatment and medical prevention of mastitis. Some answers were provided through a bibliographic review carried out by experts notably on decision-making factors: how to assess and describe an epidemiological situation, on what basis and criteria. These points are important for practitioners to determine the treatment strategy to implement in herds. Regarding efficacy of individual treatment strategies, the bibliography provided us at times with too many overly partial answers, which are difficult to include in general standards. This consensus conference was limited to an audience of French practitioners, it would be very useful to compare and contrast international experience.

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Leukocyte dynamics and cytokine expression in response to mycobacterial water soluble compounds in bovine mastitis

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Abstract

The epidemiological scenario of bovine mastitis in recent years is associated with impaired immune responses. The therapeutic and immunomodulatary potential of water-soluble fraction (WSF) of *Mycobacterium phlei* in subclinical mastitis (SCM) was studied. Forty-five cows screened for mastitis divided in three equal groups. Group I cows (15), negative for intramammary infection (IMI) served as healthy control, group II and III (30 cows) positive for IMI were injected with 100 µgm of WSF and 5 ml sterile phosphate buffer saline (PBS)via teat canal on 3 alternate days, respectively. Somatic cell count (SCC) and total bacterial count (TBC) was studied at 0,3,7,15 and 30 days post treatment (PT). Milk differential leukocyte count (DLC), phagocytic activity (PA) and phagocytic index (PI) of the milk polymorphonuclear cells and cytokine transcripts elaborated by peripheral blood lymphocytes culture (PBL) using reverse transcription polymerase chain reaction was observed at '0' and 7 days PT. Treatment with WSF significantly ($P<0.05$) reduced the SCC and TBC on 15 and 30 days PT.Milk DLC revealed enhancement of lymphocyte %, similarly the PA and PI enhanced significantly on 7 days PT. PBL isolated from blood samples in group II showed mRNA expression of Interferon- γ (IFN-γ) and Interleukin-2 (IL-2). Reduction in SCC and TBC, enhancement of lymphocyte %, phagocytic activity and phagocytic index of milk leukocytes and expression of IFN-γ and IL-2 indicates the immunomodulatary potential of the adjuvant prepared from nonpathogenic strain of *M.phlei*. This is the first preliminary study on intramammary use of water soluble fraction in bovine mastitis, hence, can be used as adjunct or alternative to antibiotics in order to reduce the antibiotic residue in human food chain.

Keywords: Cytokines, immunomodulatory, *Mycobacterium phlei*, phagocytic activity, polymorphonuclear leucocytes

Introduction

Bovine mastitis continues to be the most economically devastating disease affecting the dairy industry (National Mastitis Council, 1996). Antibiotics are the only proven method for treatment of mastitis, however, antibiotic therapy of established mammary infection is only moderately efficacious (Daley *et al.,* 1992), and depresses the activity of the immune cells(Hoeben *et al.,* 1997). Polymorphonuclear leucocytes(PMN) are the most important cellular defence during mastitis but impaired function of PMN has been observed with intramammary infection during periparturient period(Cai *et al.,* 1994). Recruitment of

leucocytes from peripheral circulation into the diseased udder is an essential part of udder defence and many cytokines are considered to be important for the accumulation of leucocytes at the site of inflammation (Persson-Waller, 1997). Mycobacteria effectively enhance immune responses and are widely used as immuno adjuvants (Wahl *et al.,* 1979). The cell wall fraction of Mycobacterium contains glycopeptides, N-acetyl-muramyl-L-anahylD-isoglutamine, which is a potent immunomodulator and inducer of several cytokines (Azuma, 1992., Stolzenberg *et al.,* 1997). One possible approach to control mastitis involves manipulation of host defence mechanism during such immunosuppressive phase to resist pathogenic infection. The objective of the present study was to evaluate the immunomodulatory potential of the WSF from non-pathogenic strain of *M. phlei* during bovine subclinical mastitis (SCM)in order to reduce the antibiotic residue in human food chain.

Materials and methods

The WSF was prepared as per the method described earlier (Adams *et al.,* 1973), with little modifications (Mukherjee *et al.,* 2004).The crude WSF was collected, reconstituted in sterile 10 mmol , phosphate buffer saline (PBS, pH 7.4) with 20 µg of WSF in 1 ml of PBS filtered through a membrane filter (pore size 0.45 mµ) and stored in a sterile container at 4 ^{0}C till further use. Forty-five, crossbred lactating cows were selected from an organized dairy farm (Cattle and Buffalo), IVRI, Izatnagar. They were maintained in the animal shed of the institute under identical environmental conditions, and divided in 3 equal groups. Group I, consisting of 15 healthy cows, served as control. Fifteen cows in group II and 15 cows in group III (30 cow in all), positive for SCM on the basis of a positive CMT reaction (Schalm *et al.,* 1971) were taken. The cows in group II received an infusion of 100 µg of WSF per teat after diluting the drug in 5 ml sterile PBS (pH7.4), on 3 alternate days, while 5 ml sterile PBS was similarly infused in group III cows. Fifty ml of milk from each cow was collected in sterile vials after cleaning the teat orifice with 70% ethyl alcohol and after discarding few streams of milk on days 0, 3, 7, 15, and 30 PT. The SCC of the milk samples was assessed by the method of Schalm and coworkers, (1971). The TBC carried out by the method of Griffin and colleagues (1977), the organisms being identified on the basis of the morphology of the colonies on selective medium, characteristic hemolytic pattern and Gram's staining.

The isolation of PMNs was carried out as described by Daley and coworkers (1991). The viability of the cells was checked by the trypan blue exclusion technique (Colligan *et al.,* 1994) and the cell suspension was adjusted to 1 x10 ^{6} PMNs / ml, in sterile PBS.

Milk DLC was done as described by Dulin *et al.* (1982). Numbers of neutrophil and lymphocyte were counted in 100 cells and expressed in percentage.

The phagocytic activity of isolated milk PMNs was conducted as per the method described by Fox *et al.* (1987). Phagocytic activity (PA) was expressed by the % phagocytosed neutrophil in 100 cells and phagocytic index was determined on the unit of Staphylococci ingested by single PMN, counted in 100 cells. Peripheral leucocytes were obtained by separation of heparinized blood from healthy and mastitic cows in Histopaque density gradient centrifugation . Peripheral blood mononuclear cells (PBL) fraction was washed twice and resuspended in RPMI - 1640 medium with 10% heat inactivated fetal calf serum(Sigma,St Louis, MO,USA).The PBL were stimulated with 10µgml^{-1} of concanavalin A

(Sigma,St Louis, MO,USA) in tissue culture flask (Nunc, Denmark) , incubated at 37° c in humified 5% CO_2 atmosphere for 24 hrs. The cells were harvested and centrifuged at 4000 x G for 10 mins. The pelleted cells were washed in 0.1 % diethyl pyrocarbonate treated phosphate buffer saline , the pelleted cells were used for RNA extraction .Total RNA was extracted from blood derived cultured mononuclear cell pellete using RN easy mini kit (QIAGEN,Max-Volmer,-Stra beta E, Hilden, Germany). The procedures were carried out according to the manufacturer's direction. Reverse transcription as well as PCR of blood lymphocyte derived RNA was done by using QIAGEN one step RT-PCR kit. The reaction mixture was performed according to the manufacturer's direction using primers for bovine IL-2 and IFN-γ. RT-PCR was performed in DNA engine, peltier thermal cycler (Model PTC - 200,Massachusetts,USA).RT-PCR products were run on 2% agarose gel in TBE buffer , the gel was stained with Ethedium bromide , and the size was determined by using suitable molecular weight markers (Gene Ruler^{TM}-100 bp DNA ladder plus,Fermentas , life science).The gel was visualized with an ultraviolet illuminator and photographed Upstream and downstream primers of bovine IL-2 and IFN-γ sequences were same as those adopted from Ito and kodama (1996),synthesized by QIAGEN, natter mannelle, cologne,Germany.

The data were analyzed by one-way analysis of variance. The mean ± SE of the data were analyzed using Duncan's Multiple Range Test and the mean± SE of different groups was analyzed by using paired students 't' tests (Snedecor and Cochran, 1980).

Results

Results pertaining to the activity of the WSF in SCM are presented in Table 1. There were no changes in mean SCC and TBC of milk in group I cows during the trial period. The intramammary infusion of WSF in group II cows significantly (P<0.05) enhanced the mean SCC to an extent of 217 % and 79 % on day 3 and 7 PT respectively, as compared to 0 day values, thereafter SCC values decreased steadily till day 30 PT. Where as the SCC increased in group III cows till day 30 PT. The TBC in group II dropped significantly (P<0.05) on day 3 PT, and there after continued to decrease significantly till day 30 PT, where it reduced to

Table 1. SCC (x 10^5cells/ml) and TBC (x 10^3cells/ml) in response to WSF treatment (group II) compared to healthy cows (group I) and cows with subclinical mastitis which received PBS (group III).

Parameters	Days post treatment				
	0	3	7	15	30
SCC					
GrI	2.87 ± 0.38^{x}	2.38 ± 0.29^{x}	2.32 ± 0.40^{x}	2.55 ± 0.17^{x}	3.07 ± 0.33^{x}
GrII	$8.53\pm 0.56^{a,\ y}$	$27.36\pm2.42^{b,\ y}$	$15.27\pm 1.97^{c,\ y}$	$7.93 \pm 0.32^{a,y}$	$4.93\pm 0.71^{d,\ y}$
GrIII	$8.71\pm0.31^{a,\ y}$	$13.28\pm1.61^{b,\ z}$	$11.72 \pm1.40^{b,\ z}$	$10.28\pm 1.34^{b,\ z}$	$9.97\pm 1.19^{a,z}$
TBC					
GrI	0.33 ± 0.15^{x}	0.40 ± 0.13^{x}	0.27 ± 0.14^{x}	0.30 ± 0.07^{x}	0.40 ± 0.14^{x}
GrII	$5.98 \pm 1.26^{a,\ y}$	$3.55\pm0.17^{b,y}$	$1.93 \pm 0.18^{c,\ y}$	$1.14\pm 0.42^{c,y}$	$0.60\pm 0.13^{d,x}$
GrIII	$3.61\pm 1.16^{a,\ z}$	$3.60\pm0.62^{a,\ y}$	$3.27\pm 0.11^{a,z}$	$3.10\pm 0.77^{a,z}$	$3.06\pm 0.33^{a,y}$

*Values with different superscripts in each rows (a, b, c, d) and in each columns (x, y, z) differ significantly (P<0.05)

an extent of 883% as compared to pretreatment values. However, the TBC in group III increased till day 30 PT and it remained >3 x10 ^{3} cfu/ ml of milk. The organism isolated from the 30 milk samples from SCM cases were, *Staphylococcus aureus* (18 %), , *Streptococcus agalactiae* (20 %) and other *Streptococci* (24 %*), Coliform bacilli* (32%), contamination (6%).

Changes in Milk DLC in response to the WSF treatment is presented in Table 2. The milk DLC in healthy cows did not show any difference in neutrophil and lymphocyte % on day '0' and day 7 PT.Intramammary infusion of WSF non significantly enhanced the neutrophil %, however significantly (P<0.05) higher lymphocyte % was observed on day 7 PT. Where as, no difference was observed in neutrophil and lymphocyte percent in group III cows on day 0 and 7 PT with PBS treatment.

The PA and PI in response to WSF treatment are presented in Table 3.The PA and PI of milk PMNs in healthy cows remained unchanged on day 0 and day 7 PT.The PA and PI activity significantly enhanced (P<0.05) to the extent of 73.5 % and 77.4% in group II cows on day 7 PT respectively. Whereas, non significant rise of PA and PI was observed in group III cows on day 7 PT. The peripheral blood lymphocyte culture of mastitic cows did not show expression of IFN-γ but poor expression of IL-2 was observed. However peripheral blood lymphocyte culture after 7 days PT with WSF showed high expression of IFN-γ and IL-2 at 270 bp and 307 bp.

Discussion

The biggest challenge facing the dairy industry is to reducing the antibiotics in dairy cows for clean milk production. The pressure on the use of antibiotics and apparent lack of progress in control of intramammary infection has led to an increase in the use of

Table 2. Milk Differential leukocyte counts in response to Water Soluble Fraction of M.phlei treatment in bovine sub clinical mastitis.

	Days PT			
	Neutrophil %		Lymphocyte %	
Groups /Treatment	0 day	7 days	0 day	7 days
GrI (healthy)	27.89±0.78 ^{x}	28.40±0.69 ^{x}	20.93±1.08 ^{x}	20.23±1.42 ^{x}
GrII (WSF)	48.50±1.19^{y}	51.00±2.10 ^{y}	19.00±1.79 ^{a,y}	26.88±2.59 ^{b,y}
GrIII (PBS)	49.67±1.59 ^{y}	46.50±1.93^{z}	18.53±1.89^{y}	19.67±0.05^{x}

*Values with different superscript in each rows (a, b) and columns (x, y) differ significantly (P<0.05)

Table 3. Phagocytic activity and Phagocytic Index in response to Water Soluble fraction of M.phlei treatment in bovine sub clinical mastitis.

	Days PT			
	Phagocytic activity		Phagocytic Index %	
Groups /Treatment	0 day	7 days	0 day	7 days
GrI (healthy)	23.88±1.30 ^{x}	22.90±1.29 ^{x}	2.46±0.02 ^{x}	2.51±0.04 ^{x}
GrII (WSF)	20.82±0.86^{a,y}	36.13±0.54$^{b,\ y}$	1.82±0.12 ^{a,y}	3.23±0.23 ^{b,y}
GrIII (PBS)	20.02±0.86 ^{y}	21.14±0.89^{z}	1.76±0.09^{y}	1.98±0.10^{z}

*Values with different superscript in each rows (a, b) and columns (x, y,z) differ significantly (P<0.05)

alternative therapies. The objective of this study, therefore, was to characterize the host response to intramammary infusion of WSF of *Mycobacterium* in bovine SCM. In the present experiment a SCC above 8 x 10^5 cells per ml. and TBC more than 5x 10^3 bacteria per ml. of milk were observed in SCM. Similarly, high neutrophil % was observed whereas low percentage of lymphocytes, reduced phagocytic activity and phagocytic index was observed in SCM. Expression of IL-2 was poorly observed but IFN-γ expression was not detected in mastitic cows. Regulation of PMNs migration from the circulation to the diseased udder is of great importance with regard to the further development of fundamental methods for treatment of mastitis. Appropriate elimination of pathogens requires both the effectiveness of the drug and optimal functioning of the host immune system. This is especially important for animals with an impaired immune function during periparturient period (Hoeben *et al.,* 1997). Reduced activity of phagocytic cells precipitates bacterial infection during periparturient period in dairy cows and lower activity of IFN-γ were reported in such animals (Shuster *et al.,* 1996). The immune system in the udder has been studied extensively by use of exogenous cytokines against mastitis (Quiroga *et al.,* 1993 ; Sordillo *et al.,* 1997). Interleukin -2 stimulates the IFN-γ , which infiltrates leucocytes at the site of inflammation and promotes cell mediated immunity (Springer 1994).The immune competence of the phagocytic cells can be modulated by number of specific and non -specific mediators (Smith 1994). It is known that administration of WSF of Mycobacterium potentiate the secretion of cytokines from mononuclear cells (Chartey 1983 ; Leneau *et al.*, 1986). The cell wall fraction of mycobacterium contains trehlose dimycolate and muramyl dipeptide both potent stimulant of immune system and stimulates the production of cytokines by activated mononuclear cells (Berstein *et al.,* 1991). In this drug trial, the SCC significantly enhanced on day 3 and 7 PT, thereafter, it declined sharply from day 15 till day 30 PT. Similarly, the expression of IL-2 and IFN-γ mRNA was clearly observed on day 7 PT with enhanced phagocytic activity and phagocytic index in group II cows. It is evident from the above results that WSF possess immunomodulatory potential which stimulated the cytokine production from the mammary mononuclear cells. Similarly sharp reduction of total bacterial count from day 3 PT indicates either the antibacterial nature of the drug or clearance of the pathogen by enhanced activity of the phagocytic cells. PMNs exist in various stages of activation varying from dormant to activated. Activation triggers the expression of microbiocidal activity of phagocytes through respiratory bust activity. Mukherjee and coworkers(2004) observed decreased bacterial count with enhanced myeloperoxidase and acid peroxidase of PMN in bovine SCM with treatment of WSF. Similarly, Tamaka *et al.* (1977),observed increased phagocytosis by reticuloendothelial system in vivo with WSF treatment.

Conclusion

It may be concluded, based on the results that, intramammary infusion of WSF shows antimicrobial and immunomodulatory activities. Both the effect appear to be related to the enhancement of the activity of the resident PMNs as well as mononuclear cells in the bovine mammary gland and thus, support its use as alternative for the treatment of bovine subclinical mastitis in order to reduce the antibiotic from food producing animal. To my knowledge, it is the first report on intramammary use of WSF and its efficacy on phagocytosis by PMNs and also on cytokine production by mononuclear cells in bovine SCM.

However, further study is needed to examine the active principle responsible for such activity in clinical mastitis.

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Direct and indirect effects of subclinical mastitis treatment in dairy herds

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Abstract

The objective of this research is to evaluate the impact of treatment of subclinical mastitis on duration of infection and mastitis transmission in dairy herds. We describe the use of a deterministic state-transition model of intramammary infections (IMI) to predict the impact of subclinical mastitis treatment programs in populations of lactating dairy cattle. Results of model simulations indicate that the parameters duration of infection and the transmission coefficients influence the steady state values for the proportion of cows infected. The theoretical model predicts that a reduction in the duration of subclinical infections caused by contagious pathogens leads to a decrease in the prevalence and incidence of new IMI among susceptible individuals. Thus the model demonstrates potential indirect effects of mastitis treatment interventions resulting from changes in the intensity of pathogen transmission within a herd.

Keywords: mathematical model, transmission dynamics, duration of infection

Introduction

Subclinical mastitis is the dominant form affecting dairy cattle yet it frequently goes undetected or is left untreated on many dairy farms (Hillerton and Berry 2003; Oliver *et al.,* 2004). Cases of subclinical mastitis constitute a major reservoir of bacteria that are an important source of infection for other cattle in a herd (Bramley and Dodd 1984, Zadoks *et al.,* 2002, White *et al.,* 2003). Hence, successful treatment of subclinical mastitis has a direct effect on the individual animal, but may also have an indirect effect on other members of a herd, in that it reduces exposure to pathogenic organisms.

As early as 1969, Dodd and others recognized the importance of population level measures and mathematical models in evaluating mastitis control strategies (Dodd *et al.,* 1969). However, it has only been recently that a small number of studies have incorporated population level mathematical models to describe the dynamics of pathogen transmission and the overall effects of interventions such as post milking teat disinfection (Lam *et al.*1996, Zadoks *et al.,* 2002, White *et al.,* 2003). To the best of our knowledge, no studies have utilized state-transition models to estimate the overall effects of diagnosis and treatment of subclinical mastitis during lactation.

The use of deterministic state-transition compartmental models to describe pathogen transmission dynamics (SIR models) allows for the estimation of the effective reproduction ratio (R) for a pathogen (Anderson and May 1991, Zadoks *et al.,* 2002). Essentially, R describes the tendency of a pathogen to spread in a population of hosts, and is a function of the probability per unit time that one infectious individual will infect a susceptible individual (the transmission parameter, β) and the duration of infection (*d*). A primary advantage of SIR models is they incorporate the effect of population level infection prevalence and thus have use in quantifying the overall impact of interventions such as vaccination or antibiotic treatment programs (Bonten *et al.,* 2001, Longini *et al.,* 2002).

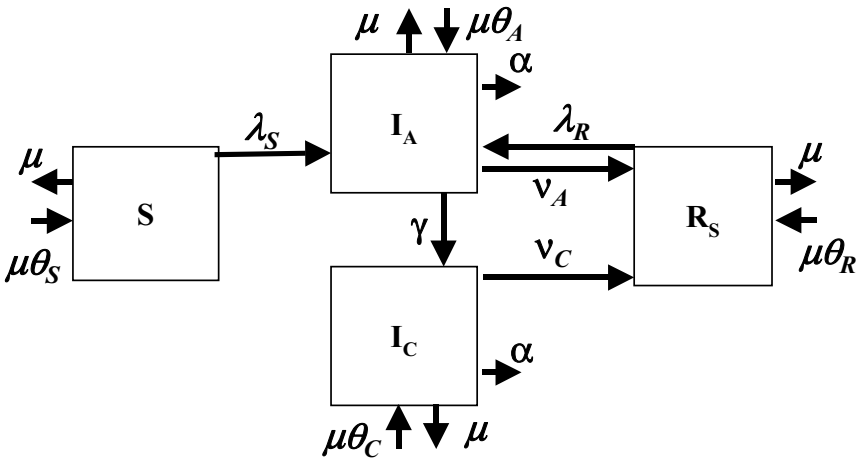
In order to estimate the overall effects of diagnosis and treatment of chronic subclinical mastitis in dairy herds, we have developed a deterministic state-transition model that describes subclinical mastitis dynamics. The purpose of this model is to enhance understanding of the impact of treatment programs on mastitis transmission and the epidemiology of subclinical mastitis. In the future, data available from an ongoing randomized, negative control, clinical field trial may be used to further explore the potential use of this model as a reasonable method to estimate of the effect of treatment programs on mastitis epidemiology in dairy herds.

Model development

We have developed a flow diagram to describe the transmission dynamics of subclinical mastitis in herds of lactating dairy cattle (figure 1). Using this flow diagram, a deterministic state-transition mathematical model has been developed. The model is defined by a set of linked ordinary differential equations to describe change in proportion of infected and uninfected cattle over time. This model builds upon previous epidemiologic models (Lam *et al.*1996, Zadoks *et al.,* 2002) by including four states: uninfected susceptible (S), transient subclinically infected (I_A), chronic subclinically infected (I_C), and recovered susceptible (R_S). Variables in the model include proportion of individuals within each state, and initial values for these variables are estimated from refereed literature. Fixed parameter estimates in the model include values for proportion of individuals entering and exiting the lactating population in each state, spontaneous recovery rates from each of the infected states, and cure rates due to antimicrobial treatment of infected individuals. Herd size is assumed to be constant with rate of new individuals replacing those that exit. Fixed parameter values are estimated using data obtained from refereed publications. Realizations of the model are obtained using the computer software package Berkeley Madonna (Version 8.0.1, Macey and Oster, University of California, Berkeley, CA, 2000).

Model behavior

Steady state conditions for the proportion of individuals in infected and uninfected states, and pathogen transmission rate parameters are estimated from the model using parameter estimates that have been previously reported (Zadoks *et al.,* 2002). We estimated initial parameters for S, I_A, I_C, and R_S to be 0.70, 0.10, 0.10, and 0.10, respectively. Thus, the total proportion of uninfected cows is 80%, while 20% of the population is infected at the start of all simulations. Parameter estimates, such as cure rate for chronic infections (1/duration of chronic infection), and transmission rate (β), are varied to evaluate model



Variables

S	Proportion of herd with no previous evidence of IMI (naïve susceptible)
I_A	Proportion of herd with new subclinical IMI
I_C	Proportion of herd with chronic subclinical IMI
R_S	Proportion of herd un-infected but with history of previous IMI (recovered)
λ_R	Force of infection – coefficient of transmission between I and R individuals
λ_S	Force of infection – coefficient of transmission between I and S individuals

Parameters

μ	Rate of turnover of lactating cows (cull, death, dry-off)
α	Rate of turnover of lactating cows attributed to mastitis (cull, death, dry-off)
γ	Rate of change new IMI to chronic IMI
β_S	Transmission rate for uninfected susceptible cows
β_R	Transmission rate for recovered susceptible cows
ν_A	Recovery rate new IMI (transient infection)
ν_C	Recovery rate chronic IMI
θ	Proportion of individuals entering the lactating herd in each state

Figure 1. A compartmental state-transition model of subclinical intramammary infection (IMI) for estimation of the impact of mastitis treatment strategies. Flow diagram illustrates conceptual framework of infection states associated with transmission dynamics of major gram-positive mastitis pathogens in a population of lactating dairy cattle. Changes in proportion of cows in each compartment or state (Susceptible, Newly Infected, Chronically Infected, and Recovered-susceptible) are modeled. The model is defined by a set of ordinary differential equations that describe changes of proportion of individuals in each state over time. (Symbols are defined).

behavior. Key variables that are examined following simulations are the maximum or steady state values for total proportion of cows uninfected and total proportion of infected cows, plus the effective reproduction ratio. The model predicts that, interventions that reduce duration of chronic subclinical mastitis lead to a reduction in the effective reproduction ratio for contagious pathogens. For example if a control program targeting diagnosis and treatment of chronic subclinical mastitis during lactation reduces duration of chronic infections by approximately 37% from a mean of 95 days to that of 60 days then the effective reproduction ratio is reduced by approximately 50%. As a result the overall effect of the treatment program is estimated to be approximately 1.5 times the direct effect of treatment, as the added benefit of curing chronic infections is the reduction in transmission of new infections to susceptible individuals (figure 2). As would be expected, the transmission rate (β) is a key parameter of concern. We examined the impact of varying the transmission parameter across a range of previously reported values. We found that when β is approximately 0.017 the model predicts a steady state of 20% of the population with IMI (i.e. no increase or decrease from initial value). Zadoks *et al.* (2002) previously described differences in the transmission coefficient for cows in the naïve susceptible (S) and recovered susceptible states (R_s), where susceptibilty to infection among recovered cows (β_R) was approximately 3 to 5 times greater then cows that had never experienced a previous IMI (β_S). We examined the effect of varying the relative difference in transmission between

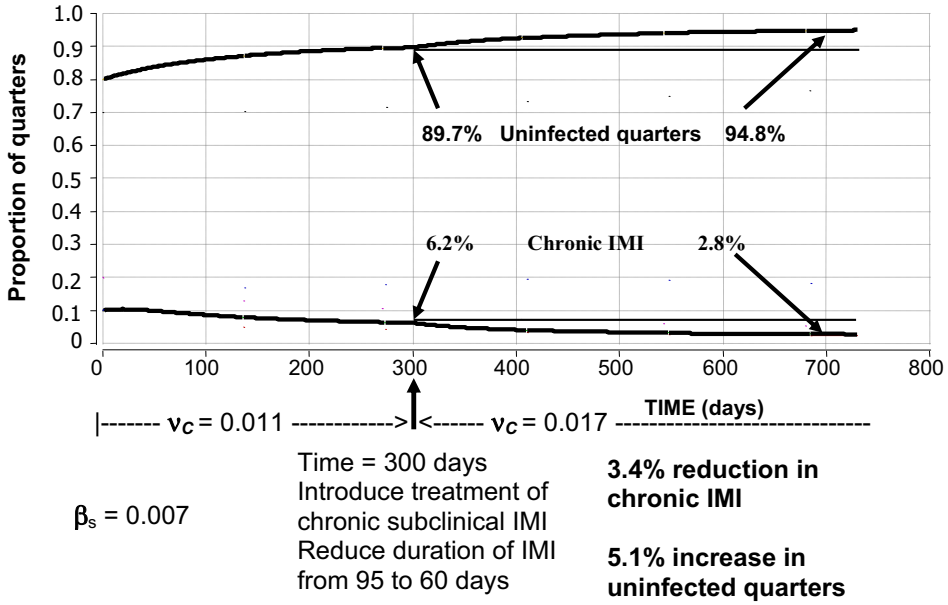


Figure 2. Change in proportion of quarters that are uninfected (clean) and chronically infected over time. At 300 days in time an antibiotic treatment program that targets chronically infected quarters is introduced. The predicted effect of this program is to reduce the mean duration of infection from 90 to 60 days, resulting in a 3.4% reduction in the proportion of chronically infected quarters (direct effect). The indirect effect of this program is a reduced force of infection on susceptible individuals in the herd. This results in a 5.1 % increase in proportion of uninfected quarters at approximately 1 year following start of the treatment program. Therefore the overall effect of the treatment program is predicted to be approximately 1.5 times the direct effect of treatment.

the two uninfected classes. We found that when β_R was greater then 2 times β_S the number of chronically infected cows in herd accumulate and recycle, creating an increasing pool of new infections. These simulations provide evidence that although other parameter estimates were not varied in our current analysis, in the future we should consider the impact of specific parameter estimates such as the mastitis associated exit rate (α). Changes in this parameter obviously influence the proportion of recovered susceptible cows, and can be used to model the effect of targeted lactation therapy in combination with selective removal of cows with non-responsive or recurrent infections.

Discussion

Previous model simulations have focussed on mastitis control practices that are designed to reduce the probability of transmission. Studies have modeled the effect of teat-end hygiene (i.e. post milking teat disinfection) on estimates for the transmission coefficient of *Staphylococcus aureus* in individual dairy herds (Lam *et al*.1996, Zadoks *et al.,* 2002). In the first of these studies, the duration of infection was estimated from field trial data and shown to be approximately equivalent for treatment and untreated control udder halves (Lam *et al*.1996). In the second of these reports, duration of infection was observed to differ among the three herds studied. In one herd, where duration of infection was shortest, lactation therapy was initiated soon after diagnosis of subclinical mastitis, and the higher mastitis cure rates were observed. The authors suggested this contributed to decreased transmission, and the lower overall prevalence of infection observed (Zadoks *et al.,* 2002). Results of our simulations are consistent with those authors' observations, where treatment of subclinical mastitis leading to a reduction in duration of infection among individual lactating cows results in decreased force of infection in the herd. This supports two important concepts that have emerged from our simulation studies. First, we believe that there is substantial value in monitoring and reporting duration of infection as an outcome measure in field trials of mastitis therapy. And, second that the indirect or population level effects of mastitis treatment programs should be evaluated as a component of therapeutic field trials.

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Therapeutic treatment with casein hydrolyzate eradicate effectively bacterial infection in treated mammary quarters in cows

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Abstract

Accumulation of casein-derived peptides to critical concentration in milk stored in the mammary gland induces the process of mammary involution in the gland. The ability of casein hydrolyzates (CNH) to dry-off milk secretion in infected mammary quarters and cure microbial infection was evaluated. Lactating cows, infected by various common pathogens in one quarter and having an average somatic cell count (SCC) of 2,000,000 were treated. An infected quarter was infused with 10 ml of CNH (prepared under GMP conditions) three consecutive times, 24 h apart, after milking. Treatment accelerated and synchronized the natural involution process as reflected by rapid dry-off of milk secretion in 35 out of 37 cows within 3 days. Dramatic changes in the composition of mammary secretion: disappearance of lactose and fat and accumulation of whey proteins with components of the innate immune system were observed. The bactericidal activity of mammary secretion of treated glands, which started within 8 h, persisted after the 3 day course of CNH treatments protocols. Immediate success resulting in SCC of less than 400,000 in the three functional glands in post-treated cows was 85%. After parturition, 78% of the treated quarters were bacteria free. Thus, CNH appears as an effective tool to improve milk hygiene in cows exposed to subclinical and chronic mastitis and to eliminate bacterial infection. The latter properties of CNH treatment also suggest that it may be used as an effective non-antibiotic dry treatment for cows.

Introduction

Clinical and subclinical mastitis are inflammatory states of the udder that affect a high proportion of dairy cows around the world. Clinical mastitis is easily diagnosed due to marked alterations in milk composition and appearance, decreased milk production, elevated body temperature and swelling, redness, or fever in the infected quarters. Subclinical mastitis, the most prevalent form of the disease, often remained undetected because signs are not readily apparent. Many subclinical intramammary infection (IMI) tend to persist, resulting in elevated milk SCC and decreased milk production, which may lead to development of clinical mastitis and the opportunity for certain mastitis pathogens to spread from infected mammary quarters to uninfected ones.

Recently, we have shown that the process of active involution in goats and cows is triggered following milk stasis as a result of induction of high rate of plasmin activity, which

in turn liberates active casein-derived active peptides (Shamay *et al.,* 2002, 2003). Infusion of casein hydrolyzates (CNH), which contain the active casein-derived peptides, dramatically accelerated the rate of involution in goats and cows, to the extent of completion within 3 days (Shamay *et al.,* 2002, 2003). At the third day post-treatment, the scanty mammary secretion was watery, almost fat and lactose-free, turbid (serum-like) and contained more then 5×10^6/ml leukocytes, mostly (90%) in the form of polymorphonuclear neutrophils. The local inflammatory response induced by CNH was associated with increases in the concentrations of components of the innate immune system: lactoferrin (an antimicrobial protein) and immunoglobulin type G (Shamay *et al.,* 2003) and enhanced the formation of hydrogen peroxide, NO and NO derived species such as nitric dioxide (Silanikove *et al.,* 2005). Thus, CNH treatment induced marked bactericidal and bacteriostatic responses in mammary secretion against a wide range of pathogens within 8 h from the first treatment as a result of the drastic reduction in nutrient availability for bacterial growth and the acute activation of the innate immune system (Silanikove *et al.,* 2005).

The aim of the present study was to find out whether the acute inducement of the innate immune system by CNH treatment can be use to eradicate bacterial infections associated with clinical and subclinical mastitis and whether milk hygenity (milk SCC) is improved following the treatment.

Material and methods

Cow study population

The study comprised 37 cows after an initial recruitment of 42. The reason of withdrawal was lack of reliable data (SCC) before treatment in 4 cows and no complain by the farmer after treatment (n=1). Cows were eligible for recruitment in this study if they had four functional quarters, had no significant teat lesion, were in good health and had not received antibiotic and or anti-inflammatory therapy within the last 30 days. No mastitis vaccines were used in any of the herds at least during the previous year. The cows in these farms were fed a typical Israeli total mixed ration that comprised 65% concentrates and 35% forage containing 17% of protein.

Preparation of casein hydrolyzate

Casein hydrolyzate was prepared from commercial casein as described (Shamay *et al.,* 2003). The procedure took place in Hy Laboratories (Rehovot, Israel) under GLP conditions, ensuring that the final product was sterile and that the product was bottled in sterile vials. Endotoxin level in the final product was 0.48 EU/ml according to the Limulus Amebocyte Lysate (LAL) test. The amount of endotoxin injected 0.0001 EU/kg body weight (assuming average cow BW of 500 kg), is $\times$2000 lower than the tolerance limit of endotoxin in intrathecal administrated human drugs (K = tolerance limit in EU/kg = 5 EU/kg for parenteral drugs and 0.2 EU/kg for intrathecal drugs). The final products for single injection into a single quarter contained 10 ml CNH with peptide concentration of 10 mg/ml.

Treatment administration

Bacteriology

Milk samples were examined at the Israeli Cow Breeding Association (ICBA) central laboratory, Caesarea, Israel or at the National Mastitis Reference Center, Kimron Veterinary

Institute. Organisms were identified and quantified by standard laboratory techniques (Leitner *et al.,* 2004a). Positive finding, were based on two consecutive identifications of known mastitis pathogens.

Somatic cell counts

Individual cow mean SCC were calculated for recording before (SCC-PRE) treatment (not more than 15 days) and after treatment (SCC-POST). After treatment mean SCC was calculated by averaging individual cow SCC at 60 days after treatment and at least two measurement or two following months of SCC. Treatment success was assessed from mean SCC below 200,000 cells/ml, as individual cow SCC greater than 200,000 cells/ml have been reported as being indicative of intramammary infection (Dohoo and Leslie, 1991). In addition, 27 cows treated in a pre-dry-off period were followed by their monthly SCC from up to 10 months after subsequent lactation period.

Result collection and statistical analysis

Data on treatment, SCC, bacteria, cow's parity and quarter treated drying-off dates were compiled. The data were analyzed by SAS/STAT package. Groups (clinical vs. subclinical mastitis, SCC and microorganisms) were compared using the chi-squared test for SCC and the one-way ANOVA for continuous variables. Pre- and post- values were compared using ANOVA for repeated measurements. All milk samples were analyzed for SCC with a Fossomatic 360 at the ICBA laboratory.

Results

Pretreatment data

Data were collected from 42 cows from 10 herds (2 to 7cows/herd) with a confirmed diagnosis of mastitis in one quarter. The most prevalent identified pathogens were *Arcanobacterium pyogenes*, *Staphylococcus aureus, Escherichia Coli* and *Streptococcus uberis*. Infection with *Streptococcus* species, *P. auroginosa, Corynobacteruim bovis* and *Micrococcus* were exclusively sub-clinical, whereas ~ 60% of infections with *S. aureus, E.Coli* and *A. pyogenes* were clinical and ~40% subclinical.

Somatic cell count after treatment

There were significant differences between SCC-PRE (average 2,210,200) and SCC-POST in the following 60 days (average 205,000) either when tested for individual pathogen or in the whole study group ($p<0.0001$) (Table 1). Pathogen effect on SCC was not significant either in the pre-treatment period (SCC-PRE, $p=0.3$) or in the post treatment period (SCC-POST, $p=0.5$).

In the clinically infected glands, SCC-POST in 75 % of the cows (n=9/12) was 201,000 cells/ml or less after treatment, and in all the treated cows SCC-POST was 401,000 cells/ml or less (Table 2). In the subclinical infected glands, SCC-POST in 57% (n=12/21) of the cows was 201,000 cells/ml or less after treatment and in 81% of the cases 401,000 cells/ml or less. When considering all the data set, SCC-POST in 63.6% (n=21/33) of the cases was below 201,000 cells/ml, which was significantly higher ($p<0.01$) than the number of cases (n=12/33) in which SCC-POST was above 201,000 cells/ml.

Table 1. Number of cows by pathogens and the SCC (×1000) before and between 15 to 60 days from treatment, pooled across herds in Israel (n=10), period 2001-2003.

	Number	Somatic Cell Counts	
		SCC-PRE	SCC-POST
		Average	Average
Pathogens			
Staphylococcus aureus	5	1,235.2	147.4
All *Streptococcus*	7	3,357.4	262.3
Escherichia Coli	5	1,781.2	275.4
Arcanobacterium pyogenes	10	1,465.1	145.6
Others	6	3,283.5	226.7
All:			
Average SCC		2,210.2	205.0
Standard deviation		2,374.3	170.2

Note: SCC-PRE denotes pre-treatment somatic cell counts; SCC-POST denotes post-treatment somatic cell count.

Table 2. Number of cows by SCC (×1000) frequencies between 15 to 60 days from treatment, pooled across herds in Israel (n=10), period 2001-2003.

Stage	Cows by SCC-POST		
	0 - 200	201 - 400	>401
Clinical	9	3	0
Subclinical	12	5	4
Average SCC-POST	95.6	329.4	530.9
Standard deviation	45.8	67.1	133.4

Note: SCC-POST denotes post-treatment somatic cell count.

Somatic cell count after parturition

In 27 of the cases information was available after parturition in the next lactation. On the average, 6.1 months had elapsed between treatments to drying-off (Table 3). In 59.3% of the cows (16/27), SCC-POST was below 201,000 cells/ml, which is not significantly different from 21/23, the number of cases in which SCC-POST was below 201,000 cells/ml in the period between treatment and dry-off. It is worth noting that in almost 26% of the cows SCC-POST were below 101,000 cells/ml during the whole follow-up observation and in 85% (23/27) SCC-POST was below 401,000 cells/ml. The latter value is not different from 29/33 (88%), the number of cases in which SCC-POST were below 401,000 cells/ml in the period between treatment and dry-off.

Discussion

A typical common confusing problem in modern dairy farming is the case of high-yielding albeit subclinically infected cow. Such a cow frequently produces a lot of milk, but the infection in one quarter increases its SCC to a level that may increase the collecting tank counts, thus, reducing the grading of the milk on the farm level. In contrast to clinical mastitis, it is not usually advisable to treat subclinical mastitis during lactation (Gruet *et*

Table 3. Post-partum mean of SCC (×1000) after cow's treatment pooled across herds in Israel (n=10), period 2001-2003.

SCC-POST	Cow			Length Observation		SCC-POST		
	#	%	(95% CI)	Months	Range	Mean	S.D.	Range
≤100	7	25.9	(9.4;42.5)	5.9	(2,9)	65.5	19.0	(31.5;91.0)
101-200	9	33.3	(15.6;51.1)	6.5	(3,10)	123.7	18.8	(103.8;158.2)
201-400	7	25.9	(9.4;42.5)	6.2	(2,9)	284.2	63.3	(203.1;378.5)
≥401	4	14.8	(1.4;28.2)	4.5	(1,6)	761.5	280.6	(436.0;1,034.7)

Note: SCC-POST denotes post-treatment somatic cell count as average between 15 up to 306 days. S.D. - standard deviation.

al., 2001) because the cure rate is low and because the cost of the treatment and a withdrawal period of 4-5 days of milk make it economically unjustified (Yamagata *et al.,* 1987). In some experiments, although intramammary therapy during lactation with antibiotics resulted in apparent bacterial cure, it did not reduce quarter or cow SCC in comparison to the pre-treatment levels (Cattell *et al.,* 2001). The ability of CNH treatment to dry off the infected gland, which contributes to elevated SCC on the cow level while continuing milking from the three other uninfected glands, improved dramatically milk hygienic immediately after treatment. CNH treatment was even more effective in reducing SCC in clinically infected cows. As the treatment effect is restricted to the treated quarter, the improvement in milk quality was obtained without the need to discard milk from the uninfected gland. This is important, because discarded milk is one of the major causes for economic losses in clinical mastitic dairy cows (DeGraves and Fetrow, 1993).

Our data clearly show that milk yield (MY) on the cow level was not affected despite of drying-off one of the quarters. Two likely explanations may be considered: i. The contribution of the infected quarter MY to cows MY was small and the decreased MY in the infected quarter was already compensated by increased MY in the uninfected ones. Sheep and goats with one infected gland compensate for its reduced milk production by increasing the output from the uninfected gland; the compensation being greater in goats than in sheep (Leitner *et al.,* 2004a, b). The cows feel better and thus their appetite improves because of termination of the acute (clinical cases) chronic (subclinical cases) inflammatory state in the infected gland.

In addition to the immediate improvement in milk quality that persisted during the concurrent lactation, another important added value from the treatment was gaining of high rate of bacterial cure, which was associated with secretion of milk with low SCC during the next lactation cycle. The level of bacterial cure-maintenance of SCC below 201,000 cells/ml is appreciably higher than typical rates of spontaneous recovery from bacterial infection (40-50%) and are comparable with the highest rates of curing obtained with extended intramammary treatment of subclinical mastitis (8-9 days) with pirlimycin (Olivier *et al.,* 2003). Such an extended antibiotic treatment would be justified only in few cases for treating highly valuable cows. Furthermore, there was no evidence to suggest that extended therapy with pirlimycin resulted in a greater reduction in SCC in milk than the 2-day treatment, in which bacterial cure rate of *S. uberis* was only 58% (Olivier *et al.,* 2003).

The numbers of individual pathogens in the study group was too small in order to derive conclusive conclusions regarding the efficacy of the method against specific pathogens.

However, the fact that the treatment was equally effective against broad range of pathogens including contagious, environmental and gram negative bacteria is consistent with its mode of action, namely, immediate activation of the innate immune system and conversion of mammary secretion into nutrient-deficient and hostile environment for bacteria. Development of pyogenic mastitis, such as those caused by *A. pyogenes* is associated with poor prognosis for cure with antibiotic treatment. In order to prevent the spread of the infection into the systemic system and total loss of the cow, the conventional treatment is to cut the teat, in order to drain the infection, or to treat the infected gland with substances such as chlorhexidine or povidone-iodine. These procedures are painful and cause irreversible loss of milk secretion functionality in the treated glands. In some cases of infection, treatment with povidone-iodine was used to control outbreak from escalating infections with *S. aureus* (Middleton and Fox, 2001). The high rate of curing infections caused by *A. pyogenes* and *S. aureus* following CNH enables saving the functionality of the infected gland and thus avoiding the use of alternative traumatic and painful treatments.

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Antibiotic resistance in *Staphylococcus aureus* isolated from bovine udders: Development related to introduction of dry cow therapy?

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Abstract

A dry cow therapy field trial started in Norway in 2002, and will end during spring 2005. Included in the trial were 203 commercial dairy herds which were randomly selected into three different dry cow regimes (n= 70, 68 and 65 herds), 772 cows have so far been given therapy. The different dry cow formula consisted of two lactations- and one long acting formula. One of the lactation formulas consisted of procainpenicillin only; the other lactation- and long acting formula consisted of benzyl penicillin and dihydrostreptomycin, the long acting formula was in an oil suspension. Selection criteria for dry cow therapy were isolation of *Staphylococcus aureus* strains (SA) in milk samples before drying off combined with SCC>100.000 and/or a previous mastitis history. There was no increase in penicillin G resistance in either SA or coagulase negative staphylococci strains (CNS) during one year of selective dry cow therapy at herd level, cure rate was high in all groups, 81.0, 79.7 and 82.2 % respectively and new infection rate was low, 2.1, 1.0, and 4.6 % respectively. There were no significant differences between the treatment groups. There was no cluster effect at herd level in any of the multivariable models, but a significant effect within cows in all models. The effect of dry cow therapy was significant lower at hind quarters compared to front quarters and the odds ratio (OR) for isolating SA or penicillin G resistant SA (PRSA) given isolation of SA or PRSA in the same quarter at drying off were 5.3 and 12.0 respectively. There was no significant probability of getting PRSA at calving given SA at drying off; meaning SA at drying off did not convert into PRSA at calving.

Keywords: dry cow therapy, penicillin G resistance, *Staphylococcus aureus*

Introduction

The use of antibiotics and the development of resistant strains of bacteria have been discussed and reported since antibacterial drugs were accepted for use in both human and veterinary medicine. Antimicrobial susceptibility is an important area in mastitis diagnostics since mastitis is one of the most common diseases in many dairy farming, and the single most common cause for antibacterial use in lactating dairy cows (Kaneene *et al.*, 1992). Increased resistance of SA strains, isolated from mastitic cows, to several antimicrobial agents have been reported (Gentilini *et al.*, 2000; Myllys *et al.*, 1998). To determine if the resistance is emerging, the resistance observed historically should be compared with that of present. Major differences in sampling strategy, bacterial identification procedures,

resistance testing, and interpretative criteria have been used, making it necessary to interpret results between different reports with caution.

Many countries have established the five-point plan in mastitis control. (Kingwill, 1981) This plan recommends dry cow treatment of all quarters of all cows (blanket dry cow therapy) with an approved long-acting dry cow formula. However, as awareness increases regarding the routine use of antibiotics in food animals, blanket dry cow therapy may be a possible factor contributing to antimicrobial resistance. The Norwegian mastitis control recommends selective dry cow therapy at cow-and/or quarter level and therapy is being conducted with lactation formulas. In fact, Norway is the only country, as we know of, that does not have any approved dry cow therapy formulas (Felleskatalogen 2004). Recent reports suggest that antimicrobial resistance can be increased using dry cow therapy with high dosage of penicillin with lactation formulas (Osteras *et al.,* 1999).

The purpose of this paper is to compare trends in susceptibility patterns over a period of two years from the same laboratory and geographical region after introducing selective dry cow therapy with both lactation- and long acting formula.

Materials and methods

Selection criteria

A total of 215 commercial dairy herds from six different geographical regions were included in a dry cow therapy field trial in Norway. Due to different reasons, 11 herds withdraw during the trial, which restricted the number of herds to 203. All participating herd took annual milk samples and the first- (at the beginning of the trial) and the second annual milk sample set (after 1 year) were used to identify an increase of penicillin G resistance at herd level. The farmers took quarter milk samples monthly. These samples were taken in connection with milking and analyzed at the district laboratories of the TINE Dairies BA using Fossomatic® instruments (Foss Electric Ltd.) according to the International Dairy Federation's (IDF) recommendations (International Dairy Federation, 1984). The selective dry cow therapy regimes were conducted in following approach; before drying off, bacteriological milk samples were taken from cows with CMSCC > 100.000 and/or a previous history of clinical mastitis. SA cows were treated at drying off according to the herd's regimen. All cows are followed up with bacteriological samples 6 days after calving.

Dry cow therapy treatment

A: Juvanesta Comp - 300.000 IU benzylpenicillin (aeqv. Penethamate hydriodide BAN 300 mg) and 300 mg dihydrostreptomycin (Boehringer Ingelheim Vetmedica AS). If 1 or 2 quarters are infected only these will be treated, if 3 or 4 quarters are infected all four will be treated. The treatment is repeated four times with 24h between each treatment. The last treatment should not be milked out.

B: Siccalactin Vet - 200.000 IU penicillinbenzatin and 400 mg dihydrostreptomycin in an oily suspension. (Boehringer Ingelheim Vetmedica AS). The dry cow therapy most commonly used in Sweden. All 4 quarters are treated once, independent of how many quarters are infected.

C: Mastipen -300.000 IU procainpenicillin (VetPharma AS). Treatment procedures were followed as in treatment A group.

Culturing techniques

Quarter foremilk samples were collected aseptically after discarding the first streams of milk. Primary culturing was done on blood agar plates (washed bovine erythrocytes) with added esculin (Blood Agar Base, Oxoid Ltd, UK). The plates were divided into quarters and β-hemolytic SA was cultured on the dividing line. 0.01 ml quarter foremilk was streaked out on each quarter before incubation at 37°C for 18-24 hours. Presence of mastitis pathogens and purity of the samples were recorded and judged depending on the appearance of the colonies. Bacteriological negative samples, from cows with clinical symptoms and/or a positive CMT-test value (CMT > 3), are preincubated in 37°C for 4 hours before 0,0075 ml of milk is streaked out onto two quarters. These samples are incubation in 37°C for another 18-24 hours before they are examined.

Growths of one or more colony forming units (cfu) of SA in pure culture, or > 5 cfu of other major udder pathogens in pure culture were recognized as positive. In cultures with more than one species, SA with typical haemolytic zone was recorded as positive. If not showing typical haemolysis, the isolates are coagulase tested and all the coagulase positive isolates were reported as SA. Typical rich growing colonies in non contaminated samples responding negative on the coagulase tes are reported as CNS. All SA and CNS strains were first tested for penicillin G susceptibility using "Clover-leaf-test system" for betalactamase production. Betalactamase positive SA and CNS strains were susceptibility tested for Streptomycin, Amoxicillin/Clavulanacid, Oxycillin and Tetracycline, using diffusion agar method on Müller Hinton agar (Difco) and Neo-Sensitabs™ (A.S. Rosco, Taastrup, Denmark). All susceptibility testing were done in accordance to the Norwegian official procedure (National Veterinary Institute, 1993), which is in accordance with IDF's procedures (International Dairy Federation, 1981).

Models and statistics

The logistic regression procedure in SAS, version 8.0, was used to identify significant candidate variables to be tested in a multivariable model. The models were fitted for dry cow treated cows, both PRSA infected quarters after calving and SA infected quarters after calving. PROC GENMOD with binomial distribution and logit link function was used (Dohoo *et al.*, 2003). PRSA infected quarters at drying off, SA infected quarters at drying off, lactation number and quarters were kept in the model as independent fixed effect and herd and cow as random effects nested within cow. The random effect was expressed as the log (OR) by using the LOGOR statement. The different models were evaluated by 2*Log Likelihood procedure (Dohoo *et al.*, 2003).

Results

Penicillin G resistant CNS strains (PRCNS) were used as indicators for development of penicillin G resistance at herd level. The annual samples were taken as a screening of the herds. There was no increase in penicillin G resistance in either SA or CNS during one year of selective dry cow therapy at herd level. Instead, there was a significant decrease in PRCSN after one year (Table 1). The third annual sample set (after 2 years) are not complete jet due to the ending of the trial in May 2005. Cure rate was defined as positive bacteriological samples before drying off and negative samples 6 days after calving. New infection rate was defined as negative before drying off and positive 6 days after calving. Before dry cow

Table 1. Quarters infected with Penicillin G resistant Staphylococcus aureus= PRSA and penicillin G resistant coagulase negative Staphylococci = PRCNS at the beginning of the selective dry cow therapy trial (n total = 16,320) and after 1 year (n total = 14,998).

Dry cow therapy	% infected quarters at the beginning of the trial				% infected quarters after 1 year			
	N	PRSA	N	PRCNS	N	PRSA	N	PRCNS
Juvanesta (A)	34	0.62	19	0.34	38	0.77	8	0.16
Siccalactin (B)	28	0.50	11	0.20	24	0.45	8	0.15
Mastipen (C)	11	0.21	10	0.19	11	0.23	6	0.12
Total	73	0.45	40	0.25	73	0.49	22	0.15

therapy was introduced, the different treatment groups had an equal amount of SA and PRSA positive samples before drying off. The cure rate was high in all groups, 81.0 % (A), 79.7 % (B) and 82.2 % (C) and new infection rate was low, 2.1, 1.0, and 4.6 % respectively (Table 2). There weren't significant differences between the treatment groups.The cluster effects at herd level weren't significant in any of the models, but there was a significant effect within cows in both models. The effect of dry cow therapy was significant lower at the hind quarters compared to the front quarters and the OR for isolating SA or PRSA at calving given isolation of SA or PRSA in the same quarter at drying off was 5.3 and 12.0 respectively compared to no such isolate at drying off. There was no significant probability of getting PRSA at calving given SA at drying off; meaning SA at drying off did not convert into PRSA at calving.

Table 2. Cure rate and new infection rate at quarter level. Staphylococcus aureus = S.aur and penicillin G resistant Staphylococcus aureus= PRSA, n total = 3084.

Dry cow therapy	Cure rate %				New infection rate%			
	N	*S.aur*	N	PRSA	N	S.aur	N	PRSA
Juvanesta (A)	184	81.0	16	87.5	532	11.3	700	0.9
Siccalactin (B)	232	79.7	17	82.4	824	5.3	1039	0.6
Mastipen (C)	180	82.2	9	66.7	584	8.2	755	0.8

Discussion

The common argument in Norway is that long acting dry cow formulas will lead to resistance bacterial strains due to subtherapeutic concentration of antibiotics. An increased risk of development of penicillin G resistance bacteria strains would be a negative consequence when introducing selective dry cow therapy and should be monitored. In our study there is no increase in penicillin G resistance, so other factors, other than selective dry cow therapy, most therefore contribute to such a development. The amount of quarters being infected with PRSA and PRCNS are in consistence with Sølverød *et al*. (2001) who screened the Norwegian cow population in a survey in 2000. The risk factors associated with PRSA in this study are consistent with Østerås *et al*. (1999), but our finding that the use of lactation formula does not increase the risk of resistance development is in

contradictory to Østerås *et al*. The cure rates are higher than Østerås *et al*. found, which can be explained by high SCC cows being culled after calving instead of treated at drying off; resulting in treating cows with a higher probability of getting cured due to a lower SCC at drying off, BMSCC in Norway has decreased the last 10 years; resulting in fewer non-responders high SCC cows and more responders with lower SCC. It is well known that treatment success is strongly dependent on host-associated factors; duration of infection, number and localization of quarters infected, age, lactation stage and somatic cell count (Østerås *et al*., 1999; Sol *et al*., 1997). The cluster effects in this study are estimated using an alternative logistic regression method (Carey *et al*., 1993) which is available in the SAS version 8.0 using the LOGOR statement. The procedure returns the logs of respective OR's within cluster and subcluster respectively. The effect is expressed as the odds of randomly selected PRSA infected cows being or not being within the same herd to the ratio of the same odds for non- PRSA infected cows, comparing cows having the same fixed effects (Dohoo *et al*., 2003).

To limit the emergence of bacterial resistance, a more responsible way of using antimicrobial agents is needed in both veterinary and human medicine. Unfortunately, relatively few studies have determined the proportion of PRSA isolates, of bovine mastitis origin, over time performed at the same laboratory and geographic region. The antimicrobial situation in Norway is as follows; the use of antimicrobials in Norwegian animal production is low. In 2003, the total sale of antimicrobial drugs approved for therapeutic use in animals was 5,787 kG. β-lactamase sensitive penicillin accounted for 84% of the veterinary penicillin preparations sold in 2003 (NORM/NORMVET). In 1995, Norwegian food animal production industries voluntarily abandoned the use of all antibacterial growth promoters. Antimicrobial resistance is still a limited problem in Norway. The relatively low usage of antimicrobial agents together with the pattern of use must be continued to preserve this favourable situation. However, the situation may rapidly change if the use of antimicrobial agents increases and/or if resistance clones are imported.

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Orbeseal® and Orbenin® EDC in combination for the treatment of intra-mammary infections at drying off and prevention of new infections during the dry period and early lactation in dairy cows

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Abstract

Three hundred and thirteen (313) dairy cows from 10 commercial farms in the UK and with somatic cell counts (SCCs) above 200,000 cells/mL were enrolled in a GCP study. For each cow, treatments were randomised such that two quarters (one fore and one hind) were infused sequentially with a combination of OrbeSeal® and Orbenin® EDC (626 quarters, T01) and two quarters with Orbenin® EDC alone (626 quarters, T02). Treatments were administered as part of the standard veterinary preventative procedures on the day of drying off (day 0) for each cow. Bacteriological samples were collected on days -2, -1 and 0, and twice after calving (C), on day C+4 and between C+8 and C+11. Quarter SCCs (QSCCs) were measured on day 0 and between C+8 and C+11 days. Cows were monitored for clinical mastitis until day C+60. Clinical mastitis developed pre-calving in one quarter in T02, and post-calving in 16 quarters in T01 and 37 quarters in T02 ($P<0.05$). Analysis showed that, for quarters infected pre-treatment, there was no significant difference ($P>0.05$) between treatments in the elimination of existing infections or the prevention of new sub-clinical infections. The mean QSCCs for T01 and T02 were lower post-calving than at drying off. The combination of OrbeSeal® with Orbenin® EDC was as effective as Orbenin® EDC alone in the treatment of existing infections and performed significantly better in the prevention of clinical mastitis during the dry period and early lactation.

Keywords: OrbeSeal, Orbenin EDC, Dry Cow Therapy

Introduction

Intramammary infusion of a teat sealant containing bismuth subnitrate (OrbeSeal®, Pfizer) in dairy cows with somatic cell counts (SCCs) at or below 200,000 cells/mL at drying-off, has been shown to be effective in the prevention of new intramammary infections (Huxley *et al.*, 2002). SCCs higher than 200,000 cells/mL on three consecutive recordings are regarded as indicating the possibility of sub-clinical infections (Green *et al.*, 2002), and the infusion of an intramammary antimicrobial is usually the most appropriate measure to control an existing bacterial infection. In the present study, the clinical efficacy of a combination of OrbeSeal® and an intramammary antibiotic containing cloxacillin (Orbenin®

Extra Dry Cow) was compared to the use of intramammary antibiotic alone in the treatment of existing intramammary infections at drying off and the prevention of new intramammary infections during the dry period and early lactation in dairy cows with SCCs above 200,000 cells/mL. The study was conducted at 10 commercial dairy farms in South-West England and the results were combined in a single multicentric analysis.

Materials and methods

Three hundred and thirteen (313) dairy cows were recruited onto the study, each had somatic cell counts (SCCs) from bulked quarter samples above 200,000 cells/mL in at least two of the three months prior to drying-off, including the final month of lactation. Cows had to be in good general health, free of clinical mastitis and had to have four functional quarters, with teats free from significant lesions. Any animal that had received antibiotic or anti-inflammatory treatment in the preceding 30 days was excluded. For each cow, the quarters were randomised to treatment in a 1:1 ratio with one front and one hind quarter being treated with a combination of OrbeSeal® and Orbenin® EDC (T01), and the other two quarters being treated with Orbenin® EDC alone (T02). Thus, a total of 626 quarters were treated with the combination of OrbeSeal® and Orbenin® EDC infused sequentially (T01) and 626 quarters with Orbenin® EDC alone (T02). Prior to infusion the teats were cleaned and treatments were administered aseptically, with one tube of each product being infused into the allocated quarters on the day of drying off (day 0) for each cow. Bacteriology was performed on three milk samples collected prior to drying-off, on each of days -2, -1 and pre-treatment on day 0, as well as two samples collected post-calving (C), one on day C+4, and one between days C+8 and C+11. For each cow monthly SCC data were obtained from farm records for at least three months preceding day 0. Individual quarter milk samples were also collected for QSCC determination prior to treatment on day 0 and post-calving between days C+8 and C+11. Each cow was monitored for clinical mastitis during the dry period and for 60 days post-calving (C+60). Efficacy was evaluated on the incidence of clinical mastitis, the prevention of new sub-clinical infections in each quarter and the treatment/elimination of existing sub-clinical infections in each quarter. The study was conducted in accordance with Good Clinical Practice guidelines, and the husbandry of all animals was in accordance with local animal welfare requirements and legislation.

Data analysis

A generalised linear model for binary data with treatment, quarter position (front or hind) and their interaction as independent variables/factors was used to analyse success/failure in the elimination of pathogens present pre-treatment and prevention of new infections post-treatment. Animals for which there were incomplete data sets due to missing data and/or withdrawal from the study were not included in these analyses. For all quarters, failure to prevent clinical mastitis from day 0 to day of calving +60 was analysed using a generalised linear model for binary data with treatment, quarter position (front/hind) and their interactions as independent variables/factors. For animals with clinical mastitis, the frequency of epidemiologically important bacterial pathogens isolated from mastitic milk samples were analysed separately. For animals with mastitis the unaffected quarters were excluded from the analyses from the same timepoint as the affected quarter(s). Geometric mean quarter milk SCCs determined from the second milk samples after calving were

summarised. All treatment differences were assessed at the two-sided 5% level of statistical significance ($P \leq 0.05$).

Results

There were no treatment-related adverse experiences. A number of concurrent diseases were diagnosed during the study, and in total 27 animals were withdrawn and thus failed to complete the study (54 quarters in T01 and 54 quarters in T02) leaving 572 evaluable quarters in each treatment.

Bacteriology

Pre-treatment infections were diagnosed retrospectively in 413 quarters treated with the combination (T01) and 410 in quarters treated with Orbenin EDC alone (T02). The most prevalent infections were Corynebacterium spp. and coagulase negative (coag -ve) Staphylococci (see Table 1). Post-calving, the incidence of these species showed reductions, most marked for Corynebacterium spp., as did the incidence of all the more commonly

Table 1. Bacteria isolated pre-treatment (day 0) and post-calving (days C+8 to +11).

	OrbeSeal+Orbenin EDC		Orbenin EDC only	
	Pre-treatment	Post-calving	Pre-treatment	Post-calving
Corynebacterium spp.	37.4%	2.0%	36.7%	4.0%
Coag -ve *Staph*. spp.	17.8%	13.3%	16.6%	16.3%
Bacillus spp.	7.2%	4.2%	6.1%	3.8%
Streptococcus uberis	5.1%	2.2%	6.3%	3.4%
Staphylococcus aureus	3.1%	0.8%	3.0%	0.6%
Coag +ve *Staph*. spp.	2.1%	1.8%	2.3%	1.2%
Other *Strep*. spp.	1.6%	0.4%	1.6%	0.8%
Escherichia coli	1.9%	2.4%	1.7%	3.2%
Enterococci	1.6%	1.0%	1.9%	0.8%
Enterobacter spp.	2.1%	0.2%	1.2%	0.4%
Proteus spp.	0.7%	0.6%	1.0%	0.6%
Aerococcus spp.	1.0%	0.8%	0.9%	0.0%
Streptococcus dysgalactiae	1.0%	0.2%	0.7%	0.2%
Escherichia spp.	1.0%	0.0%	0.2%	0.2%
Pseudomonas spp.	0.9%	0.0%	0.3%	0.2%
Yeast	0.0%	0.2%	0.3%	0.4%
Citerobacter spp.	0.5%	0.2%	0.2%	0.0%
Lactococcus spp.	0.3%	0.0%	0.5%	0.0%
Aspergillus spp.	0.0%	0.6%	0.0%	0.0%
Others	1.7%	0.4%	0.7%	0.2%
No growth	34.8%	73.8%	35.0%	68.3%
Total no. of quarters sampled	572	496	572	496

Percentages do not add to 100 as quarters could have mixed infections. Others included *Enterococcus faecalis*, *Citerobacter diversans*, *Gemealla morbillorum*, species of *Klebsiella*, *Bordetella*, *Micrococcus*, *Serratia*, *Hafnia*, *Kluyvera*, and *Mucor* together with a non-fermenter and non-specific Gram -ve bacteria.

isolated pathogens, e.g. Bacillus spp., Strep. uberis, Staph. aureus, coag+ve Staph. spp., and other Strep. spp. The only exception to this trend was for E. coli which showed slight increases, from 1.7-1.9% at drying off to 2.4-3.2% post-calving.

Clinical mastitis

Significantly more cases of clinical mastitis occurred in quarters treated with Orbenin EDC alone (T02) than with the combination (T01) (38 vs. 16, *P*=0.0022) (see Table 2). One of the quarters in T02 developed mastitis during the dry period. For the 46 cows that developed mastitis post-calving, 11 were affected in one T01-treated quarter only, five were affected in both one T01- and one T02-treated quarters, 28 were affected in one T02-treated quarter and two were affected in two T02-treated quarters. Overall, there was no significant difference in incidence of mastitis between front and hind quarters (*P*>0.05). There was a significant difference (*P*=0.0137) between treatments in the combined incidence of mastitis associated with the following important pathogens: *Strep. uberis*, *E. coli* and *Staph. aureus* (eight cases in T01 vs. 21 in T02) and significantly more of these infections occurred in the hind-quarters (20 cases) compared with the front quarters (nine cases) (*P*=0.0388).

Treatment/elimination of pre-existing sub-clinical infections

For those quarters found to have been infected pre-treatment, there was no significant difference (P>0.05) between treatments in the elimination of pre-existing infections (92.3% in T01 vs. 88.9% in T02) (see Table 3). No significant differences were found between front and hind quarters (P>0.05).

Table 2. Bacteria isolated from cases of clinical mastitis.

	OrbeSeal+Orbenin EDC (T01)	Orbenin EDC only (T02)
Streptococcus uberis	5	14
Escherichia coli	3	5
Staphylococcus aureus	0	2
Coag +ve Staph. spp.	(1)[a]	4
Other Strep. spp.	0	1
Bacillus spp.	0	1
Citerobacter spp.	1[b]	0
Enterococci	1[c]	0
Escherichia spp.	1[c]	1
Proteus spp.	0	1[d]
Serratia spp.	0	1[b]
No growth	6	10
Total	16 of 572 (2.8%)	38 of 572 (6.6%)
P-value T01 vs. T02	0.0022	

[a]Isolated from quarter prior to onset of mastitis, no mastitic sample available.

[b]Present as a mixed infection with Strep. uberis.

[c]Present as a mixed infection in the same quarter.

[d]Present as a mixed infection with E. coli.

Table 3. Summary of treatment effectiveness: Elimination of infections present at drying-of.f

Treatment	No. of quarters	Treatment Failure	Treatment Success
OrbeSeal+ Orbenin EDC (T01)	326	25	301
	100%	7.7%	92.3%
Orbenin EDC only (T02)	325	36	289
	100%	11.1%	88.9%
P-value			NS

NS=not significant (*P*>0.05).

Prevention of new sub-clinical infections

For all quarters included in the analysis, there was no significant difference (*P*>0.05) between treatments in the prevention of new sub-clinical infections (77.8% in T01 vs. 74.6% in T02) (see Table 4). No significant differences were found between front and hind quarters (*P*>0.05).

Table 4. Summary of treatment effectiveness: Prevention of new infections

Treatment	No. of quarters	Prevention Failure	Prevention Success
OrbeSeal+ Orbenin EDC (T01)	496	110	386
	100%	22.2%	77.8%
Orbenin EDC only (T02)	496	126	370
	100%	25.4%	74.6%
P-value			NS

NS=not significant (*P*>0.05).

Somatic cell counts

The mean SCCs for T01 and T02 post-calving (213,200 and 222,500 cells/mL, respectively) were considerably lower than at drying off prior to treatment (mean quarter SCCs of 994,700 (T01) and 978,600 (T02) cells/mL, respectively. The distribution of the quarters across the SCC bands was similar for each treatment at drying off and showed similar improvements with a general lowering of SCCs post-calving.

Discussion and conclusions

In cows with SCCs above 200,000 cells/mL, the combination OrbeSeal + Orbenin EDC performed significantly better than Orbenin EDC alone in the prevention of clinical mastitis during the dry period and early lactation. The majority of the clinical mastitis cases were due to environmental pathogens. The combination of OrbeSeal® with Orbenin® EDC was found to be safe under field conditions.

Acknowledgements

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The safety and efficacy of the immunotherapy treatment Y-Complex in a clinical mastitis study under field conditions

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Introduction

Immunotherapy is a novel medical approach that is gaining growing interest (oncology, rheumatology, viral diseases etc.) and could offer an alternative for conventional medications. Immunotherapy is defined as a process designated to stimulate the defensive immunological system in confronting substances, such as bacteria and viruses as various disease causes.

Mastitis counts for the most of the economic losses to the dairy industry and despite the numerous endeavors invested in its prevention; hitherto, treatments resulted only in partial success. Recently, the awareness for healthy food has grown and became evident in the aim to minimize the usage of antibiotics among farms, including mastitis in dairy farms.

The current state of affairs could be summarized as following:

1. partial success rates, with overall disappointment form present medications.
2. increasing the risk for selection of resistant bacterial strains as a result of excess and repeated use of antibiotics.
3. upsurge of the danger posed to public health due to antibacterial materials residues in the milk following treatment.

Hence, arise the need for expanding the arsenal of medical formulations available for the treatment of bovine mastitis (as well as other diseases), resulting in a better cure rate, reduced bacterial resistance rate and minimal, if any, harm to consumers.

Y-Complex is a biological, broad spectrum antimicrobial agent based on specific antibodies activity combined with an inducer, which stimulates phagocytes (Figure 1). Currently, specific antibodies are directed against: *Escherichia coli, Streptococcus dysgalactiae* and *Streptococcus uberis*. When it is administered to an infected quarter the mode of action could be described schematically as follows:

1. Bacteria are bound to the antibodies on the surface of the carrier to create units of complex with pathogens while several pathogens are bound to each carrier.
2. The inducer of the complex adheres to the phagocytes and stimulates them.
3. The phagocytes engulf the complex and digest the pathogens bound to it.
4. Pathogens are destroyed and removed from the udder by milking.

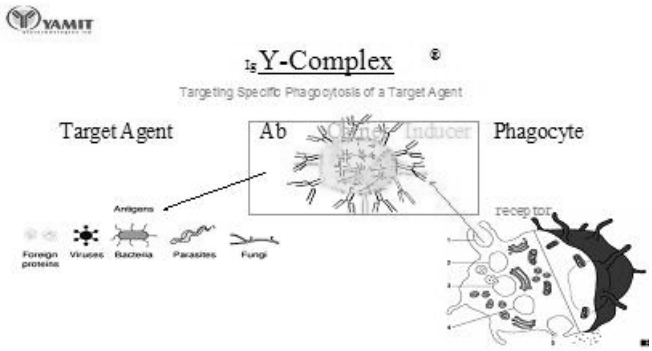

Figure 1. Schematic figure of Y-Complex.

The Y-Complex was tested in various controlled experiments in cows to demonstrate its safety and efficacy. To establish that Y-Complex does not adversely affects the udder, it was administered into healthy udders in various doses. No adverse systemic effects were observed, except for a slight increase in SCC throughout 24 hours following administration and a negligible temporary reduction in milk yield. Several preliminary challenge studies were performed with different pathogens (*E. coli, Strep. dysgalactiae*) in twelve cows (six for each pathogen) at the Volcani Center herd (Pinchasov *et al.*, 2004). Cows enrolled in the study were challenged with one of the mentioned pathogens in two quarters in parallel (either frontal or rear). Following 6-8 hours with *E. coli* or 3 days with *Strep. dysgalactiae*, one quarter was intra-mammary injected with Y-Complex and the counterpart quarter in each cow was left untreated. Curing was defined as: a) Time of bacterial eradication; b) Improvement in clinical symptoms, and c) Return of SCC to basal level prior to challenge. Y-Complex was found to be effective in all the three mentioned parameters.

The objective of the present study was to examine the safety and efficacy of Y-Complex in comparison to other medications (standard of care, SOC) in cows with spontaneous mastitis under field conditions.

Material and methods

The study was conducted at the Kazir Va'Keshet herd (~ 500 cows) throughout three months between November 2003 and January 2004.

The local facilities were:

- Housing: Outdoor open barns.
- Bedding: Straw.
- Milking parlor - Herring-bone type.
- Milking regime: 3 times a day (4 hrs each).
- Feeding (general): Total Mixed Ration distributed 5 times a day.
- Average milk yield in 2003: 11,244 liters per cow.

The treatment consisted of two intra-mammary administrations of 10 ml per quarter of sterile pre-filled syringe in intervals of 18 to 24 hrs. The Y-Complex was given immediately after milking and the next milking was avoided to allow sufficient drug concentration in the quarter. For securing a proper and balanced assignment, cows were a priori designated

into two experimental groups (A, B), based on their lactation number, days after calving, previous level of SCC and average milk yield. Once mastitis was detected, a cow was treated according to its pre assigned group (the blocks): Treatment (A) or Control (B). This enabled the examination of Y-Complex efficacy in more homogeneous experimental groups. The study investigational Y-Complex formula (Group A), contained 60 millions units (MU)(anti-*Strep* and anti *E. coli* properties). The milk of the Y-Complex treated group was withdrawn for three days according to instructions of the ethical committee for this study. The Control group (B) was treated with a combination of either antibiotics or sulfatrimethoprim for three days, with anti-inflammatory agents in most cases (SOC). In some cases cows were milked additionally as an exclusive treatment.

Overall 26 cows were reported with clinical mastitis (mostly with infection in one quarter only and some in two quarters). Three cows from the Y-Complex group were excluded in the first 24 hours due to severe infection and the need to avoid risks to animal welfare. A total of 23 cows completed the full course of the study - 10 in the Y-Complex group and 13 in the SOC control group.

Prior to treatment and throughout 28 days following administration the infected udder appearance was clinically examined. Changes in milk texture (normal, small flakes, clots, hemorrhagic) CMT, SCC and bacteriological analysis were monitored as described (Leitner *et al.*, 2004a, b). Clinical examinations were conducted by the sponsor's qualified veterinarian, including rectal temperature, heart and respiratory rates, rumen activity and hydration status (Anderson *et al.*, 1986). Physical examination of the udder included: evaluation of the quarter and various milk characteristics. Milk yield and conductivity and SCC prior to mastitis episode up to three months thereafter were also recorded. Experimental data were statistically analyzed using the t-Test.

Results

Bacteriology

The proportion of positive bacterial isolates from initial tests (day of infection appearance) was 45.5% out of which *E. coli* was the dominant bacteria (54%) and the rest were *streptococci* species. In more than 50% of the samples no bacteria was identified, assuming (according to clinical examination) that infections were caused by environmental gram negative bacteria. Similar rate of bacterial isolates in infected cows were found for Y-Complex and control groups.

Pretreatment clinical observations

Pathological changes in the infected udders of cows (swelling, edema, stiffness) were observed before treatment among 70% of the Y-Complex group and in 54% of the control group. No significant differences were found between experimental groups in systemic reactions prior to the treatment. However, in the Y-Complex group higher heart rates and rumen activity were recorded.

Milk parameters

Changes in CMT, SCC and normal milk characteristics were observed in all cows. CMT values were greater than 3 prior to treatment and declined steadily throughout the study. After 28 days, CMT remained relatively high (±2) with no significant differences between

the experimental groups. Average daily milk yields were similar in the two groups 10 days prior to infection. On day 0 (treatment), milk yield reduced in both groups by more than 30% as compared to day -10 yields. However, 7 days after treatment, milk yield returned to the pre-infection level in both groups, with no significant differences. Similar trend was observed at 28 days of the trial.

Due to the fact that reduction in SCC may persist several weeks following clinical mastitis, which could not be detected during the follow up period, cow's data were retrieved from the farm records (Herd Book) a month prior to, and up to three months post treatment (Figure 2).

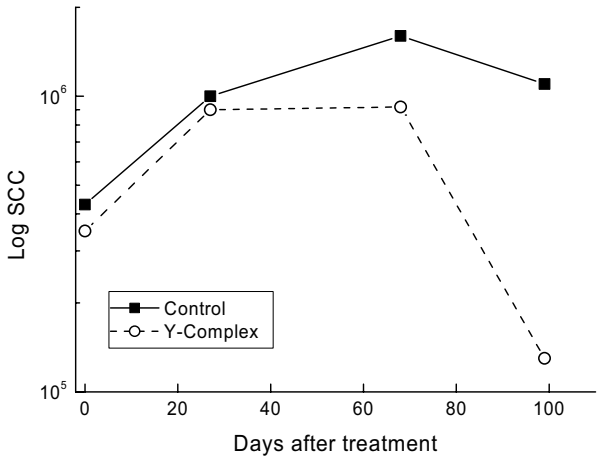

Figure 2. Mean SCC of Y-Complex and Control groups, as recorded in the Herd Book prior (day 0), during the study (75 d) and after the treatment was given to the experimental cows (99 d).
Comments: day 0 - SCC at 17/11/03 prior to infections; day 99 - SCC at 20/2/04 after treatment.

Discussion and conclusions

The prime objective of the study was to examine the safety and efficacy of the Y-Complex as compared to standard of care controls, comprising antibiotics and supportive anti-inflammatory treatment in cows with spontaneous mastitis. The premise for the success of the treatment were: clinical cure - relief of local or systemic clinical symptoms, functional recovery - return to original milk yield or at least an increase in yield in comparison to the day of infection, bacterial cure - bacterial eradication, and tissue recovery - significant reduction in SCC during the course of time, that is similar to the one observed following the treatment of antibiotics. This premise was found to be correct, such that no significant differences between the two groups in overall parameters were examined during the course of study.

One of the problems every farmer encounters is the lack of identification of the causative agent at the time of diagnosing the clinical symptoms of infection. Proper identification of the pathogen requires at least 24 to 36 hours, and the establishment of sensitivity test for a specific antibiotic may take another 24 hours. This is the major drawback in taking a decision which antibiotic to use. Therefore, most decisions to treat are based on prevalence

of pathogens on the site from previous mastitis episodes. The formulation of Y-Complex used in this study was destined to treat the most prevalent pathogens known at the site prior to the study. In fact, those pathogens were identified in only less than 50% of mastitis episodes during the study. It is recommended to consider better formulations of the Y-Complex with broader antibacterial activity.

Episodes caused by gram negative bacteria may last few days. However, the recovery time in terms of milk yield and reduction in SCC may take few months. In this study, recovery time among cows treated with Y-Complex was significantly shorter (Figure 1), returning to normal SCC level prior to infection (100,000±), as opposed to control group that were with SCC over 1,000,000 cells after three months past treatment.

The Y-Complex was found to be safe for intra-mammary administration. No major adverse events were observed. A slight temporary reduction in milk yield was observed due to local reaction, in accordance with results of previous studies, following administration of either recommended dose or ten folds higher than that.

Results of previous controlled studies and the present field study indicate that Y-Complex is a friendly treatment for both animals and consumers, and is superior to antibiotics with regards to public health issues. On the long run, the product brings forward two major advantages over antibiotics: a) minimizing the chance for selecting resistant and more violent strains of microorganisms as a result of repeated use, and b) neutralizing the risk for consumers due to residues in milk. Further to those advantages, there is an economical benefit to the farmer in the short run, due to the intra-mammary treatment given to the infected quarter by enabling normal milking of the other uninfected quarters. Basic calculation of milk loss from systemic antibiotic treatment till complete clearance of medication indicates 5 days of milking. According to milk records received in this study, each cow lost in an average 125 liters (25 L/d x 5 days) or 177 NIS as a result of milk withdrawal, whereas in the Y-Complex group, milk was withdrawn from the infected quarter only for three days (i.e., 25 x 3 x 0.25 = 19 L).

In summary, no significant differences were noted between the treatment groups during the first month of the mastitis episode. However, an advantage was noted for the Y-Complex treated cows in the rapid return of SCC to normal values, in contrast to cows of the control group. The decrease of SCC along with the return to normal milk yield indicates a functional recovery of the udder. Furthermore, farms where a problem of high SCC in milk tanks exist, returning to normal SCC affects the entire herd rather than the individual cow only. Surely, minimizing the chance for medication residues in milk and the ability to sell the milk during treatment and afterwards, portrays Y-Complex as a good substitute to standard antibiotics in treating clinical mastitis. Yet a word of caution as these are preliminary results, obtained from a small number of cows in a single herd that participated in the study. Further studies are required to elucidate and accredit the encouraging results of this study in larger number of cows at various sites.

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Therapeutic effect of systemic or intra-mammary antibiotic treatment of bovine sub-clinical mastitis during lactation

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Abstract

The short- and long-term treatment efficacy of five days systemic (intramuscular (IM)) or intra-mammary (IMM) penicillin treatment of bovine sub-clinical mastitis during lactation was compared with a control (C) group receiving no treatment. One hundred twenty-six cows met the inclusion criteria, i.e. lack of clinical symptoms, no recent treatment with antibiotics, and findings of *Staphylococcus aureus, Streptococcus dysgalactiae,* and/or *Streptococcus uberis* in combination with an inflammatory reaction. At follow-up six to eight weeks after treatment, the proportion of cows negative for the original infection was significantly higher in IM and IMM groups than in C, but the difference between IM and IMM groups was not significant. The same differences between groups were observed when evaluating presence of any major pathogens. The culling rate during ten months after treatment was significantly higher in the IMM group than in the other groups. No difference in cow milk yield was observed between the groups. Cow composite SCC was significantly lower in IM and IMM groups than in C. The cure rate was significantly affected by lactation number (lower in older cows), breed (lower in the Swedish Holstein breed), pathogen (lowest for *S. aureus*), and pre-treatment SCC (higher for above average SCC).

Keywords: sub-clinical, therapy, lactation, intra-mammary, intra-muscular

Introduction

To improve milk quality, cows with sub-clinical mastitis are sometimes treated with antibiotics during lactation. It is utterly important to evaluate the circumstances when such treatment may have a value from an economical point of view. In general, antibiotic therapy of sub-clinical mastitis during lactation has not been considered to be cost-effective as many of these cases are chronic infections (e.g. Erskine *et al*., 2003). This standpoint has recently been questioned for chronic sub-clinical infections caused by *Streptococcus (Str.) dysgalactiae* and *Str. uberis* (Swinkels *et al*., 2005). However, the risk for increased use of antibiotics resulting in the development of bacterial resistance against antibiotics must also be considered.

An important part of the strategy to reduce the use of antibiotics in dairy production is to refrain from treating cases of mastitis with poor prognosis. Cure rates vary depending

on infectious agent and duration of infection, but also on cow characteristics. In a meta-analysis of lactation therapy trials of sub-clinical *Staphylococcus (S.) aureus* mastitis, the cure rate was reported to depend on age of the cow, SCC at the time of treatment, quarter position and stage of lactation (Sol *et al.*, 1997). However, the cure rate was based only on bacteriological findings up to 60 d after treatment. To get an optimal evaluation of the cure rate, long-term follow-up of factors like SCC and culling rate is necessary, but very few studies include these variables in their study design. Moreover, little is known about the relationship between cow characteristics and the prognosis of sub-clinical streptococcal mastitis.

The route of administration is crucial to minimize the use of antibiotics as systemic therapy uses more antibiotics than local therapy, but clinical studies comparing the efficacy of these treatments of sub-clinical mastitis during lactation are rare. Owens *et al*. (1988) reported a better cure rate of sub-clinical *S. aureus* mastitis when combining intramammary and parenteral treatment compared to intramammary infusion only. This finding was supported by Erskine *et al*. (2003), who also suggested that therapy should be extended for a longer period of time. It is essential that the cure rate of antibiotic therapy is much better than that of spontaneous cure. Thus, an untreated control group is a necessary part of trials on therapeutic efficacy.

In this study, we have investigated if intra-mammary (IMM) antibiotic treatment is sufficient in cases of sub-clinical mastitis caused by the common penicillin sensitive udder pathogens *S. aureus, Str. dysgalactiae* and *Str. uberis* by comparing short and long term results of intramuscular (IM), IMM therapy, and an un-treated control (C) group during lactation. Another aim was to evaluate the effects of cow characteristics on the therapy result to identify cases where treatment is of no use either due to high self-cure rate, or poor prognosis.

Material and methods

Twenty-two farms, participating in a routine udder health service program, were included in the study. Cows between 20 and 300 DIM (with more than 150 days before expected calving) with a composite milk SCC above 300 000 per ml, and bacterial growth of *S. aureus* (β-lactamase negative), *Str. dysgalactiae* and/or *Str. uberis*, in combination with an inflammatory reaction on at least one occasion, were included in the study. Cows were excluded from the study if diagnosed with clinical disease or treated with antibiotics during the 30-day period before inclusion. Cows were randomly allocated to the following treatment groups; IM injection of 9.5 mg of benzyl penicillin potassium per kg body weight twice daily for five days (n=40), IMM treatment with 0.3 g benzyl penicillin penethamat esther after evening milking in all udder quarters for five days (n=42), or C, i.e. untreated controls (n=44). Follow-up examinations were performed 6-8 wks after treatment. The effect of treatment on cow level was evaluated in the following ways: Outcome 1) Presence vs absence of the original pathogen in any udder quarter; Outcome 2) Presence vs absence of major udder pathogens in any udder quarter; Outcome 3) New case of veterinary treated mastitis (NCVTM) within five and ten months; Outcome 4) Being culled or sold at five and ten months after treatment; Outcome 5) Cow milk yield at the second monthly milk recording after the start of the trial and at the first recording after the following calving; and Outcome 6) Cow composite SCC at the second monthly milk recording after the start of the trial. All

statistical analyses were made using a multivariable generalised linear mixed model, where the herd variation was handled by including a random herd effect in all models.

Results

One hundred twenty six cows were included in the study. The results for each outcome were as follows: Outcome 1) The estimated percentages of cows with the original pathogen still present at follow-up are given in Table 1. Treatment had a significant (P=0.001) effect on the outcome, which was also affected by lactation number (P=0.049), breed (P=0.005), bacteria (P=0.009), and pre-treatment SCC (PTSCC; P=0.031). C cows had a significantly lower cure rate than IM and IMM cows, but the latter groups did not differ. The highest cure rate was found in young animals of the Swedish Red and White breed (SRB) having above average PTSCC and infection by *Str. dysgalactiae*. The cure rate for *S. aureus* was significantly poorer than for *Str. dysgalactiae* and *Str. uberis*. As an example, the estimated cure rates for first lactation SRB cows infected with *Str. dysgalactiae* were 98, 97, and 80% in IM, IMM and C, respectively, while the corresponding cure rates for *S. aureus* infected Swedish Holstein (SH) cows in lactation ≥3 were 13, 8, and 1%.

Outcome 2) Treatment (P=0.003), bacteria (P=0.029), and PTSCC (P=0.012) significantly influenced the presence of any major udder pathogen at follow-up. The cure rate was

Table 1. The estimated percentages of cows with the original pathogen at follow-up evaluated for each variable.

Variable[1]		Number	%[2]	Conf Int
Lactation number	1	33	26.0[a]	10.1-52.3
	2	34	50.2[ab]	27.4-72.9
	≥3	59	66.7[a]	41.8-84.8
Breed	SRB	68	24.3[a]	12.1-42.8
	SH	45	72.6[b]	52.4-86.4
	Other	13	45.6[b]	13.2-82.2
Treatment	IM	40	22.7[a]	9.4-45.2
	IMM	42	35.2[a]	17.9-57.6
	C	44	81.6[b]	58.9-93.2
Season	Jan-Apr	43	35.3[a]	17.1-59.0
	May-Sep	34	37.0[a]	15.9-64.7
	Oct-Dec	49	68.9[b]	46.7-84.9
Bacteria	*S. aureus*	48	74.8[a]	49.8-89.9
	Str. dysgal	43	26.5[b]	12.4-47.8
	Str. uberis	35	40.0[b]	18.8-65.8
PTSCC	<247	31	64.5[a]	36.3-85.2
	247-287	32	64.4[a]	35.6-85.6
	388-713	30	14.1[b]	4.4-36.7
	>713	33	54.0[a]	27.1-78.8

[1]SRB=Swedish Red and White, SH=Swedish Holstein; IM = intramuscular and IMM = intramammary treatment with antibiotics, C= untreated control; PTSCC = the geometric mean of three monthly SCC in cow composite milk samples before the beginning of the trial;

[2]Values with different superscripts within variable differ (P<0.05).

significantly lower in C than in IM (P<0.001) and IMM (P=0.013). The estimated proportions of success were 66, 52 and 21% in the IM, IMM and C groups, respectively. Outcome 3) Treatment (P=0.008), lactation number (P<0.001) and PTSCC (P=0.012) had significant effects on NCVTM up to five months. The estimated proportions of cows with a NCVTM within five months after treatment were 1, 9 and 4% in the IM, IMM, and C groups, respectively. Thus, the proportion of cows with a NCVTM was lower in IM than in IMM and C, but the two latter groups did not differ. Cows in lactation number 1 and 2, and cows with PTSCC above average had the lowest risk of new treatment. No statistically significant effects were observed at ten months. Outcome 4) The estimated proportions of animals still present in the herd at five months was significantly affected by treatment (P=0.029) and breed (P=0.041) (Table 2). The proportion at five months was significantly higher in IM than in IMM, but no other differences were found. SRB cows were more likely to remain in the herd than SH cows and cows of other breeds. At ten months, treatment (P=0.022) was the only variable with a significant effect. A significantly higher proportion of cows remained in the herd in IM and C groups than in IMM. Outcome 5) Treatment did not have a significant effect on daily milk yield at two months after treatment or at the first test after the following calving. Outcome 6) Treatment (P=0.033) and lactation number (P=0.003) had significant effects on the cow composite milk SCC. The estimated SCC was significantly lower in IM and IMM than in C (210, 215 and 397 x 10^3 /ml, respectively). The SCC was higher in older cows (lactation number ≥3) than in younger cows.

Table 2. Estimated percentages of cows (n=112) still present in the herd five and ten months after treatment for sub-clinical mastitis.

Variable[1]		Five months		Ten months	
		%[2]	Conf Int	%[2]	Conf Int
Lactation number	1	96.7[a]	88.6-99.2	NS	
	2	84.3[b]	66.2-93.6		
	≥3	85.1[b]	68.5-93.7		
Breed	SRB	96.8[a]	89.4-99.1	NS	
	SH	88.7[b]	75.2-95.3		
	Other	78.7[b]	46.9-93.9		
Treatment	IM	95.8[a]	85.7-98.9	75.9[a]	55.9-88.6
	IMM	77.6[b]	58.5-89.5	49.7[b]	31.0-68.5
	C	91.8[ab]	77.9-97.3	76.4[a]	57.7-88.5
Kg milk	<26	82.3[a]	62.7-92.8	NS	
	26-30	94.1[ab]	79.5-98.5		
	30-36	80.4[a]	57.6-92.6		
	>36	96.5[b]	85.6-99.2		

[1]SRB=Swedish Red and White, SH=Swedish Holstein; IM = intramuscular and IMM = intramammary treatment with antibiotics, C= untreated control.

[2]Values with different superscripts within variable differ (P<0.05); NS = not significant.

Discussion

The results of this study indicate that five days IM or IMM antibiotic therapy of sub-clinical cases of mastitis infected with penicillin-sensitive *S. aureus, Str. dysgalactiae* or *Str. uberis* were equally efficient and gave a better cure rate on cow level than no treatment when estimated by the short term outcomes bacteriological cure and SCC at 1.5-2 months after treatment. However, on a long term basis antibiotic therapy did not give a better result on culling, new mastitis treatment and milk yield than no treatment, except for the number of new treatments at five months in the IM group. The cure rate was significantly affected by lactation number, breed, pathogen, and pre-treatment SCC.

In general, bacteriological cure rates were higher in this study than in several other studies (Seymour *et al.*, 1989; McDougall, 1998; Shephard *et al.*, 2000). However, Wilson *et al.* (1999) reported a higher overall cure rate after use of non-penicillin antibiotics. The reason for the relatively high cure rates in this study may be the extended treatment period, its importance has also been stressed by others (Erskine *et al.*, 2003, Deluyker *et al.*, 2005). In line with other studies (e.g. Wilson *et al.*, 1999; St Rose *et al.*, 2003; Deluyker *et al.*, 2005) a higher cure rate of *Str. uberis* and *Str. dysgalactiae* than of *S. aureus* was observed in the present study.

The cow composite SCC two months after treatment was lower in cows treated with antibiotics compared with in controls. This was in contrast to several other studies reporting no decrease in SCC after therapy with antibiotics (Seymour *et al.*, 1989; McDougall, 1998; Shephard *et al.*, 2000). However, a decrease in SCC after treatment has also been observed previously (Shephard *et al.*, 2000; St. Rose *et al.*, 2003; Deluyker *et al.*, 2005). Treatment had no effect on daily milk yield two months after treatment and at first recording in the following lactation, which is in line with the literature (McDermott *et al.*, 1983; Seymour *et al.*, 1989; St. Rose *et al.*, 2003).

The culling risk was significantly higher in the IMM group than in IM and C, as was the percentage of animals diagnosed with mastitis within five months compared to the IM group. Of the animals culled before ten months from the trial start, only one out of nine cows in the IM group and one out of 18 cows in the IMM group could be regarded as cured in accordance with the parameters bacteriological analysis, veterinary treatment, and cow composite SCC. Thus, it is likely that the animals in the IMM group were culled for udder health reasons. These results may suggest that IMM therapy increased the risk of introducing new infections in the udder, which is in line with findings by Shephard *et al.* (2000). The observations emphasise the importance of studying economically important long-term effects, like risk of new infections and culling, when evaluating treatment efficacy.

The cure rate varied markedly depending on cow characteristics, and especially on lactation number (lower in older cows), breed (lower in Swedish Holstein cows), and PTSCC (highest for above average SCC). This is partly in line with others (Sol *et al.*, 1997; Deluyker et.al., 2005) who also found lower cure rates in older cows. They also found that a high SCC at treatment decreased the probability of cure. In the present study, a PTSCC of 388 to 713 x 10^3/ml resulted in a better bacteriological cure rate than higher or lower SCC. The positive effect of a moderately elevated SCC is unexpected and has to our knowledge not been reported before.

The minimum demand of an antibiotic treatment is to achieve a reasonable additional bacteriological cure compared to no treatment. If the lowest acceptable level of

bacteriological cure after treatment is set to 40% (Sears, 1999), a guide when selecting cows for antibiotic therapy is given in Table 3. With this line of reasoning, treatment would not have been worthwhile in approximately 30% of the cases, and only 36% of the cases would have cured from the infection as a result of therapy. Recently, interesting models for calculating mastitis costs have been presented. Putting economic weights, as suggested by Swinkels *et al*. (2005), on the results from the present study into the described model reveals that the cost to treat a case for sub-clinical mastitis according to the IM and IMM protocols is ~60 € and ~200 €, respectively, higher than no treatment.

In conclusion, the results indicate that antibiotic treatment of sub-clinical mastitis during lactation is not economically justifiable as such therapy in many cases is useless or unnecessary. The study showed no increased rate of survival or higher milk yield, and no decreased long-term risk for new mastitis treatment for cows treated with antibiotics compared to untreated controls. IMM treatment was actually associated with a higher risk for culling compared to IM and C. Factors that determined the outcome of therapy were lactation number, breed, infectious agent, and pre-treatment SCC. Thus, in certain small groups of animals, antibiotic therapy, applied together with preventive measures, can help bring sub-clinical mastitis under control. If antibiotic treatment is used, systemic treatment with benzyl penicillin potassium seems preferable to intra-mammary treatment with benzyl penicillin penethamat ester.

Table 3. Proposed guideline for intramuscular antibiotic treatment of cows with sub-clinical mastitis according to breed, bacteria and lactation number.

Breed[1]	Bacteria[2]	Lactation 1	Lactation 2	Lactation ≥3
SRB	*S. aureus*	Treatment	Treatment	Treatment
	Str. dysgalactiae	No treatment	No treatment (High self cure)	Treatment (High self cure)
	Str. uberis	No treatment	Treatment (High self cure)	Treatment
SH	*S. aureus*	Treatment	No treatment (Low cure rate)	No treatment (Low cure rate)
	Str. dysgalactiae	Treatment	Treatment	Treatment
	Str. uberis	Treatment	Treatment	No treatment (Low cure rate)

[1]SRB=Swedish Red and White, SH=Swedish Holstein.

[2]Penicillin sensitive strains only.

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An alternative treatment trial for *Staphylococcus aureus* mastitis in organically managed dairy cattle

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Abstract

We evaluated the effect of non-antibiotic treatment of *Staphylococcus aureus* mastitis in a controlled, randomized trial conducted on two organic dairy farms in New York State. Cows were treated (n = 20) with two immune stimulants (Immunoboost™ and and Biocel®CBT) and a *Staph* nosode, or left untreated (n = 10). Milk samples were collected three times at weekly intervals both before and after treatment to determine infection status and quarter milk SCC using standard methods. There was no significant difference between treated cows and controls for cure rate or SCC. Management of *S. aureus* mastitis on organic dairy farms must continue to rely on strict segregation at milking time, excellent milking technique, and appropriate culling decisions. Ribotyping of isolates showed a predominant strain type in each herd, underscoring the importance of control of contagious transmission, and illustrating the difficulty in achieving this. Early detection of infected cows may improve prevention of contagious transmission and cure rates obtained after treatment.

Keywords: organic, alternative, homeopathic, clinical trial, *S. aureus*

Introduction

Staphylococcus aureus is the most common contagious mastitis pathogen in dairy cattle. Control of contagious transmission is achieved through segregation of infected cows, use of post milking teat disinfection and proper milking machine maintenance and use. On conventional farms, intervention with antimicrobials during lactation or with long-acting antibiotics at dry-off is recommended to treat *S. aureus* infections (Schukken *et al.*, 1993; Sol *et al.*, 1997). These methods are not permissible on organic dairy farms. Therefore, alternative treatments are recommended.

Many veterinarians and producers have designed alternative treatment protocols. However, few randomized trials to determine their efficacy have been performed using untreated or placebo-treated controls. An Ethiopian trial evaluated an herbal remedy in vitro and in vivo and demonstrated a 52.8% cure rate for subclinical mastitis in the treated group vs. 40% in the negative control group (Abaineh and Sintayehu, 2001). Ginseng was evaluated as an immunostimulant against *S. aureus* subclinical mastitis vs. a saline control. The number of quarters infected with *S. aureus* and SCC decreased in the treated group although the results were not statistically significant (Hu *et al.*, 2001). An observational study in India evaluated the effect of a homeopathic treatment on clinical mastitis in riverine buffaloes (Varshney and Naresh, 2004) but no controls were used. Without comparison of treatment and control, it is impossible to know whether animals benefit from

a treatment. Lack of such knowledge may result in use of ineffective treatments and withholding of other beneficial treatments.

Because few treatment options are available in organically managed herds, great efforts are made to prevent cow-to-cow transmission. Even so, new infections continue to occur. It is often unclear where these infections come from. Gaps in the prevention of contagious transmission could be the reason. Alternatively, new infections may arise from the environment (Zadoks *et al.*, 2000). DNA-based strain typing methods can help to differentiate between contagious transmission and so-called environmental transmission. In addition, use of strain typing allowed us to differentiate between re-infection and failure to cure in cows that are *S. aureus* positive after treatment.

The primary objective of this study was to evaluate an alternative treatment regimen for *S. aureus* mastitis in dairy cows against no treatment. Both positive effects (cure) and negative effects (positive milk residue tests or side effects of treatment) were monitored. There are few theoretical grounds to expect positive residues tests after alternative therapies but the consequences of positive residue tests are too detrimental to the farm to ignore the possibility. The secondary goal of the study, utilizing molecular typing, was to characterize the epidemiology of *S. aureus* on organic dairy farms as contagious or environmental and to determine whether 'non-cures' are persistent infections or re-infections with a different strain.

Materials and methods

Herd selection

Prospective certified organic herds with known histories of *Staphylococcus aureus* and past herd mastitis survey data with QMPS were contacted and invited to participate in the treatment trial. Three herds accepted the invitation. Informed consent to participate was provided in writing by responsible parties for each farm.

Cows from Farm A and B were selected for enrollment in the trial based on a past history of *Staph aureus* infection or if two of the past three linear scores on monthly DHIA testing were greater than 4.5. For Farm C, no regular DHIA testing was performed, and cows were selected based on a positive culture for *Staph aureus* on a recent (August, 2004) QMPS herd survey.

Sample size calculation

The chance of cure in untreated and treated quarters was estimated at 10% and 35%, respectively, based on results from conventional trials (Deluyker *et al.*, 2001; Sol *et al.*, 1997). Because there was more uncertainty about the probability of cure for the treatment group, more cows were included in the treatment group than in the control group. Because we were only interested in treatment being more successful than no treatment, sample size calculation was based one-sided statistical testing. Common values for power and confidence level in sample size calculations are 0.8 and 0.05. Given the constraints of the study (number of herds and cows available for participation, funding), we decided to accept lower levels, i.e. power = 0.6 and confidence level = 0.10. Using the SISA sample size calculator (Simple Interactive Statistical Analysis, http://home.clara.net/sisa), sample size was estimated at 16 control cows and 32 treatment cows, i.e. ca. 5 and 10 cows per herd, respectively, if each of the enrolled herds contributed equally to the trial population.

Randomization, treatment and sampling

Eligible cows on each farm were randomized into blocks of 15 (ten treated, five controls) by lottery. Treatment consisted of a single 5 cc dose of Immunoboost® (Bioniche Animal Health, Bellville, Ontario, Canada) given subcutaneously on Day 1, 30 cc BioCel-CBT (Agri-Dynamics, Easton, PA) given subcutaneously on Days 1, 2, 3 and 8, 9 and 10, and Schaeffer *Staph* nosode *30C* (Washington Homeopathic, Berkeley Springs, WV) given intravaginally twice daily on Days 1, 2, and 3. QMPS personnel performed treatment in the presence of the producer on Day 1. The farmer or her/his trained personnel were responsible for subsequent treatments.

General cow health and injections sites were monitored daily for adverse reactions during the treatment period and for the week following the end of treatment. Abnormal findings or adverse reactions were recorded by the farmer on individual animal treatment records designed for the study.

Milk sample collection and processing

For all eligible cows, quarter milk samples for aerobic culture and for SCC were taken on three occasions before treatment (at weekly intervals) and again after treatment (at two-week intervals) following standard guidelines (NMC, 1999). Samples were cooled immediately and transported to a QMPS laboratory for culture and pathogen identification. Somatic cell counts were performed for all quarters by Dairy One (Ithaca, NY) using Fossomatic methods.

Aerobic culture was performed according to NMC guidelines. Bacterial growth was recorded a '+' for < 3 colonies, '++' for 3-5 colonies, and '+++' for > 5 colonies. Representative colonies of *S. aureus* from each half plate were subcultured onto TSA and incubated at 37^0C for 24 hours. Colonies from these plates were frozen at -80 ^{0}C in Microbank™ sterile vials containing porous beads which serve a carriers to support microorganisms (ProLab Diagnostics, Austin, TX) until molecular typing could be performed. For strain typing, *S. aureus* isolates were grown overnight on brain-heart-infusion (BHI) agar and subjected to automated ribotyping with EcoRI using the RiboPrinter® Microbial Characterization System (Qualicon, Wilmington, DE, USA). A quarter was determined to be cured if all three post-treatment cultures were negative for *S. aureus*. Treatment was determined to be a failure if one of three post treatment cultures yielded *S. aureus* and if ribotyping confirmed that both pre- and post treatment strains were identical. *S. aureus* colonies from post-treatment cultures were subcultured and stored as described previously.

Composite milk samples were taken at the second milking on Day 3 of treatment for milk residue testing. Since no commercial kits or research protocols exist for the detection of residues of alternative treatments, the most commonly used on-farm kit for the detection of growth inhibitors in milk was used. (Delvotest® *Mini*, DSM Food Specialties, Menomonee Falls, WI).

Statistical analyses

Fisher exact test (Statistix 8.0) was used to compare cure rates between treated and control quarters. For analysis of SCC before and after treatment, raw SCCs were transformed to linear scores (LS) using the formula: $\{[\log_2(SCC/100{,}000)] + 3]$ (Dabdoub and Shook, 1984) and then averaged for the three pretreatment samples and also for the three post treatment samples. Pre- and post-treatment mean LS were compared for each quarter using

a paired t-test. This analysis was done within and across herds. The significance level was set at $P < 0.05$.

Results

Farm A, B and C enrolled 15 (Treated n=10, Control n=5), 14 (Treated n=10, Control=4) and 18 cows (Treated n=11, Control n=7), respectively. The number of infected quarters (two of three culture samples positive with greater than 5 *S. aureus* colonies) was 18, 16 and 45 for farm A, B and C, respectively. Treatment was completed for all enrolled cows on Farms A and B. On both farms, one cow was lost to follow up because sample collection could not be completed. On Farm C, Immunoboost® and the first two doses of BioCel-CBT® and *Staph* nosode were administered but the owner felt that continued treatment would be too upsetting for the cows and so he withdrew from the study. None of the cows showed any adverse reactions physically or at the injection site post-treatment.

Bacteriologic and SCC cure

A total of 32 *S. aureus* infected quarters and 80 non-*S. aureus* infected quarters were available for analysis. Most cows were infected with *S. aureus* in a single quarter. No effect of treatment on cure rate was observed. Cure was observed for one control quarter on farm A and for two treatment and two control quarters on farm B. Mean LS for *S. aureus* infected quarters was 6.6 pretreatment and 6.7 post treatment; mean LS for non-*S. aureus* infected quarters was 1.9 pre-treatment and 2.1 post treatment. There was no effect of treatment on LS for infected or non-infected quarters of treated cows. All post treatment milk residue tests on Farms A and B were negative.

Strain typing

Among 53 isolates from herds A and B, seven ribogroups were detected. Six ribogroups were documented in the DuPont database. One ribogroup, 116-1106-3, was not listed in the DuPont database but had been detected before in a dry-cow treatment trial of Canadian dairy cattle. One of the cured cows (Cow 9507, Farm B) had a different ribotype before than treatment. DUP-4025 was the most common strain in both herds. In each, several other strains that were detected. The majority of non-cured quarters were infected with DUP-4025, while the majority of cured quarters were infected with other strains. Three of 5 isolates that had been collected from cows in herd A in April, three belonged to DUP-4025. These cows were included in the treatment trial, which was conducted in the fall, implying that these quarters had been infected for many months before treatment was initiated.

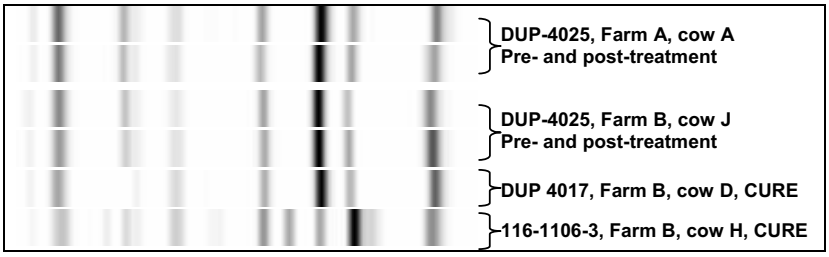

Figure 1. Example of ribotyping results for cured and non-cured cows with DuPont- based or new ribogroups.

Discussion and conclusion

In this paper, we describe the first controlled clinical trial of an alternative treatment of subclinical *S. aureus* mastitis in North America. Treatment was based on a combination of non-specific immune stimulants, colostral whey products, and Staph nosode. Treatment had no effect on bacteriological cure or somatic cell count of infected quarters. In addition, the treatment procedure was so time-consuming and invasive that two of three participating producers said they would not consider it for use, unless it could be administered by an alternate route such as feed, and one producer discontinued participation in the trial.

Immunoboost®, derived from a mycobacterium cell wall fraction (MCWF), is a non-specific stimulant of the bovine immune system. It is currently in use in the USA as treatment for calf scours due to *E. coli* (Worthington and Nosky, 2000). Colostral-whey products (Biocel-CBT®) are produced from hyperimmunized dairy cattle. In vitro, ruminant whey has been shown to enhance neutrophil function (Mercier *et al.*, 2004.) Kerhli *et al.* (1989) evaluated the use of a parenteral colostral whey product in cows with and *S. aureus* mastitis. A slight favorable change in several immune parameters was noted but infections were not eliminated. Peer-reviewed publications on homeopathic nosodes are scarce but anecdotal information is available in veterinary texts (Detloff, 2004; Karreman, 2004) and includes favorable results. An Irish study evaluated three commercial homeopathic preparations, none of which showed any efficacy (Egan, 1998). Thus, the poor cure rates observed in our trial are similar to results found in other organic studies (Hu and Du, 1997; Egan, 1998).

The fact that the majority of our cows had chronically infected quarters with high SCC may have contributed to the low success rates (Sol *et al.*, 1997). Specifically selecting cows that had more risk factors for cure or using a treatment protocol at dry off rather than during lactation may enhance cure rates. The majority of cows in the two herds were infected with *S. aureus* in just one quarter and these infected quarters were responsible for the majority of the cows' elevated SCC counts. Selectively drying off these quarters or milking them separately with a quarter milker would have a beneficial impact on bulk milk SCC and milk quality.

Ribotyping showed a dominant strain, DUP-4025, in both herds. This can be due to contagious transmission, lack of discriminatory power of *EcoRI* ribotyping, or host adaptation of the strain resulting in chronic infections. When incidence of infection is similar for host-adapted and environmental strains, longer duration of infection could cause a higher prevalence of host adapted strains. Detection of multiple strains in each herd supports the concept that some infections may be of environmental origin. *S. aureus* has been detected on the skin of cows, people and dogs, in bedding material, etc. so that complete eradication of *S. aureus* is impossible. Similar results, i.e. predominance of one strain and presence of a variety of other strains in low numbers, have been obtained in numerous studies around the world using a variety of techniques, including ribotyping, PFGE and, most recently, MLST (Smith *et al.*, 2005).

In conclusion, this particular immunomodulatory protocol was ineffective against chronic *S. aureus* mastitis. Prevention of infection continues to be the mainstay of *S. aureus* control in organic dairy herds. Selection of cows with higher chances of cure and treatment at different times or with different protocols may contribute to improved response rates.

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Prepartum intramammary antibiotic therapy: Effects on udder health, milk production, and reproductive performance in dairy heifers

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Abstract

Preparturient heifers (n = 545) from nine herds in the US and Canada were enrolled in a study testing the hypothesis that prepartum antibiotic intramammary therapy would cure existing intramammary infection (IMI) resulting in increased milk production, reduced somatic cell count (SCC), and improved reproductive performance. Mammary secretions were collected 10-21 d prior to expected calving, and heifers were systematically assigned to receive antibiotic treatment using a commercially available lactating cow intramammary product (Tx) , or no treatment (Cx). The percentage of mammary quarters infected prior to parturition in Tx and Cx groups was 32.9 and 35.4. The coagulase-negative staphylococci accounted for the majority of prepartum IMI (74.8%). *Staphylococcus aureus*, environmental streptococci and coliforms accounted for 24.5% of prepartum infections. Mammary quarters that were infected prepartum and treated with antibiotics had a 79.9% cure rate. Control mammary quarters had a spontaneous cure rate of 31.7%. Treatment had a significant effect ($P<0.001$) on the cure rate of infected quarters at parturition and a decrease in the number of new IMI during the first 21 days in milk (DIM), but not a reduction in linear (L)SCC ($P>0.22$) during the first 200 DIM. The reduction of IMI was not associated with a significant change in milk production during the first 200 DIM. No significant effect on services per conception or days open between treatment and control groups was observed. Prepartum intramammary antibiotic therapy reduced the number of heifer IMI, but this reduction in IMI did not translate into a significant improvement in milk production, SCC , or reproductive performance.

Keywords: heifer mastitis, prepartum antibiotic therapy, efficacy, intramammary infections (IMI), and linear milk somatic cell count (LSCC)

Introduction

Control of mastitis during the nonlactating period in multiparous cows has relied upon dry cow intramammary antibiotic treatment which has been widely practiced for more than 50 yr. Several investigators have studied the practice of using similar approaches during the nonlactating period of heifers before first parturition. The use of lactating intramammary antibiotic therapy in heifers preterm has been investigated. A report from Oliver *et al.*, 2003 indicated that in one herd of Jersey cows such therapy had a significant positive economic impact as evidenced by an increase in milk production. Moreover mastitis has been shown to have a negative impact on reproductive performance of cows. Thus we hypothesized that preterm intramammary antibiotic therapy of primiparous cows would significantly decrease IMI at parturition resulting in increased milk yield, reduced SCC, and improved reproductive performance, during the first lactation.milk.

Materials and methods

Seven dairy herds in seven states (US), and two herds in one province (Canada), 547 heifers in total, were enrolled in the study. All herds utilized a production testing program. Heifers were assigned to treatment group by identification number with odd numbered heifers serving as untreated controls. Collection of mammary secretions from all heifers occurred between 10 and 21 d before expected date of parturition using aseptic procedures. Even numbered heifers received intramammary instillation of a commercial lactating cow preparation containing cephapirin sodium (Cefa-Lak, Fort Dodge Animal Health, Fort Dodge, IA) immediately after sample collection in all four mammary quarters. Such treatment was only given once and not repeated. Controls were not treated. Following intramammary infusion, and sampling in control animals, each teat was immersed in a polymer solution that creates a physical barrier on the teat after application (Stronghold™, West Agro Inc., Kansas City, MO).

Quarter samples of mammary secretion were collected aseptically within the first 3 d postpartum. Aseptically collected mammary quarter foremilk samples were also collected at 1 to 7 d postpartum, 7 to14 d postpartum, and 14 to 21 d postpartum. Milk samples were stored at -5° C until cultured. A 10 µl aliquot from each milk sample thawed at ambient temperature was spread on blood agar plates and incubated for 48 h at 37°C. After incubation, plates were observed and organisms identified presumptively according to procedures described by Hogan *et al.* (1999). Organisms were identified as *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus species*, and coliforms based on colony morphology, reaction on CAMP and MacConkey agars, catalase and coagulase test reactions, and Gram stain. Isolates not identified by these presumptive techniques were classified as "other." A sample was considered contaminated when isolation of three or more dissimilar colony types was made.

A mammary quarter was considered infected prepartum if the same pathogen was isolated from duplicate samples that were taken prior to treatment. If only a single prepartum sample could be obtained, results of the single sample were considered to determine the presence of an IMI. A mammary quarter was characterized as having a new infection if a pathogen was isolated from two out of three samples taken in the postpartum period that was not present in the prepartum samples. A mammary quarter remained infected if the same

organism that was present prepartum was isolated postpartum. If any quarter sample taken on day 7 or 14 was contaminated, a new sample was not taken, and the mammary quarter was not disqualified. If the final milk sample taken 3 wk postpartum was contaminated, a new mammary quarter milk sample was taken immediately following diagnosis of contamination A mammary quarter was considered cured if the pathogen present before calving was not isolated in any of the samples obtained during early lactation.

The SAS analytical statistics software package (SAS, Cary, NC) was used for data analysis. Frequency tables of cure by treatment by herd were prepared, and the Cochran-Mantel-Haenszel Chi-square test with herds as strata was used to analyze the significance of intramammary antibiotic treatment on bacterial cure. Milk production and LSCC were analyzed according to a linear model for repeated measures (PROC MIXED, SAS, Cary, NC). New IMI in lactation after calving were determined and the difference in new infections between treatment and control groups was evaluated using Chi-square analysis.

To test the significance of differences between treatments in calving to conception interval (days open), survival analysis (PROC PHREG) with herds as strata was used. To test for significance between treatment groups in number of services per conception, a frequency table of number of services per conception was prepared and difference tested for significance using a Cochran-Armitage trend test.

Results

There was a significantly higher cure rate in mammary quarters treated with antibiotics before calving (79.9%) than spontaneous cures in control quarters (31.7%). Treatment efficacy for all IMI was 59.5% (Table 1). Although treatment efficacy was significant in all herds, there was a significant herd effect. When analyzed by pathogen type including *S. aureus*, environmental streptococci, coliforms and CNS, the CNS caused the vast majority of IMI, 570 out of 762 infected quarters (74.8%). Treatment had a significant effect ($P<0.0001$) on curing CNS infections; treatment efficacy was 57.5%. Four of the nine herds had significantly ($P<0.05$) higher cure rates in treated mammary quarters than controls. The remaining five herds had too few major pathogen IMI to provide meaningful inferences. Overall, when controlling for herd effects, treatment of major pathogen infected mammary quarters was significant ($P<0.0001$) with an efficacy of 60%. Five heifers had IMI with pathogens in the "other" category. Two heifers had IMI with *Pseudomonas sp*., 1 with a *Lactobacillus sp*., and all three IMI were cured. One heifer had a *Pasteurella sp*. IMI and one a *Serratia marcescens* IMI, neither were cured by treatment. Overall, treatment was significantly associated with a reduction in new IMI during the first 21 d of lactation.

Treatment had no significant effect on milk production during the first 200 DIM ($P = 0.7839$). Treated heifers produced an average of 28.08 kg on test day while control animals produced an average of 27.77 kg of milk on test day. The effect of interval, the time between treatment and first sampling, on milk production was significant ($P=0.0350$). There was a significant DIM effect on production ($P<0.0001$), as milk production over time appeared to resemble a typical lactation curve for first lactation animals. There were significant differences in milk production between herds ($P<0.0001$), as some herds had higher mean milk yields than in other herds. Moreover, there was a significant herd by treatment interaction ($P<.03$). In 5 of 9 herds, the control cows had measured mean milk production yields greater than treatment cows. There was no significant difference in the SCC linear

score of animals that were treated when compared to control animals (P=0.2209). The mean LSCC was 2.85 for control heifers during the first lactation as compared to 2.53 for the treated heifers.

Pathogen type (major or minor) and whether the mammary quarter was cured, did not significantly affect milk production, although cured quarters had greater production means. Heifers with mammary quarters cured of a minor pathogen IMI at parturition averaged (+/- SD) 27.83 (+/- 8.74) kg milk per day compared to production of 26.86(+/- 9.05) of those heifers not cured. Likewise, heifers cured of a major pathogen at parturition produced on average 26.35 (+/-9.27) kg milk per day as compared to those not cured with an average of 23.40 (+/-7.21). Effects of cure and pathogen type did significantly affect LSCC. Heifers with mammary quarters with major pathogen IMI that were not cured had the highest LSCC, while those with cured minor pathogen IMI had the lowest LSCC.

Intramammary antibiotic therapy did not significantly effect reproductive performance as measured by days open. The median days open for control animals was 128 days (95% C.I., 106,144) and 136 days (95% C.I., 122,152) for treatment animals. Services per conception was 1.88 ± 1.26 and 2.02 ± 1.15 services per conception for control and treatment animals, respectively, and not significantly different.

Treatment was significantly (P<0.001) more efficacious in preventing new IMI in the first three weeks of lactation as contrasted with control. Heifers treated with antibiotics prepartum had fewer new IMI in the first three weeks of lactation, 2.6% in treated quarters, with 7.8% of control quarters with new IMI in the first three weeks postpartum.

Discussion

It was hypothesized that treated mammary quarters would significantly improve cure rate resulting in increased milk production, decreased SCC, and improved reproductive performance as compared to control animals during the first lactation. Intramammary antibiotic therapy did cure significantly more prepartum IMI.

The majority of IMI were caused by CNS and cure rates of CNS IMI in treated glands was approximately 57.5% greater than in control glands. Others reported similar findings (Oliver *et al.*, 2004; Oliver *et al.*, 1997; Oliver *et al.*, 1992; Owens *et al.*, 2001; Trinidad *et al.*, 1990). Although major pathogen infections were not frequent in most herds, the overall cure rate was greatest in treated quarters, consistent with other reports (Oliver *et al.*, 2004; Oliver *et al.*, 1997; Owens *et al.*, 2001; Owens *et al.*, 1991; Trinidad *et al.*, 1990). Moreover, prepartum antibiotic therapy also had a prophylactic effect with fewer IMI in the first 21 d of lactation for treated as compared to control mammary quarters.

The effect of fewer IMI with improved prepartum cure and the prophylactic effect of treatment did not result in an improved postpartum milk yield in treated animals nor a significant reduction in LSCC. This is in partial agreement with a previous report on intramammary antibiotic therapy in prepartum heifers (Oliver *et al.*, 2003). They also reported that heifers treated with intramammary antibiotic 7 and 14 d prior to expected calving had significantly fewer IMI, but also significantly higher milk production and lower LSCC than control animals (Oliver *et al.*, 2003). In the current study heifers cured of IMI had greater daily arithmetic mean milk production records, although the coefficient of variation of production within group (cured or not cured of IMI) was as high as 35.12%.

This suggests that cure of IMI prepartum is desireable, but that spontaneous cures and factors other than treatment might significantly affect milk yields.

We hypothesized that coupled with the reduction in IMI postpartum, treated heifers would have improved reproductive performance, consistent with previous observations in studies that used multiparous cattle (Barker *et al*., 1998; Schrick *et al*., 2001). However, the response in primiparae may be sufficiently different as younger cattle did not benefit reproductively in this study by reduced IMI similar to that reported by Schrick *et al*. (2002).

Summary

Intramammary antibiotic therapy administered to heifers 10-21 d prior to expected calving aides in curing IMI prior to the first lactation. Such therapy also led to a significant decrease in new IMI postpartum. However, milk production and milk SCC were not significantly affected by treatment during the first 200 DIM. Given these mixed results, fewer postpartum IMI due to treatment as opposed to no change in milk production and SCC, suggests that preterm therapy should be practiced with care. Findings of this study would suggest that pre-term intramammary antibiotic therapy would not likely yield an economic benefit in many herds and thus should not be universally practiced on par with dry cow antibiotic intramammary therapy. However, intramammary antibiotic therapy administered to heifers within 1-3 weeks before expected calving might have value in a herd when a significant proportion of animals have IMI with at parturition. Intramammary antibiotic therapy administered to heifers 10-21 days prior to expected calving date may have both a curative as well as a prophylactic value.

Table 1. Intramammary infections (IMI) in heifers pre- and post-partum: Efficacy of treatment on cure rate of all IMI.

Herd[a]	Infected Quarters		Cured Quarters		Efficacy[b]	Quarters at Risk	
	Tx	Cx	Tx	Cx		Tx vs	Cx
A	51	49	39	15	60.0%	208	176
B	12	19	11	3	82.8%	96	96
C	6	8	6	3	62.5%	32	20
D	57	77	43	7	88.0%	112	140
E	25	23	23	5	76.4%	48	40
F	137	126	106	58	40.5%	202	198
G	26	34	24	15	52.1%	220	208
H	27	33	23	11	60.9%	76	76
I	33	19	24	6	56.6%	144	140
TOTAL	374	388	299	123	59.5%	1138	1094

[a]Herds included in study: Louisiana State University; The Ohio State University; University of Tennessee Dairy Experiment Station; University of Tennessee Middle Tennessee Experiment Station; University of Connecticut; Washington State University; University of Guelph (Ponsonby and Elora); and Cornell.

[b]Efficacy = 100*(1 -Relative Risk Ratio) where Relative Risk Ratio = percentage control quarters cured/ percentage treated quarters cured.

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Milking technology

Milking machine test survey

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Introduction

One of the recommendations of the NIRD/CVL Five Point plan for mastitis control was a regular milking machine test. An annual milking machine test is a recommendation of the UK Farm Animal Welfare Council and a requirement of the UK National Dairy Farm Assured Scheme.

Materials and methods

A sample of milking machines test reports from January and April 2004 has been analysed to show the quality of milking machine operation on UK dairy farms. The milking machines were all tested by Genus Milking Systems staff and were randomly distributed between England, Wales and Scotland with no pre-selection for machine manufacturer, configuration or size. Data for 1000 tests were collected; they were analysed in confidence using the post code as the unique identifier. The data from the 2004 tests have been compared with findings from a previous survey in 1997 using 2,500 tests from herds in England and Wales, made from November 1996 to February 1997 (Berry and Scrivens, 1998). At that time the tests were made to BS5545 (1988). A new minimum standard (ISO 5707E, 1996) was introduced in April 1997, for machines installed or modified after this date. This has been used in the current survey which also includes data on the number of people milking, parlour automation and milk quality performance. The aim was to determine how many and why milking machines failed to reach the minimum standard recommended and any changes in systems on farms since the last survey.

Results

All the main manufacturers were well represented but many of the milking machines tested were composite systems with a variety of manufacturers' parts. Milking machines on a large number of farms in areas affected by the Foot and Mouth Disease epidemic of 2001 were upgraded or replaced, overall approximately 30 % of units have been upgraded or refitted. However, many others have received little attention; possibly due to the current economic state of dairying.

Since the survey in 1997 the general trend in the UK has been an increase in average herd size. Of those milking machines not replaced in order to accommodate this increase in herd size most have been increased by adding extra units on to existing parlour systems, rather than 'doubling up' i.e. number of milking units equals number of cow standings. Over 70% of farms milk in a herringbone parlour.

Some 61% of units tested in 2004 failed to comply with the minimum requirements for operation of a milking machine test compared to 70% in 1997 (Table 1). Machines in both

surveys may have failed on only one aspect of the test but many failed for several reasons so the reasons for failure total more than 100%. Whilst the number of machines failing may have decreased, in 1997 some 9% of those failing had an inadequate vacuum reserve increasing to 25 % of all machines failing in the current survey. Inadequate vacuum reserve compromises milking performance and effective cleaning of the milking machine (Tan *et al.*, 1993 a/b). The number of machines where no pulsation faults were identified was not recorded. However, it is rare for pulsation not need modification at the time of the test (Ohnstad, 1997). In 1997 pulsation ratio ranged from 50% to 70% (open to closed ratio). In the current survey most were generally set around 60- 65%, with a few manufacturers still recommending ratios of 56% and 67%. Liner shell compatibility was not examined in this survey. In 1997 53% of units used an alternate pulsation configuration and this had increased to 61% in the current survey. Only one milking machine manufacturer in the UK currently supplies a simultaneous pulsation system.

In the current survey, more than 80 % of machines failing had excessive vacuum losses and 20 % had excessive milk line air ingress. Some 25 % of machines had no or inadequate drainage and 25 % failed to comply with the requirements for either the site or the accuracy of the vacuum gauge. While some of these faults may be perceived as less important all can have an effect on milk quality.

One milker now manages an average of 10.4 units (an overall increase of 9 % since 1997). As the number of staff involved in milking has reduced by 30 % since 1997 the impact of large milking machines appears small. Of the farms surveyed some 47 % did not use automatic cluster removers (ACR) at the end of milk flow. Since no particular milking machine supplier was over represented this was not all due to an individual milking machine philosophy or manufacturer. Lack of ACR may indicate an economic consideration or limitation, or a reluctance to apply technology. Failure to use ACR increases the potential that cows are over milked at the end of milking with possible welfare implications (Natzke *et al.*, 1978; Shearn and Hillerton, 1996; Hillerton *et al.*, 2002). Combined with an increase in parlour sizes and a reduction in staff numbers, the potential benefits of increased automation for both cow welfare and milk quality should receive more consideration as parlours increase in size and fewer staff are employed.

Table 1. Reasons for a milking machine failing to comply with the recommended minimum standard (% machines failing).

Reason for failing	Test		Consequences
	1997	2004	
Excessive vacuum	55	80	Poor milking machine
Line loss			performance
Inadequate	9	25	Poor milking machine
Vacuum reserve			performance
Excessive airline loss	37	20	Degradation of milk quality
Drainage problems	Not available	25	Poor machine cleaning
Vacuum gauge	Not available	25	Potentially poor milking
Problems			performance
Total failure	70	61	

Bulk milk cell count data were collected. However, no inference was drawn between cell count and whether a machine passed or failed as there are many other potential factors which could also affect this.

Conclusions

Whilst the milking machine test standards have increased performance requirements since 1997 and there has been a slight reduction in the number of milking machines failing the test, there is still considerable room for improvement in all areas of milking machine performance. The National Dairy Farm Assured Scheme and contractual arrangements require an annual milking machine test and currently more than 60 % of parlours fail. This is failure to meet the minimum, far lower than optimum, requirements for milking performance and cleaning. It is perverse that the machine is required to be tested but not to pass that test given that the milking machine has long been recognised as an important risk factor in transmission of mastitis and contributor to poor milk quality.

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Machine milking risk factors for teat end callosity in dairy cows on herd level

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Abstract

The objectives of this study were to evaluate the effect of milking on teat-end callosity (TEC) at herd level. In total, 192 farms were visited once to collect data on the milking management, teat condition and milking machine function. Teat-end callosity was grouped into four categories: no callosity, smooth callosity, rough callosity, and very rough callosity. Teats in the rough and very rough callosity category were regarded as exhibiting a higher risk for clinical mastitis. Therefore, the percentage of teats within a herd with rough and very rough callosity (%ROUGH) was used as the dependent variable in a basic generalized linear model with a logit link in which the effects of single independent variables were analysed. Farms with milking parlours using a milk meter had on average higher %ROUGH than farms with high mounted milk recorder jars or without milk measurement. Longer machine-on time resulted in higher %ROUGH. Higher vacuum differences in the short milk tube were associated with lower %ROUGH as were increasing diameters of the short milk tube. At the same time feeding of concentrates in the milking parlour was associated with lower %ROUGH than individual feeding. There was an association between teat cup liner brand and %ROUGH. More concave and flat teat-ends showed lower %ROUGH. Post-milking teat disinfection was associated with higher %ROUGH compared to no post-milking teat disinfection.

Increases in rough and very rough teat-end callosity are associated with increased incidence of clinical mastitis, and therefore teat-end callosity can be used as a monitoring tool for assessing the quality of milking, management and machine handling.

Keywords: teat condition, milking machine, liner

Introduction

The teat of a cow is the first line of defence against mastitis. The ability of the teat to prevent infections is associated with the condition of the teat end (Michel *et al*., 1974; O'Shea *et al*., 1987). Therefore, teat end condition, often measured in terms of teat end callosity (TEC), is an essential physiological parameter in order to get more insight in the first line of udder defence. Moreover, a direct relation between TEC and incidence of clinical mastitis was found in a large study on 15 dairy farms. Roughness and thickness of the callosity ring are indicators for increased risk of clinical mastitis (Neijenhuis *et al*., 2001; Neijenhuis *et al*., submitted). Teats with a smooth thin callosity ring had the lowest risk

of clinical mastitis. Teats without any callosity ring had a higher risk of clinical mastitis compared to teats with a thin callosity ring. On the other hand, thicker and rough callosity also increased the incidence of clinical mastitis. Mein *et al*. (2001) simplified the TEC scoring system on basis of the relationship with mastitis into four categories in order to standardize classification of TEC in the field.

Cow factors (parity, lactation stage, udder anatomy) influence TEC (Michel *et al*., 1974; Sieber and Farnsworth, 1981; Neijenhuis *et al*., 2000). It is expected that besides cow factors, the functioning of the milking machine, and milking methods applied by the milker (milking technique) also influence TEC (Ziesack *et al*., 1989; Hamann, 1987; Rasmussen, 1993; Shearn and Hillerton, 1996). Changes in TEC may result from mechanical forces exerted by vacuum and the collapsing liner during machine milking (Mein *et al*., 2003). If there is a direct relation between the functioning of the milking machine and TEC, an increase in TEC can be used as an early warning signal of milking machine failures.

Until now, the relations between different settings of the milking machines or different milking systems and TEC have only been studied in relatively small experimental trials (Hamann, 1987, Ziesack *et al*., 1989). These field trials hardly provided any information on the relation between the level of TEC at the herd level and machine milking. Therefore, the objectives of this study were to evaluate the association between TEC and machine milking parameters at the herd level.

Materials and methods

A total of 200 dairy farms, spread throughout The Netherlands were asked to participate in this study. Inclusion criteria were a minimal herd size of 35 lactating cows, and the use of a milking parlour. Farms with a tie-stall or an automatic milking system were excluded from participation. Of the 200 dairy farms that were asked to participate, 192 farms entered the study.

From January to June 1998, each farm was visited once. At the farm visit, photographs of all four teats were taken of on average 35 randomly selected cows (range from 18 to 42 cows per farm) directly after milking. The teat photographs were interpreted by one trained technician who determined TEC in terms of TEC thickness and TEC roughness using a standardized classification system (Neijenhuis *et al*., 2000). In order to standardize the scoring to the international standard (Mein *et al*., 2001), the results were grouped in 4 categories:

N = no callosity (N), S = smooth callosity (1A, 1B and 2A), R = rough callosity (1C and 2B), and VR = very rough callosity (2C and 2D). Additionally, teat end shape was scored as round, pointed, flat or concave.

Besides the TEC-measurements, farm and machine milking data were collected (Table 1). The most recent milking machine maintenance report available on the farm is the result of a yearly check of the milking machine by the dealer and is a standardized report (De Koning and Huijsmans, 2001). Measurements in this report are not carried out during milking and include parameters such as vacuum level, capacity, pulsation rate, and pulsation ratio (Anonymous, 1996). Information on milking technique and milking machine were obtained by a questionnaire and observations in the milking parlour. Functioning of the milking machine was furthermore evaluated with dynamic measurements during the evening milking. The questionnaire and observations in the milking parlour included information

Table 1. Variables taken into account in the statistical analysis to identify the association with teat end callosity R and VR (%ROUGH).

Category	Variables
General farm description	Herd size (lactating dairy cows), type and size of milking parlour, feeding of concentrate during milking, automatic cluster removal, diameters short and long milk tube, length long milk tube, long milk tube support
Liners	Brand, material and replacement strategy
Measurements during one evening milking	Milk production, pre-treatment strategy and time, air sucking, cluster attachment, machine on-time, moment cluster detachment, post-milking teat disinfection, hygiene during milking, milking high SCC cows last, proportion of teat ends classified as pointed, proportion of teats classified as thick or long.
Dynamic measurements	Average, minimum, maximum, and difference in vacuum in the short and long milk tube, difference in vacuum between long and short milk tube, vacuum decrease in the short milk tube at the beginning and half way milking
Maintenance report milking machine	Air usage and vacuum measurements
Pulsator maintenance report	Pulsation rate and ratio, and phases

about pre-milking treatment, method of milking cluster attachment and detachment (improper detachment was defined as detachment later than 30 sec. after ceasing of milk flow), method and materials used during post-milking teat disinfection, hygiene during milking (scored as very clean, normal or very dirty), and maintenance of the milking machine (e.g. time between replacement of liners). Replacement of rubber liners was considered to be on time when they were replaced before 2500 milkings and for silicone liners before 7500 milkings.

Duration of pre-milking treatment, intervals between pre-milking treatment and cluster attachment, machine-on time, and milk production were measured for two batches of cows during milking. Depending on the size of the milking parlour, these two batches of cows comprised between 6 to 40 cows. Additionally, the vacuum level under the teat in the short milk tube and vacuum level in the long milk tube was measured with two vacuum gauges connected to a recorder at one milking unit during a complete milking of all cows milked with that unit.

All data were collected by four technicians who were specifically trained to make the photographs of the teats, fill out the questionnaire, and to carry out this standardized short dynamic milking evaluation.

Statistical analysis

As a descriptive analysis, for all variables the frequency of occurrence (categorical variables) or average value (continuous variables) was calculated. Because TEC categories R and VR are regarded as a higher risk for clinical mastitis (Neijenhuis *et al.*, submitted), per farm the following dependent variable was calculated: percentage teats with TEC category

R or VR (%ROUGH). Before statistical analyses were carried out, observations were checked for unlikely values. No data were excluded for this reason.

A generalized linear basic model with a logit link function for the binomial distributed outcome variable %ROUGH was formed with type of milk measurement and average machine-on time (McCullagh and Nelder, 1989). Type of milk measurement included in this study was: milk recorder jars with or without a milk lift from the cluster to the milk recorder jars, milk meter with low line plant or no milk recording system with low line plant. On top of this basic model, added single effects of independent variables were analyzed. Independent variables which contributed to the dependent variable with a level of significance $P < 0.25$ in the basic model were selected for multivariable analysis (Hosmer and Lemeshow, 1989). Forward stepwise selection of these variables was performed ($P<0.05$). The variable that accounted for most variance, as measured by the deviance ratio, was added to the model.

Results

Descriptive statistics

From the initial 192 farms, 184 farms were used in analysis. Six farms were dropped because the breed was not Holstein Friesian or Holstein Friesian cross breed. Another two farms were dropped because the milking frequency was 3 times a day instead of twice daily. The 184 farms had on average 65 lactating cows (range 31 to 350). The recorded cows produced during the visit on average 13 kg milk (range 8 to 19 kg milk) in 383 seconds (range 258 to 611). The mean percentage of teats within a category of TEC on a farm was: 1.7% N, 81.4% S, 16.8% R and 0.1% VR. On average 17% (range from 0 to 51%) of the teats were scored as %ROUGH. The distribution of %ROUGH is shown in Figure 1.

Model results

Milk measurement type and machine-on time were always forced into the regression model. Of the 70 variables offered to the model, 23 variables were associated with %ROUGH ($P < 0.25$). After forward stepwise introduction of the 23 selected variables of the first step,

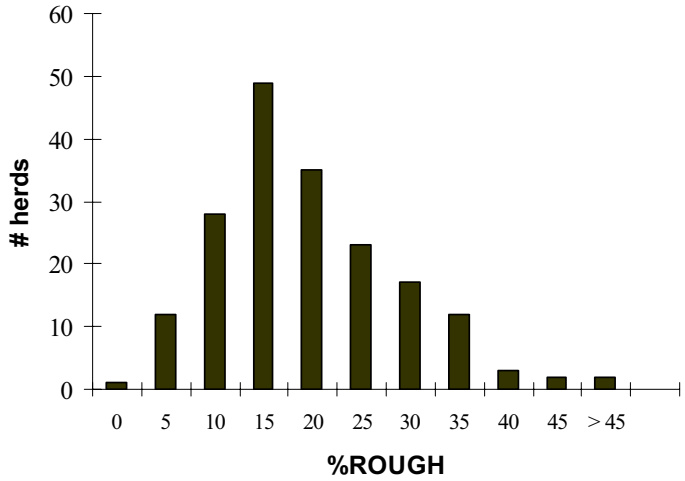

Figure 1. Frequency distribution of %ROUGH teat ends per farm.

6 remained significant ($P < 0.05$) and were added to the final model. The final model contained information of 163 farms.

Based on the fitted model, average %ROUGH was 16.8%. Machine-on time was 6.4 minutes, percentage of concave and flat teat ends was 11%, difference in the short milk tube during milking was 10.9 kPa, and diameter of the short milk tube was 10.6 mm. The final model had 144 df and a deviance of 789.

Farms with milking parlours using a milk meter had more %ROUGH than farms with high mounted milk recorder jars or without milk measurement. Longer machine-on time resulted in higher %ROUGH. Higher vacuum differences in the short milk tube were associated with lower %ROUGH as was an increasing diameter of short milk tube. Central feeding of concentrates in the milking parlour was associated with lower %ROUGH compared to individual feeding. Farms using teat cup liners brand A had on average more %ROUGH than farms using brand F or H. Farms with a higher proportion of concave and flat teat ends had less %ROUGH. Practicing post-milking teat disinfection was associated with higher %ROUGH compared to no application of teat disinfectant after milking.

Discussion

In the statistical analysis, several variables showed a significant relation with %ROUGH. Only the relationship between teat end callosity and liners, and the associated variables, will be discussed in depth in this paper because this is the only part in direct contact with the cows' teat.

The association between higher %ROUGH scores and the brand of liners may be due to differences in the liner dimension and through different pulsation and other milking characteristics between the brands. Mein *et al*. (2003) found that different liners apply a range of different over-pressures on the teat and that, with increasing vacuum levels and decreasing length of the C-phase of pulsation overpressure will be increased. In this study, no direct relationship between vacuum level or pulsator phases and callosity was found. However, variance component analysis for differences between the brand of the liner with the highest %ROUGH scores compared to the brands with significantly lower %ROUGH scores revealed a higher operating vacuum (42.8 versus 41.1 kPa) and a shorter A-and C-phase of the pulsation (14.6 vs 17.3% and 10.6 vs 13.4%). The reason for increasing over-pressure and increasing %ROUGH may lay in the more rapidly compressed teat-end (Mein *et al*., 1987). Liner shape, dimensions and material, in combination with dimensions of cow teats, should be looked upon more closely. Vacuum and pulsation characteristics should be taken into account to unveil the underlying combinations that cause the teat condition to deteriorate.

Earlier research showed that TEC is a risk factor for clinical mastitis (Neijenhuis *et al*., 2001). Furthermore this research showed that %ROUGH is influenced by the milking machine and milking management on the herd level. Given this circumstantial evidence, TEC, and more specifically %ROUGH, can be used as an early warning signal for an increased risk of clinical mastitis in milking machine research and as a monitoring tool for the quality of milking, including the machine and management, in the field.

Conclusions

Milking machine and milking management influence %ROUGH. Variation in %ROUGH between farms is explained by cow factors such as teat-end shape and machine-on time and milking machine factors such as the liner and the vacuum. Cows with more TEC have also an increased risk of clinical mastitis, and because of this relationship, %ROUGH can be used as an early warning signal for increased risk of clinical mastitis, and therefore can be used as a monitoring tool to assess the quality of machine milking.

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Long-term effects of different pulsation characteristics on teat thickness, teat skin moisture and teat skin pH of dairy cows

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Abstract

Ten identical twin sets, located at the research station of Dexcel Ltd., Hamilton, (New Zealand), were submitted to two different pulsation modes in a long-term trial, in split twin set design. The group, treated with the 'fast' milking mode, with a dynamic [b] phase, exhibited significantly higher teat thickness changes than the group treated with 'slow' pulsation mode. The teat end thickness change decreased throughout the lactation for both treatment groups. The milk yield was significantly correlated with the teat end thickness change for the treatment group 'fast'.

The pulsation treatment had no significant effect on teat skin moisture or pH.

Keywords: teat thickness, teat skin moisture, teat skin pH, pulsation

Introduction

In a conventional double-chambered teat cup, effective pulsation reduces the risk of new infection, since milking without pulsation produces a significant increase in the rate of new intramammary infection (IMI) (Bramley *et al*. 1978). The pulsation rate (cycles per minute) is of importance for cow comfort, teat condition and new infection risk (Walser 1966). An optimum pulsation rate of 50 to 63 cycles/min has been recommended (O'Shea 1981) to minimise teat damage and cow discomfort. An insufficient duration of liner closure per pulsation cycle contributes to the incidence of new IMI (Reitsma *et al*. 1981).

If liner pulsation is faulty or ineffective, the liner applies insufficient compressive load to the end of the teat during the collapsed phase of each pulsation cycle, resulting in congestion and oedema of the tissue surrounding the teat canal (Williams and Mein 1980), which can be determined as teat thickness. Depending on the liner type, the teat thickness increases considerably with decreasing [d] phase or increasing [b] phase (Hamann and Mein 1996) and takes more than 8 h to recover after milking (Neijenhuis *et al*. 2001b).

The teat skin condition also plays an important role in preventing new IMI. Teat skin moisture and pH provide information about the integrity of the bovine teat skin (Hansen 2002). For the period of one lactation, the effects of different pulsation modes on teat tissue and teat skin parameters were examined in this study.

Materials and methods

The trial layout is described in Table 1.

Table 1. Trial layout.

Design	Split twin set
Animals	10 identical twin sets, 5 sets Frisian, rest Frisian cross-breeds
Duration	One complete lactation, approximately 8 months
Milking	Ruakura Milk Harvester (RMH), mounted on rotary system
Treatment	One twin treated with 'fast' mode, the other with 'slow' mode
Parameters	1. Teat skin moisture and pH of back teats, prior to milking, barrel and tip of teat
	2. Teat end thickness of all four teat ends, before and after milking
Teat sanitation	All teats were sprayed with Teatguard Plus® (Ecolab, Hamilton, N.Z.) after every milking.
	The ready to use mixture had a pH of 3.3.

Treatment

The 'fast' mode was a dynamic pulsation mode, during which the open phase was controlled by the milk flow of the cow (pulsation rate 22 - 55 cycles/min, pulsation ratio 66 - 81 % open). The higher the flow rate, the more frequent [b] phase extensions became. During a pulsation cycle with extended [b] phase, the pulsation ratio was increased and the rate decreased, yet the next one or two cycles could be completely normal. The 'slow' mode had a pulsation rate of 47 cycles/min with a pulsation ratio of 43 % open.

Teat tissue and skin parameters

The teat end thickness was determined eight to 12 mm above the teat apex with a 'cutimeter', altered after Hamann *et al*. 1996. Measurements were carried out before attachment of the teat cups and immediately after cluster removal and the percentage change in teat end thickness was calculated after Hamann *et al*. 1996.

Teat skin moisture was measured using the Corneometer CM 820® (Courage and Khazaka electronic GmbH, Cologne, Germany). Teat skin measurements were performed before the teats were touched. Disposable rubber gloves were worn to ensure that the moisture of the fingers did not influence the moisture of the teat skin during measurement. Skin moisture is expressed in arbitrary units (Hamann *et al*. 2004).

The teat skin pH was determined using the flat surface Skin-pH-Meter PH 900® (Courage and Khazaka electronic GmbH, Cologne, Germany) (Hamann *et al*. 2002). On all occasions where a pH of '8' was exceeded, teats were visually soiled with faeces or soil, therefore this data had to be excluded from the analysis. During the first eight weeks and the last three weeks of lactation of every cow, all parameters were measured weekly (W1 - 8 and P1 - 3), in the main part of the season, monthly (M1 - 5).

Results

Effect on teat tissue

Only the data of 5 twin-sets, determined by similar calving dates could be included into the statistical analysis, because a time effect on teat thickness changes was discovered.

The teat thickness data is summarised in Table 2, including the information for milk yield and cups-on-time. The analysis of variance with repeated measurements revealed significant differences between the treatment groups for teat thickness change ($P \leq 0.001$). An influence of 'stage of lactation' on the parameters was found, yet no interaction between 'stage of lactation' and 'treatment'.

Overall, the teat thickness changes decreased through the lactation in both treatment groups. Milk yield and teat thickness change were significantly correlated ($r=0.247$, $P \leq 0.001$) for treatment group 'fast'. For the 'slow' treatment, no correlation was found.

Table 2. Teat thickness change (%), milk yield (kg) and cups-on-time (min) of cows milked with two different pulsation modes (sub-set of 10 cows).

Treatment	Thickness		Milk yield		Cups-on-time	
S. of	(% change)		(kg)		(min)	
lac.	Fast	Slow	Fast	Slow	Fast	Slow
W1	5.9	2.3	11.2	10.8	5.0	4.7
W2	4.4	0.4	12.8	12.0	5.1	5.6
W3	6.3	1.1	13.3	11.9	5.2	5.8
W4	1.6	-3.1	13.1	12.9	5.0	6.6
W5	5.2	-1.5	12.9	12.0	4.8	6.3
W6	11.5	-0.1	14.1	13.2	5.3	6.6
W7	11.7	-0.8	15.0	13.9	5.5	6.9
W8	6.8	0.1	14.4	13.8	4.8	6.9
M1	7.6	-4.3	10.8	11.5	4.9	5.6
M2	3.3	-6.6	11.2	10.8	4.6	5.5
M3	1.1	-2.2	10.4	10.4	4.4	5.7
M4	1.2	-1.3	8.2	8.5	4.0	4.8
M5	-0.6	-6.9	9.0	9.1	4.1	4.6
P1	2.2	-5.2	6.9	7.8	4.2	4.9
P2	7.0	-7.7	7.6	6.7	3.5	5.4
P3	-0.6	1.9	7.6	5.8	3.4	5.1

S. of lac.: Stage of lactation

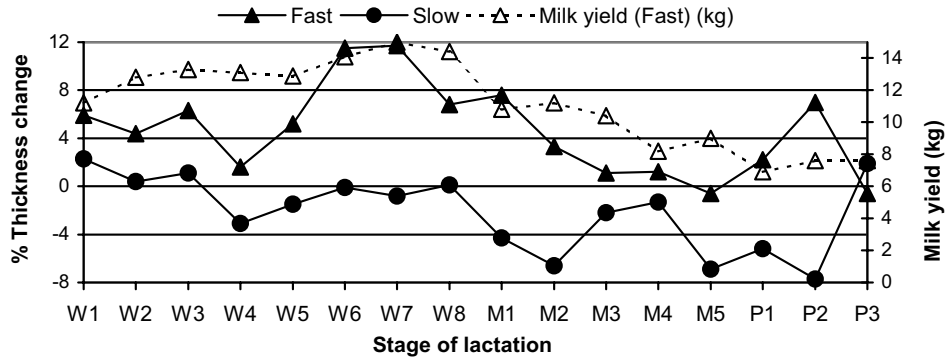

Figure 1. Mean teat thickness change (%), 10 cows treated with pulsation modes 'fast' or 'slow' and milk yield for treatment group 'fast'.

Effects on teat skin

The data was analysed by stage of lactation according to the measurement scheme. Only a sub-set of ten cows was included, that had calved within a period of 24 days, in order to minimise not only temperature but also time effects. The teat skin data of these ten cows is summarised in Table 3, grouped by parameter, location and treatment.

Teat skin moisture values fluctuated from 23 to 72 arbitrary units, the teat skin pH exhibited values between 6.53 and 6.59. The lactational development can be observed in Figure 2. No significant effect of pulsation treatment on teat skin moisture or pH was found (Table 3).

Table 3. Mean teat skin moisture and teat skin pH, for cows treated with two different pulsation modes (sub-set of 10 cows).

Parameter	Moisture				pH			
Location	Barrel		Tip		Barrel		Tip	
Treatment	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
S. of lac.								
W1	54.1	36.8	48.0	34.8	7.12	7.17	6.87	6.92
W2	36.2	33.9	32.4	39.4	7.01	6.95	6.86	6.90
W3	28.1	31.5	35.6	23.5	6.75	6.86	6.55	6.72
W4	58.2	61.3	50.8	58.0	6.43	6.14	6.36	6.07
W5	50.1	54.0	55.5	43.7	6.67	6.59	6.82	6.57
W6	72.3	60.6	59.1	65.4	6.20	6.40	6.17	6.42
W7	38.8	47.2	41.9	38.8	6.33	6.51	6.52	6.79
W8	45.6	71.1	42.3	52.6	7.06	7.33	7.10	7.21
M1	52.3	47.1	41.8	59.6	6.28	6.34	6.30	6.28
M2	53.8	70.3	56.8	69.8	6.83	6.59	6.87	6.78
M3	61.8	57.9	68.9	52.9	6.55	6.30	6.50	6.16
M4	51.4	53.3	58.1	50.9	6.30	6.60	6.17	6.47
M5	67.2	61.3	67.0	68.3	6.48	6.53	6.29	6.34
P1	54.4	66.2	55.9	56.1	6.11	6.10	6.10	6.02
P2	52.8	66.5	44.8	61.8	6.13	5.83	6.25	5.73
P3	31.5	34.3	37.5	34.0	6.30	6.20	6.23	6.13
P-values	NS		NS		NS		NS	

*P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, NS: not significant; Analysis of variance with repeated measurements, S. of lac.: Stage of lactation

Discussion

The long-term effect of different pulsation modes on teat tissue was examined. The pulsation mode had a significant effect on teat thickness during milking. This was expected, since teat thickness has been shown to increase with increasing [b] phase length (Hamann and Mein 1996). Although the [b] phase of the pulsation mode 'fast' was variable in length, it was always longer than the [b] phase of the 'slow' mode. It is likely that this increased the degree of congestion or oedema in the teat tissue during milking, resulting in an increase in teat thickness at the teat tip after milking.

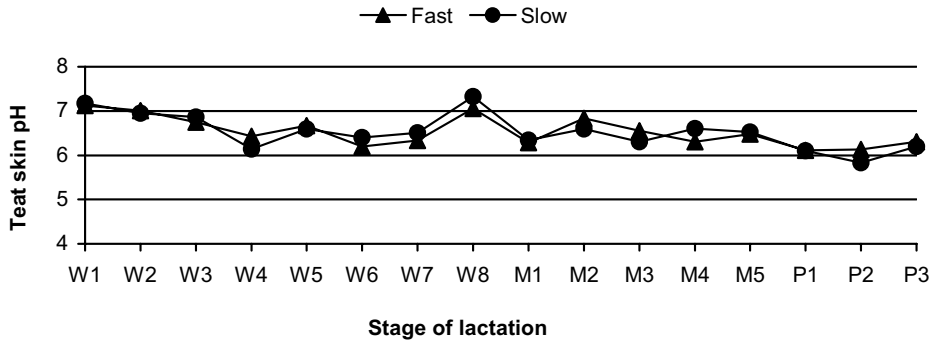

Figure 2. Lactational development of teat skin pH (barrel). Five twin sets milked with two different pulsation modes.

In the subsequent analysis, a significant correlation was found between teat thickness change and milk yield, for cows milked with the 'fast' mode. This finding was confirmed by the milking rate and milk yield increases. It was observed that higher milking rates were achieved using the 'fast' pulsation mode, as would be expected by the findings of Woolford and Sherlock (1987) who observed an increase in average milking rate of 23.8 per cent when using the Ruakura Milk Harvester (RMH). For both treatment groups, a reduction in teat thickness change was found during lactation, which was consistent with a lactational decrease in milk yield and milking duration. This confirms further the relationship between milking rate, teat thickness changes and pulsation mode.

Milking machine parameters, such as vacuum level and pulsation are associated with teat condition (Hamann 1997). Good teat condition is crucial for good health of the udder and the prevention of mastitis (Francis 1984; Fox 1992; Neijenhuis *et al*. 2001a; O'Shea 1981; Sieber and Farnsworth 1981). Teat skin moisture and pH are parameters that may provide some information about the condition of the teat skin (Hamann *et al*. 2002; Hamann *et al*. 2004). The teat skin moisture and pH levels observed in this study were within the range found in other studies. The teat skin pH was typical for cows treated with a sanitiser of low pH (Hansen 2002).

No significant effect of the pulsation treatment on teat skin moisture or pH was found. Unfortunately the literature provides no information about the influence of milking machine parameters on teat skin moisture or pH. It is possible that the pulsation treatment had no impact at all on the two teat skin parameters, although an influence on teat tissue has been found. On the other hand the treatment could have caused only subtle changes in skin moisture or pH that were not detectable with the devices used here, or did not last long enough to be picked up before the next milking. The sample size may have been too small to obtain a significant effect, but identical twins were used to compensate for small sample numbers (Carter 1954). Unfortunately it is not useful to determine teat skin moisture or pH directly after milking, because the teat skin is moist and the pH was influenced by the milk surrounding the teat surface during the milking process. Therefore the direct influence of the different pulsation modes on teat skin is undeterminable.

Conclusion

The long-term effect of the pulsation treatment on teat tissue was not surprising, although it has not been shown over an entire lactation like this before. Yet, it was disappointing that this effect could not be connected to the teat skin parameters. If that were possible, some of the visual and subjective parameters used in mastitis research could be superseded.

The lactational changes in teat skin pH and moisture were interesting findings but a more complicated trial design would be required to discover subtle teat skin alterations caused by machine parameter changes.

Acknowledgements

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Details of some of the forces applied to the cow's teat during milking

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Abstract

This work details some of the mechanisms of teat damage to help with diagnosing problems and to design better milking machine components. Various cases found in the milking machine are discussed. Results from mathematical models using large strain elasticity are used to understand the interaction between the teat and the liner. Biomechanical models are proposed for the teat barrel and the teat end. Experimental verification of the models is ongoing and some of the results are presented. Various mechanisms of the mechanical behaviour of the teat are discussed in the context of commonly encountered problems. A future target of the programme is to propose numerous tissue failure criteria.

Keywords: teat damage, biomechanical models, liner design, hyperkeratosis

Introduction

The forces applied to the teat of the dairy cow during machine milking are low and do not cause immediate damage, however they are repeated many times over days and years. The cumulative damage amounts to a form of Repetitive Strain Injury (RSI). Over a typical lactation the teat will be exposed to 250,000 cycles of loading as the liner closes and opens.

Typical machine induced teat problems are abnormal teat colour, oedema and congestion of the teat, haemorrhage of small blood vessels, excessive physical changes to the orifice such as hyperkeratosis or gross changes in pathology such as eversion of the end. It is necessary to consider the stresses when the liner is open, when the liner is closed, when the liner is in the process of opening and when the liner is closing. The rubber and the teat are, most simply, both elastic at the loading frequencies in machine milking. The elasticity of large multiaxial strains must be used to give an understanding of the mechanisms of different types of teat damage. The work has been verified by pressure and displacement measurements.

Teats vary in shape and tone within the herd, with parity and through lactation. Some of the less desirable changes in the teat over parity and through lactation are as a result of the physical adaptation of the teat to the forces applied during milking.

Case 1. The open liner

When the liner is open the teat is exposed to milking vacuum (typically 40 kPa or more). Figure 1 shows the system of forces on the teat end during the open phase. A more extreme

form of damage due to vacuum such as haemorrhaging is due to the same mechanism as an aneurysm. Some congestion is common in the teat tissue and the teat wall is typically 40% thicker after milking (Hamann *et al.*, 1994. The pressure seen by the barrel of the teat varies between slightly above atmospheric (typically 5kPa), when the liner is closed, to the milking vacuum of -40 kPa (relative to atmospheric pressure). Therefore, the pressure on the teat averaged over time is around -20 kPa. The tissue can dilate with fluids in response to the vacuum.

For a 22 mm diameter liner the downwards force due to vacuum is 15 Newtons. The force will pull the teat into the liner. If the liner is narrower than the teat then the radial compression will also cause the teat to elongate. Van Der Merwe (1985) showed the teat wall as being made of 2 layers of relatively inelastic parallel cords embedded in an elastic matrix. Using such a model the stress / strain behaviour of the teat barrel can then be investigated (Figure 2). The cords form a network where the angle ϑ controls the length and diameter of the teat according to the formulae below.

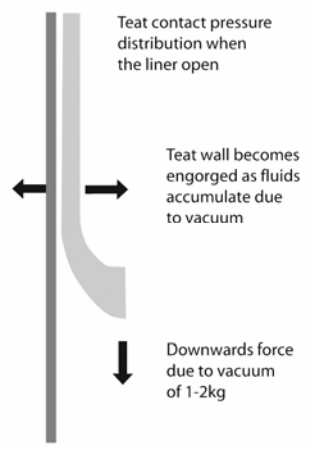

Figure 1. Axial forces on the teat.

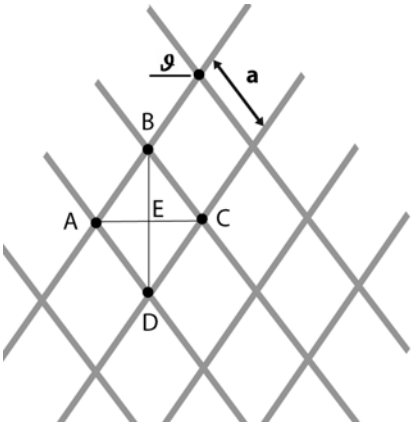

Figure 2. Geometry of teat structure.

$AE = a*\cos\vartheta$ (1)

(AE is proportional to diameter)

$BE = a*\sin\vartheta$ (2)

(BE is proportional to length)

Where ϑ is the angle defining the orientation of the 2 layers. The axial strain is

$$e = \sin\vartheta_2/\sin\vartheta_1 - 1 \quad (3)$$

The radial strain is

$$e = \cos\vartheta_2/\cos\vartheta_1 - 1 \quad (4)$$

Subscripts 1 (initial angle) and 2 (angle in strained material) refer to angles at different loadings. A Poisson's ratio can then be derived and is a function of strain not a singular material constant.

Data from Lees *et al.*(1991) suggest initial cord angles of 30 - 40º. The relationships, combined with the elasticity of the surrounding tissue, can be

used to calculate the barrel strains during pulsation.

The pressure difference across the teat barrel will cause axial tension in the teat barrel, which becomes radial and circumferential at the teat end. These tensions are resolved at the teat end. The resulting tension causes the teat orifice to stretch open. Overstretch could occur in the elastic tissue and the smooth muscle around the teat end. High levels of stretch can both fatigue and relax the teat (relax here is meant in the sense of both a physical change, as creep, and changes under nervous control (Butler *et al.*, 1992). These can account for the teat being left open after milking. At a vacuum level across the teat end of 40kPa and for a high flow rate cow (up to 12 l / min peak flow), assuming the milk is flowing for 0.5 seconds per cycle then there must be an orifice diameter at least 4.75 mm. If the orifice is reduced to 4.5 mm diameter the milking vacuum needed to achieve the same flow rate is 48 kPa. This indicates how crucial it is for the tissues in the orifice structure to be in good repair.

The teat orifice "cord reinforced structure" has been described (Van Der Merwe, 1985) as a "multi spiralled, net-like, integrated musculo-elastic system". The angle of the spiral relative to the axis was reported as low and, therefore, the axial stiffness and strength of this structure must be low. The hoop stiffness and strength will be high, as it has evolved to resist the tensions induced in suckling. Two different shaped teats have been modelled (Figure 3) and the orifice strain calculated. Figure 4 shows stretching forces applied to the orifice. An amplification of the barrel tension occurs at the orifice and it can be seen that the mechanism is a rather elegant and simple tap or switch that opens the duct at the orifice and allows milk to flow to the calf. However, the strains can be large and the orifice tensile hoop strain differs markedly between two teats of identical properties except for the teat end shape (Figure 5).

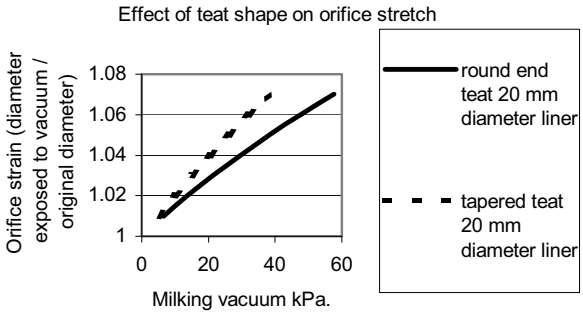

Figure 3. Shape of the modelled tapered and round teats.

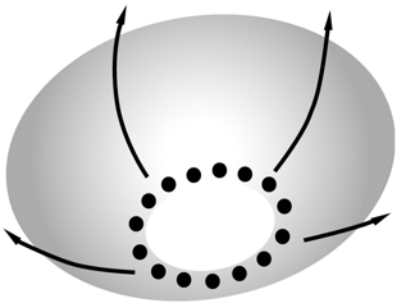

Figure 4. Forces stretching open the teat end.

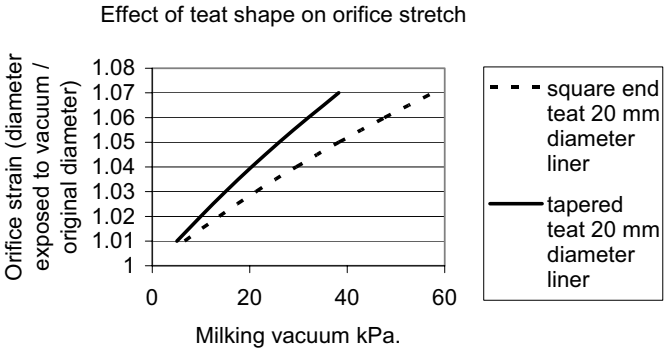

Figure 5. Teat orifice strains with tapered and round teat ends.

Case 2. Compressive forces on the teat end when the liner is closed

With reference to Figure 6 and from basic membrane theory

teat contact pressure = liner tension /deformed teat radius (6)

The tension is the liner tension and increasing it will increase the pressure, The radius is the *deflected* shape of the cow's teat and as this is on the denominator of the equation reducing the radius increases the contact pressure. Importantly, away from the teat end

where there is little axial curvature, the liner applies little pressure to the teat. Pressures over the teat end are typically 5 to 10 times greater than those applied to the wall of the teat barrel. The increase in teat wall thickness noted by Hamman *et al*. (1994) can occur, as the liner is not being effectively massaged along the barrel. The pressure due to tension is more important contributing to the small forces due to bending in the axial direction.

Figure 7 shows the pressure distribution over 2-4 mm at the teat end. Note that a vertical section through the pressure map gives the pressure profile indicated in Figure 6.

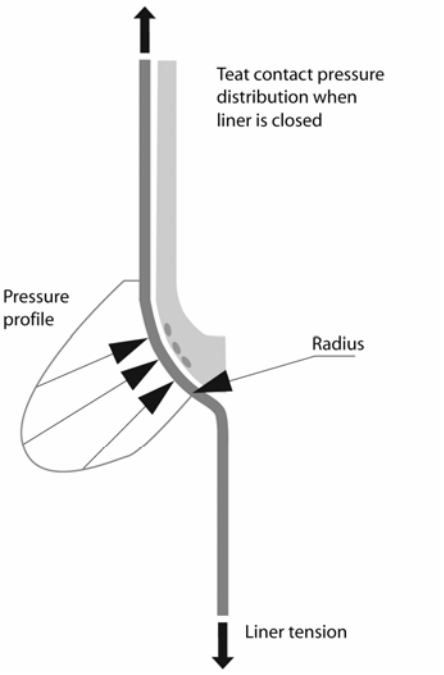

Figure 6. Compressive forces on the teat end when the liner is closed.

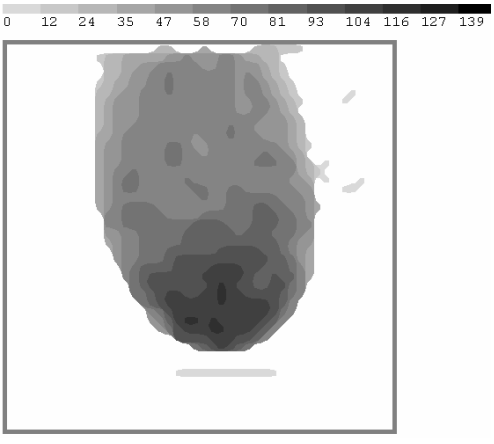

Figure 7. Typical pressure map of the teat end pressures (kPa) compressive and extensional.

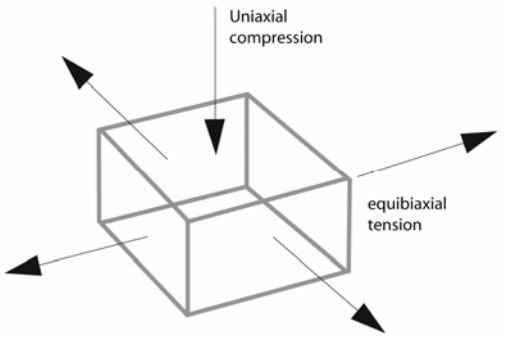

Figure 8. Uniaxial compression is identical to the equibiaxial tension so compression gives an axial strain in the teat tip.

Discussion

Once numerical values for the stresses and strains have been determined then it is possible to validate a variety of failure criteria for the different stress systems. Strain rather than stress criteria should be used, but further experimental investigation is needed. As an example, hyperkeratosis develops where the tissue is sheared. A maximum principal strain can be derived from the shear strain. The maximum principal strain is then the elongation stimulus of the *stratum germinatum* layer of the skin, stimulating the production of keratin.

Uniaxial compression and equibiaxial tension are equivalent stress systems (apart from the hydrostatic pressure which can be ignored). The action of the pressure localised around the teat end results in both a radial and axial extension of the teat orifice structure. In the final stage of closure of that part of the liner below the teat it extends, the resulting extra tension gives a further axial strain to the teat end. The tissue structure around the teat orifice is arranged to close the orifice and not to resist axial extension. Excessive extension of the teat orifice due to the forces outlined could contribute to teat end hyperkeratosis.

A single tissue type can have different failure criteria for different force systems. For the teat end tissue under uniaxial compression and axial strain it is possible to propose strain-based failure criteria, i.e. limits beyond which the teat will be susceptible to cumulative damage.

Conclusions

- A biomechanical model for the teat barrel and end structure allows accurate modelling of the teat barrel and end structures, to understand the response of the teat the forces applied to it.
- High orifice stretch and axial strain (as a result of compressive forces) are identified as significant sources of teat end attrition.
- Failure criteria allowing hyperkeratosis and other tissue damage are proposed.
- This paper deals only with the forces. The movement between the teat and liner will be the subject of a further paper.

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Effects of lactic acid teat dip on chapped teat skin

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Abstract

The objective of this study was to evaluate the effects of postmilking teat dip containing lactic acid and glycerine formulation on chapped teat skin. Thirty lactating cows with severely chapped and cracked teat skin in three commercial dairy herds in Argentina were included. This teat condition was a consequence of using chlorine compounds for postmilking teat disinfection. Teat skin condition of the cows was evaluated 5 days on chapped teats and 15 days during the use of lactic acid teat dip, using a visual score system. The daily average teat condition score, milk production and milkout time was computed for all cows in the study. The average teat skin condition before and after the study was 4.68 and 1.29, respectively. After teats heal at the end of the study, average milk production increased by 7.52% and average milkout time decreased by 30.58%. The prevalence of intramammary infections with *Staphylococcus aureus* in chapped teats was 73.33%. Severely chapped and cracked teat skin was associated with and increased prevalence of intramammary infections with *Staphylococcus aureus*. Application of a lactic acid postmilking teat dip resulted in improved teat skin condition on chapped teat skin. Poor teat condition had a negative influence on milk yield and milkout time. The maintenance of good teat skin condition is important to prevent intramammary infections, improve milk yield and reduce milkout time.

Keywords: teat chapping, lactic acid, *Staphylococcus aureus*

Introduction

The maintenance of healthy teat skin condition is a key requirement for an effective mastitis prevention program. Chapped teat skin has been positively correlated with increased colonization by *Staphylococcus* aureus and an increased risk of intramammary infections (IMI) (Fox, 1995). Some germicides in teat dips can have irritating effects on teat skin, causing chapping, lesions, drying, or a caustic reaction. Two studies (Burmeister *et al.*, 1998; McKinzie *et al.*, 1995) showed an impact of teat skin condition on milk yield and milkout time. The use of iodine teat germicides in one of these studies (McKinzie *et al.*, 1995), resulted in improved teat skin condition on artificial chapped teat skin. However, these studies involved artificial chapping of teats and no information is available under field conditions and the effects of lactic acid teat dip. The objective of this study was to evaluate the effects of postmilking teat dip containing lactic acid and glycerine formulation on chapped teat skin under field conditions on milk yield and milkout time.

Materials and methods

Thirty lactating cows with severely chapped and cracked teat skin in three commercial dairy herds in Argentina were included. This teat condition was a consequence of using chlorine compounds for postmilking teat disinfection. Teat skin condition of the cows was evaluated 5 days on chapped teats and 15 days during the use of lactic acid teat dip, using a visual teat evaluation score system (Goldberg *et al*., 1994). The degrees of teat skin chapping were: 1) teat skin is smooth and free from scales, cracks, or chapping; 2) teat skin shows some evidence of scaling; 3) teat skin is chapped, and some small warts may be present; 4) teat skin is chapped and cracked, and redness is present; numerous warts may be present; and 5) teat skin is severely damaged and ulcerative with scabs or open lesions, and large or numerous warts are present. The daily average teat skin condition score, milk yield and milkout time was computed for all cows in the study. Quarter milk samples were collected from all lactating cows before the use of lactic acid teat dip, to examine the relationship between teat skin chapping and IMI by *Staphylococcus aureus*. Isolates were identified according to the procedures of the National Mastitis Council (Hogan *et al*., 1991). Differences between teat skin condition score, milk yield and milkout time before and after the implementation of lactic acid teat dip were analyzed by Repeated Measures/General Linear Model.

Results and discussion

Summary of results before and after the implementation of lactic acid teat dip is shown in Table 1. Differences between teat skin condition score, milk yield and milkout time before and after the implementation of lactic acid teat dip were significant ($P < 0.01$). After teats heal at the end of the study, average milk production increased by 7.52% and average milkout time decreased by 30.58%. The prevalence of intramammary infections with *Staphylococcus aureus* in chapped teats was 73.33%.

Findings of the present study were in accordance with the results of Burmeister *et al*. (1998) and McKinzie *et al*. (1995), who showed that chapped teat skin decrease the milk yield and increase milkout time. This study is in agreement with Fox (1995), who found that chapped teat skin has been positively correlated with increased colonization by *Staphylococcus* aureus and an increased risk of IMI. Severely chapped and cracked teat skin presented in cows of this study, was a consequence of using chlorine-based compounds for postmilking teat disinfection. Skin conditioning agents are not included in the types of chlorine products used, because of associated formulation problems (Pankey *et al*., 1984). Therefore, the use of such products is not recommended. Because of the potential for causing irritation, skin conditioning agents are often added to teat dip formulations to serve as

Table 1. Mean teat skin condition score, milk yield and milkout time before and after the study.

Parameter	Chapped teats	Lactic acid
Teat skin condition	4.68 ± 0.16	1.29 ± 0.08**
Milk yield (Kg)	20.46 ± 0.65	22.00 ± 0.79**
Milkout time (min)	6.31 ± 0.35	4.38 ± 0.19**

**P < 0.01

humectants, like glycerine, to draw water onto the teat skin surface, or as emollients, like lanolin, which serve to coat the teat skin and prevent evaporative water loss (Pankey *et al.*, 1984). McKinzie *et al.* (1995) showed that the use of iodine teat germicides with advanced conditioning technology improve the teat skin condition, milk production and milkout time on experimental chapped teats. Our study evaluated the effects of postmilking teat dip containing lactic acid and glycerine formulation on chapped teat skin under field conditions. Lactic acid, one of the more effective ingredients developed for teat skin care, is an alpha-hydroxy acid (AHA). AHAs have been used in cosmetic live-on products intended for daily application to skin. The functional benefits provided by AHAs are skin moisturizing and exfoliating (Wickett, 1996). For this reason they are formulated into skin creams and lotions to help mitigate the appearance of wrinkles and the signs of aging skin. Lactic acid is inhibitory to both Gram-positive and Gram-negative organisms, and has also been effective as a teat germicide (Boddie *et al.*, 1992). This teat disinfectant is a food-grade chemical and nonirritating to teat skin. This product leave no harmful residues in milk (EMEA, 1990) and is tolerant of organic matter. Therefore, lactic acid teat dip is antimicrobial, naturally compatible with milk and skin conditioning. The addition of glycerine help to maintain teat skin condition at an acceptable level. This humectant attract moisture to the outer layers of the skin to keep it soft and supple. In addition, a study (Orth *et al.*, 2000) demonstrated that the skin care benefits of glycerine include osmoregulation of the intracellular milieu, maintenance of liquid cristallinity and fluidity of cell membranes and intercellular lipids. The combination of lactic acid and glycerine provided better teat skin condition compared to chlorine-based compounds for postmilking teat disinfection. According to Rasmussen *et al.* (1998), this study shows the benefit of skin conditioning like glycerine and also the humectant germicide combination. Therefore, teat conditioning properties are a result of the teat dip composition and not the specific germicide or the skin conditioning (Hemling, 2002). Others advantages to improve teat skin condition were the increase of milk yield and decrease of milkout time. The negative influence of chapped teat skin on milk production and milking speed can be a consequence of the disturbance release of oxytocin in the milk ejection reflex. The inhibition occurs in response to a stress reaction, because of the cow discomfort during milking. Bruckmaier *et al.* (1998) reported that the disturbance of milk removal can be a consequence of peripheral inhibition of the reflex and inhibition at the level of the central nervous system. In practice inhibition can have enormous effects on milk production both in the short-term and long-term perspective. Peripheral inhibition of the milk ejection reflex is characterized by the lack of an oxytocin effect at the udder level under conditions of normal milking related release of oxytocin from the pituitary. The inhibition occurs in response to catecholamines and as a result of a blockade of oxytocin receptors. Catecholamines stimulate α-adrenergic receptors, causing a contraction of the teat and cisternal area whereby the milk removal is inhibited in spite of a normal milking-related release of oxytocin. As long as milk is available in the cisterns the milk flow is not reduced. However, the effect of the inhibition of the ejection occurs when the milk travels from the alveolar area into the cistern through the contraction of milk ducts. During central inhibition, the disturbed milk ejection reflex was a lack of oxytocin release in response to pre-stimulation and milking. Disturbance of milk removal has been observed in primiparous cows immediately after parturition, during oestrus and during milking in unfamiliar surroundings. The basal concentrations of cortisol and β-endorphin were higher in cows milked in unfamiliar surroundings in comparison with cows milked in familiar environment. The elevated

concentrations of these substances indicate that the cows were subjected to some kind of emotional stress. Elevated cortisol levels can be considered as a stress reaction in cows. This study also suggest that during machine-milking, the physiological requirements of the cows need to be considered, and, most importantly, stressors must be minimized. Negative effects on comfort of the cows should be avoided during milking to maintain optimal milk let-down, milk production, milkout time and parlor throughput.

Conclusions

Application of a lactic acid postmilking teat dip resulted in improved teat skin condition on chapped teat skin. Teat dip containing appropriate amounts of skin conditioners, such as glycerine, can help maintain teat skin condition at an acceptable level. The teat conditioning properties are the result of the teat dip formulation and not the specific germicide or the skin conditioning agent. Severely chapped and cracked teat skin was associated with and increased prevalence of intramammary infections with *Staphylococcus aureus*. Poor teat condition had a negative influence on milk yield and milkout time. The maintenance of good teat skin condition is important to prevent intramammary infections, improve milk yield and reduce milkout time.

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Technical success and effectiveness of teat cleaning in automatic milking in Finland

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Abstract

Proper milking hygiene is essential for good quality raw milk and udder health of the cows. Technical success and effectiveness of teat cleaning and the management factors associated with them were evaluated in nine automatic milking herds. In total 616 teats cleaned with cleaning cup and 716 teats cleaned with rotating brushes were included. Technical success and the effectiveness of teat cleaning, including the location and nature of the dirt were evaluated visually. On average 79.9% of the teat cleanings with a cleaning cup and 85 % with brushes were technically successful ($P = 0.012$), i.e. the teat was correctly positioned in the cleaning device throughout the whole cleaning process. Factors associated with technical success of teat cleaning were the herd, behaviour of the cow, days in milk, teat cleaning method, teat location, udder and teat structure and teat colour. Excessive udder hair and technical failure of the automatic milking machine also caused a few technically unsuccessful teat cleanings. From originally dirty teats, 79.8% became clean or almost clean during the cleaning process with the cleaning cup and 72.9% with the brushes ($P = 0.024$). The main factors associated with the efficiency of teat cleaning (evaluated for technically successful cleanings only) were the cleanliness of the teat before cleaning, the herd, teat cleaning method, and teat condition. Teat orifice was the most ineffectively cleaned site of the teat (50.5%/43.8 % teat orifices cleaned).

Keywords: automatic milking, effectiveness of teat cleaning, technical success of teat cleaning

Introduction

Proper milking hygiene is essential for the production of good quality raw milk and for the udder health of the cows (Pankey *et al.*, 1989). Raw milk may become contaminated by bacteria from teat surfaces, mastitic milk or contact surfaces of the milking equipment (Galton *et al.*, 1982). Mastitis pathogens may enter the teat canal during milking in suboptimal milking conditions (Rasmussen *et al.*, 1994). According to EU legislation, the udder and teats of a cow must be visually clean before milking (Council Directive 89/362/EEC).

Automatic milking systems (AMS) have no method for distinguishing between dirty and clean teats before cleaning, or for monitoring the effectiveness of the cleaning. They have no sensors to detect whether the teat is in the cleaning device during cleaning, which is contrary to Finnish legislation (8/EEO/2002). The only published study of technical success

of teat cleaning is from Norway (Hvaale *et al.*, 2002). In some experimental studies, teat cleaning with AMS has been less effective than manual cleaning (Knappstein *et al.*, 2004), as effective as manual cleaning (Ten Hag and Leslie, 2002) or even more effective (Melin *et al.*, 2002). In field studies with both visual and bacteriological evaluation of teat cleanliness, teat cleaning with AMS was less effective than manual cleaning (Knappstein *et al.*, 2004; Tangorra *et al.*, 2004). The aim of this study was to evaluate the technical success of teat cleaning in AMS herds and possible reasons for failures. Another objective was to evaluate the effectiveness of teat cleaning and potentially related management factors.

Materials and methods

Herds and teat cleaning systems

Nine commercial dairy herds with automatic milking system were visited once from September to December in 2003. Group A consisted of 161 cows with 616 milking teats, and Group B of 184 cows with 716 milking teats.

Teat cleaning system of Group A has a separate cleaning cup which uses warm water, variable air pressure and vacuum to clean the teats and afterwards dries the teats with warm air. Teats are located by lasers and a camera prior to cleaning.

Teat cleaning system of Group B uses wet rotating brushes to clean the teats. After cleaning the brushes are sprayed with warm water and disinfectant.

In this study, cows in Group A had normal teat washing regimens and cows in Group B had two brushing sequences. Teat cleaning devices were visually clean and undamaged.

Technical success of teat cleaning (TSTC)

TSTC was evaluated visually and recorded as successful, partly unsuccessful or totally unsuccessful. Cleaning was successful if the teat was straight and completely in the cleaning device throughout the entire cleaning process (for Group B throughout both cleaning sequences). Cleaning was partially unsuccessful if the teat was folded against udder basis or otherwise only partially in the cleaning device, or not in the cleaning device for the whole time of the cleaning. Cleaning was totally unsuccessful if the teat was not in the cleaning device at all or if the cleaning process never took place at all for that particular teat.

Effectiveness of teat cleaning (ETC)

All four teats of each cow were visually evaluated by the same experienced person. The side of the teat facing the researcher was evaluated with the help of a flashlight. Teat end was evaluated with the help of a mirror without touching the teat. A five-category scoring system for teat cleanliness before and after cleaning was created. The location and nature of the dirt were recorded. Cleanliness score was also treated as a dichotomous variable by classifying teats as clean if they were in the category “clean” or “almost clean” and as dirty otherwise.

Statistical analysis

Pearson`s chi-square test was used to test the independence of different variables between groups or the interdependence of the covariates. Factors associated with TSTC were studied with a binary logistic regression model:

Successful teat cleaning = μ+ group + herd + teat location (fore, hind) + parity + days in milk + milking frequency + time since last milking + cow behaviour + teat colour + udder and teat structure + udder hairiness. (1)

Interactions were tested and included in the model if necessary.

Cow and herd characteristics associated with ETC were studied with an ordinal regression model. Only teats with technically successful cleaning and teat cleanliness before cleaning > 0 were included in the analysis. The initial model was:

Teat cleanliness after cleaning (0-4) = group + herd + teat location (fore, hind) + teat cleanliness before cleaning + parity + days in milk + milking frequency + time since last milking +teat colour + udder and teat structure + teat condition + udder hairiness + group*teat cleanliness before cleaning + herd*teat cleanliness before cleaning. (2)

Results and discussion

Technical success of teat cleaning

For Group A on average 79.9% of teat cleanings were technically successful, and for Group B 85% (P = 0.012) (Figure 1). This is much less than in a Norwegian report, where AMS failed in 10 to 20% of the teat cleanings per cow (Hvaale *et al.*, 2002).

Herd was the most important factor affecting the technical success of teat cleaning, which varied between herds from 62.9 to 95.8% of the teats in Group A and from 80.4 to 89.9% in Group B (P < 0.001). The type of the teat cleaning system used affected TSTC to a lesser extent (Table 1)**.** In Group B, 63% of the partly unsuccessful and 80% of totally unsuccessful cleanings were unsuccessful in both brushings, as also reported by Hvaale *et al.* (2002).

The causes of most of the unsuccessful teat cleanings could not be determined (Table 2). Some of them may have resulted from incorrect programming of the teat coordinates. Of defined causes device failure concerning 6 cows in one herd in Group A and restless behaviour in Group B caused most of the totally unsuccessful teat cleanings. Cows may move after the teat is located by the teat cleaning system in the Group A, and teats may slip away from the cup or the cow may kick the cleaning cup off. With the teat cleaning system

Figure 1. Technical success of teat cleanings per teat with two different cleaning methods; cleaning cup (Group A) and rotating brushes (Group B) (with CI).

Table 1. Factors associated with technical success of teat cleaning (TSTC).

Factors associated with TSTC (reference group)	OR[1]	95% CI of OR
Group (A)	0.41**	0.23 - 0.74
Teat location (Hind)	0.43***	0.31 - 0.59
Restless behaviour	0.28***	0.18 - 0.43
Days in milk (after first 30 days postpartum)	2.89***	1.93 - 4.34
Abnormal udder and teat structure	0.51**	0.32 - 0.80
Group A * teat colour	0.38**	0.21 - 0.68

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

[1]odds ratio of the factor

used for the Group B, cows are expected to stand always in the same position and remain still. Some of the restless behaviour might have been caused by the presence of the researchers.

Partly unsuccessful teat cleanings were mostly caused by abnormal udder and teat structure. Closeness of the hind teats, very thick teats and oblique position of the teat were the most problematic abnormalities in both groups. More failures in TSTC were found in early lactation (Table 1), which may be related to restless behaviour in some individual postpartum cows, udder edema or changing udder shape. Resistance to mastitis is at its lowest in early lactation and milking hygiene is important at that time.

Black teat pigmentation was associated with unsuccessful teat cleanings in Group A, where the teats are located with lasers before cleaning. Excessively long udder hair was related to a few cases of partially unsuccessful cleanings in one herd of Group A, where an excessive amount of clean, dry bedding material was attached to long udder hair. The AMS pointed the lasers towards the bedding particles and ended up folding the teats against the udder base.

Effectiveness of teat cleaning

After cleaning, only 33.1% of the teats in Group A and 37.1% in Group B were totally clean ($P = 0.168$). If a dichotomous scale was used, 84.5% of the teats in Group A and 80.6% in Group B were considered clean ($P = 0.094$). According to Knappstein *et al.* (2004), using visual evaluation of cleanliness, as much as 69% of the teats appeared visually clean

Table 2. Observed reasons for partly and totally unsuccessful cleanings of Group A (cleaning cup) and Group B (rotating brushes).

Observed reason for failure	Partly unsuccessful		Totally unsuccessful	
	Group A	Group B	Group A	Group B
Unknown	65%	54%	34%	42%
Behaviour of the cow	12%	21%	7%	52%
Udder and teat structure	14%	25%	7%	6%
Udder hair	9%			
Device failure			52%	
Total number of failures	76	76	44	31
(% of all cleanings per teat)	12.7%	10.7%	7.4%	4.3%

after teat cleaning. Ordinal regression model showed that ETC differed between herds slightly more than between groups, which agrees with the results of Knappstein *et al*. (2004). The group was associated with ETC ($P = 0.003$). Interaction of group and teat cleanliness before cleaning was also associated with ETC (Group A, $P < 0.001$ and Group B, $P < 0.000$).

Figure 2 shows that in Group A, a larger proportion of the extremely dirty teats were clean after cleaning ($P = 0.002$). Using a dichotomous approach, of the originally dirty teats 79.8% in Group A and 72.9% of those in Group B were clean after teat cleaning ($P = 0.024$). However, Knappstein *et al*. (2004) found that, based on bacterial counts on the teat skin before and after teat cleaning, extremely dirty teats were cleaned more effectively with brushes, while slightly soiled teats were cleaned more effectively with a cleaning cup.

Teat cleanliness after cleaning varied mainly according to the cleanliness of the teat before cleaning ($P < 0.000$). Almost clean and slightly dirty teats were cleaned well, but dirty and, especially, extremely dirty teats were not cleaned well enough, as around 45% of them were left dirty. Only four teats were dirtier after cleaning than before. Bedding material on the teats was cleaned almost completely. Cleansing of teat orifices was least effective compared to teat barrel and apex, as only 50.5% (Group A) and 43.8 % (Group B) of teat orifices were cleaned ($P = 0.039$). This is critical, as bacteria and sediment on the teat orifice have direct access to the teat canal and also to the raw milk collected. Teat condition was also associated with ETC ($P = 0.006$).

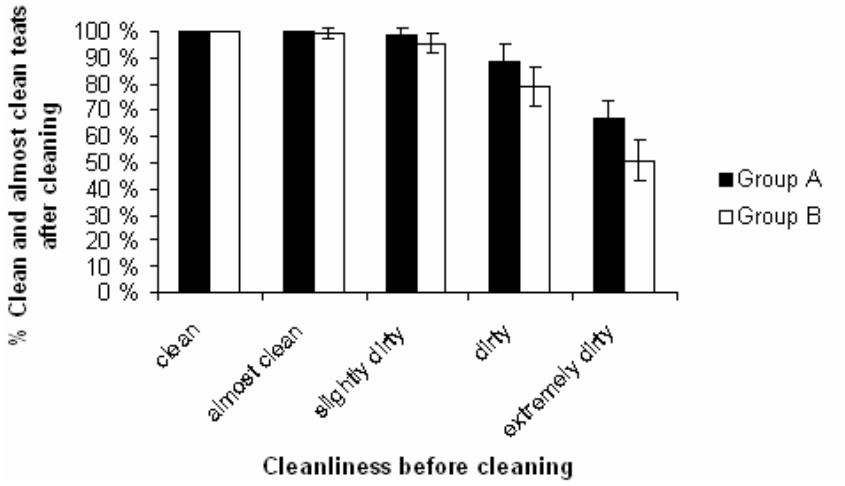

Figure 2. Proportion of clean teats after cleaning in different categories of teat cleanliness before cleaning with two different cleaning methods; cleaning cup (Group A) and rotating brushes (Group B) (with CI).

Conclusions

There is clearly a need to improve the reliability and monitoring methods of TSTC in AMS. The great variation between herds shows that TSTC can be improved by management. The function of the cleaning device can be evaluated by observing teat cleaning of several cows repeatedly. If there are many unsuccessful teat cleanings (less than 95% of teats successfully cleaned), a management action is necessary. Teat coordinates programmed in the computer should be re-evaluated. Cows with poor udder structure should be culled.

Milking should occur smoothly so that it does not cause distress and restlessness. Laser lenses should be kept clean and bright, and udder hair short. If these management actions are not enough, consideration should be given to requesting technical service in order to address technical issues.

As only half of the extremely dirty teats became clean or almost clean in the automatic cleaning process, hygienic measures are of utmost importance. The adjustability of the brush- or cup-cleaning mechanism to suit herd or cow characteristics available in AMS should be exploited. A hygienic standard of the milking stable and automatic milking machine can do much to prevent teats from becoming soiled in the milking stall. Good condition of teat skin and opening should be maintained in the herd, and the teat cleaning device should be clean, intact and used according to the recommendations of the manufacturer.

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Diagnosis of mastitis and indicators for milk quality

Use and interpretation of bacteriology in the diagnosis of bovine intramammary infection

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Abstract

The interpretation of bacteriology and the diagnosis of intramammary infection has been an area of intense debate over many years and positions have become polarised with regard to the merits of different techniques. This paper discusses the relative advantages and disadvantages of different approaches to the diagnosis of intramammary infection. A variety of datasets were available for analysis. The results of bacteriological culture of a total of 25,652 quarter milk samples were available for analysis. Concurrent quarter somatic cell counts were available for 7732 quarters samples. In addition, data from serial milk samples collected from 1252 quarters of 313 cows, once daily, over 3 days were available. Accredited laboratories were used for all bacteriology and somatic cell counting; the laboratories followed recognised standard operating procedures and were blinded to both cow and quarter identity. Bacteriology results were reported objectively, both as the species of bacteria identified and the number of colonies cultured; no subjective interpretation of the plates was permitted. Analysis of the datasets available revealed that 35.9% of samples yielded a bacteriological growth, a major pathogen was isolated in 12.7% of samples and a minor pathogen in 25.6% of samples. The vast majority of isolates were in pure culture. Concurrent somatic cell count data did not support the hypothesis that environmental pathogens were more likely than contagious pathogens to be representative of contamination. The pattern of culture positive findings that led to a diagnosis of intramammary infection using the 2 of 3 positive criterion was significantly different between contagious and environmental pathogens. When compared to a historic dataset the patterns of diagnosis were noticeably different for the environmental pathogens. In the light of these findings it may be necessary to reassess our approach to defining intramammary infection, in particular with reference to the environmental mastitis pathogens.

Keywords: diagnosis, bacteriology, intramammary infection

Introduction

The interpretation of bacteriological findings and the diagnosis of intramammary infection (IMI) has been an area of intense debate over many years. The current guidelines that are followed in both scientific and regulatory studies are largely based on very historic data, often collected over 25 years ago. Over the time since these definitions were developed there has been a dramatic change in both the prevalence and aetiology of bovine mastitis in the developed world (Bradley, 2002), and it is now arguably uncertain whether the same diagnostic criteria hold true. In particular, the historic criteria favour the diagnosis of long

term, persistent intramammary infections of the type characterised by contagious pathogens. The current criteria may be resulting in an underestimation of the importance of the environmental pathogens and thereby detracting from the development of techniques and therapies for this group of pathogens. This paper attempts to discuss the relative advantages and disadvantages of different approaches to the diagnosis of intramammary infection.

Materials and methods

Data was collated from a number of studies conducted at the University of Bristol, School of Veterinary Science between 10th February 1997 and 27th February 2004.

Sample collection

A stringent regime for the collection of samples was followed. In all cases samples were collected by veterinary surgeons. Teats were initially wiped to remove gross contamination and dipped in a solution containing 2800ppm available chlorine. Following a minimum 30 second contact time, the teats were wiped dry. Each teat was subsequently scrubbed with a cotton wool swab soaked in 70% ethanol and allowed to dry. Prior to collection of the first sample the teat ends were scrubbed for a second time using 70% ethanol and foremilk was discarded (except from udders assessed as having little secretion present during the dry period, when foremilk was collected). Following a third scrub of the teat ends, duplicate samples were collected. When quarter milk samples were collected for somatic cell counting, these were collected prior to the first sample collected for bacteriology. Milk samples were immediately stored in a cool box and maintained at or below 4ºC. Bacteriology was usually performed within 24 hours, occasionally samples were stored frozen, prior to analysis, but typically for less than 2 weeks. Disposable gloves were worn throughout the sampling process and were changed between cows.

Bacteriology

In all cases bacteriological analysis was undertaken by an accredited laboratory following recognised laboratory techniques. 10µl of secretion was inoculated onto sheep blood agar and Edward's agar; 100µl of secretion was inoculated onto MacConkey agar to enhance the detection of *Enterobacteriaceae*. Plates were incubated at 37ºC and read at 24 and 48 hours. Organisms were identified and quantified using standard laboratory techniques. Colonies were enumerated on blood or Edward's agar; if coliforms were only present on the MacConkey agar then colony counts were adjusted to reflect the greater volume inoculated. All colony types appearing within a sample were reported, and input into an Access database. Subjective evaluation of the bacteriological plates was not undertaken - the aim being to report all findings, thereby allowing a full interpretation of the colony types isolated. Samples containing more than 3 colony types were deemed contaminated and excluded from the analysis.

Somatic cell counting

All somatic cell counting samples were submitted weekly to ON MeRiT Laboratories, Newbury, UK for analysis using the Fossomatic method.

Data analysis

Data was analysed to determine the impact of colony numbers on IMI diagnosis as well as to determine the distribution of diagnoses according to quarter somatic cell count. Finally, a dataset comprising samples collected on three consecutive days from 1252 quarters, of cows with elevated somatic cell count, was analysed to allow investigation of the relative merits of different approaches to the definition of intramammary infection.

Results and discussion

Bacteriology was available for analysis on a total 25,652 quarter milk samples from 29 farms. Concurrent, pre-milking quarter somatic cell counts were available on 7732 quarter samples.

A breakdown of the number of colony types identified on individual cultures is outlined in Table 1. 35.9% of samples yielded a bacteriological growth, a major pathogen in was isolated in 12.7% of samples and a minor pathogen in 25.6% of samples. The vast majority of isolates were in pure culture. Only 0.1% of samples was categorised as contaminated, this rose to 0.7% of samples if contamination was classified as more than two colony types - this more harsh definition of contamination resulted in the exclusion of 138 additional samples, 99 of which would only have been considered contaminated because of the presence of a minor pathogen.

The impact of colony number on 'diagnosis' of an 'intramammary infection', when interpretation of infection status was determined on the basis of isolation of a pathogen in a single sample, is illustrated in Table 2. The impact of increasing the threshold of the number of colony forming units for 'diagnosis' of an 'intramammary infection' appears to be more marked on gram negative than gram positive major pathogens. This is not surprising and fits with the findings of other authors who have described the coliforms as often being shed in low numbers (Smith *et al.,* 1985).

Figure 1 outlines the relationship between isolation of a mastitis pathogen and quarter somatic cell count. This figure demonstrates the proportion of cultures that were positive for a pathogen that were in each of the cell count categories. One may expect environmental pathogens to be relatively over-represented in the low cell count categories as a result of

Table 1. The number and type of pathogen isolated from individual samples.

Total number of colony types	Number of cultures	Proportion of quarters (%)	Number of major pathogen types	Number of minor pathogen types	Number of cultures	Proportion of quarters (%)
0	16,450	64.1	0	0	16,450	64.1
1	7948	31.0	1	0	2202	8.6
			0	1	5746	22.4
2	1090	4.2	2	0	365	1.4
			1	1	545	2.1
			0	2	180	0.7
3	138	0.6	3	0	39	0.2
			2	1	78	0.3
			1	2	21	0.1
>3	26	0.1	-	-	26	0.1

Table 2. The impact of colony numbers on diagnosis of intramammary infection, based on a single culture and isolation.

Number of colony forming units / ml	Pathogen					
	Coag +ve Staph	*S. uberis*	*S. dysgalactiae*	*E. coli*	Coag -ve Staph	Corynebacterium spp
Number of isolates						
≥100 cfu/ml	706	561	69	597	2860	3927
% ≥100 cfu/ml	100.0	100.0	100.0	100.0	100.0	100.0
% ≥200 cfu/ml	94.8	98.9	100.0	80.7	81.2	96.5
% ≥500 cfu/ml	87.0	92.9	95.7	63.5	60.8	84.8
% ≥1000 cfu/ml	78.0	82.9	85.5	53.9	47.8	70.2

the increased likelihood of them being found coincidentally as a contaminant. However, this was not the case, and in fact the environmental pathogens (*E. coli* and *S. uberis*) were less likely to be identified in lower cell count quarters than the Coagulase +ve *Staphylococci*. This finding suggests that when an environmental isolate is identified in a fastidiously collected, quarter sample it is likely to be of intramammary origin, and is less likely to be associated with a low SCC than a Coagulase +ve *Staphylococcus*!

The 1252 quarter dataset, in which samples had been collected on each of three consecutive days, was further analysed to investigate the influence of different diagnostic criteria on the apparent prevalence of intramammary infection. Table 3 outlines the proportion of quarters, identified as culture positive in 1 of the 3 samples collected, that were found to be positive using a variety of other diagnostic criteria.

The most dramatic reduction in the proportion of quarters defined as positive is with *E. coli*. It could be argued that this is not surprising and is a reflection of the likelihood of the presence of *E. coli* being more likely to be contamination. However, another interpretation could be that this is a reflection of intermittent shedding of this pathogen, or the short duration of such infections - this view could be supported by the findings

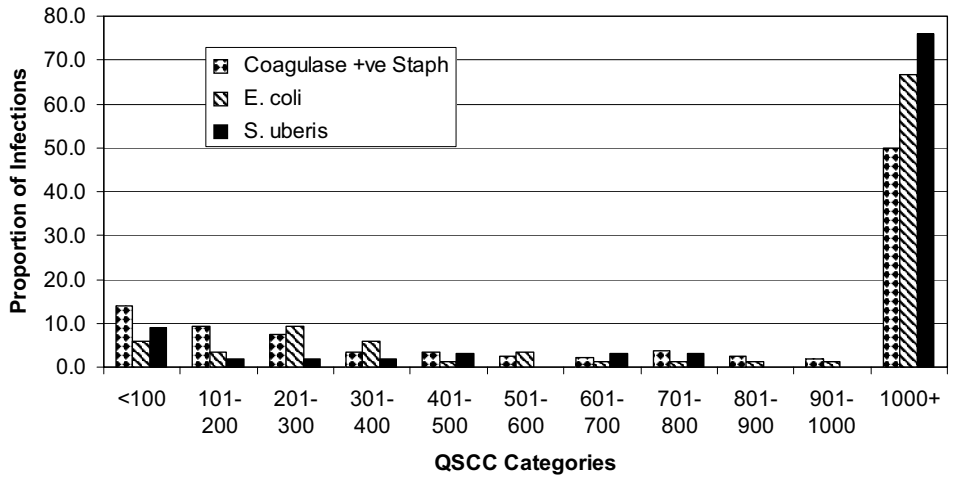

Figure 1. An illustration of the proportion of positive cultures occurring in different somatic cell count categories for some of the most commonly isolated major pathogens.

presented in Figure 1, which suggest that *E. coli* was most likely to be recovered from very high SCC quarters. It would appear from the data that the currently used diagnostic criteria may underestimate the true prevalence of the gram negative mastitis pathogens.

A major issue when trying to unravel the complexities of interpretation of bacteriological data is how one should go about defining the 'gold standard' test. Undoubtedly, taking 1 of 3 samples as a positive result will be the most sensitive approach, though not the most specific. Conversely 2 of 3 samples being positive, though more specific will be less sensitive. It may in fact be that a single culture of a well collected sample may give the 'truest' indication of infection status? It is important that these issues are considered and arguably, when considering all of the data, in its entirety, the isolation of a pathogen from a single, well collected sample is sufficiently similar to the 'Gold Standard' of 2 of 3 to justify using this approach in large field studies. This is not at odds with the conclusions of the IDF Bulletin 211 published in1987. Additionally, the use of single samples in positive control efficacy studies may have merit, as the error inherent in this approach will be duplicated in both treatment groups. However, when a 'greater specificity' of diagnosis is required then a diagnostic approach using more frequent (and expensive!) sampling and complex diagnostic criteria may be required.

In addition, the impact of setting a 'colony forming unit' threshold was examined on the proportion of quarters identified as being infected. Interestingly, the application of a threshold of 500 cfu/ml for the definition of infection resulted in a significant decrease in the number of *E. coli* and coliform infections diagnosed when using a '2 of 3' or '3 of 3' criteria whilst not having a significant effect on the number of quarters defined as infected on the basis of a single isolation - this may be a reflection of the intermittent shedding of these species (which may be occurring at very low levels?).

Table 4 makes a direct comparison with the findings of this dataset and that presented by Griffin *et al.* in 1977. This table examines the proportion of quarters diagnosed as infected or uninfected on the basis of bacteriological findings in each of three tests (the results of the third test are only shown when the first two disagree).

A few major differences and similarities are worthy of comment. There is a substantial difference in the prevalence of infection with major pathogens in the two datasets (25.8% *cf* 9.4%) with *S. aureus* in particular, but not surprisingly, being far more prevalent in the 1977 dataset. Despite this large difference in prevalence it is interesting to note that the

Table 3. An illustration of the impact of different intramammary infection definitions on the number (proportion) of quarters identified as infected compared to the definition of any one of three cultures revealing the presence of a pathogen (n=1252).

	Definition of infection					
Pathogen	1 of 3	2 of 3	3 of 3	1 of 2	2 of 2	1 of 1
Coagulase +ve Staph	65	41 (63.1)	30 (46.2)	57 (87.7)	34 (52.3)	45 (69.2)
S. dysgalactiae	10	7 (70.0)	6 (60.0)	9 (90.0)	6 (60.0)	8 (80.0)
S. uberis	70	41 (58.6)	25 (35.7)	64 (91.4)	31 (44.3)	45 (64.3)
E. coli	21	5 (23.8)	5 (23.8)	18 (85.7)	5 (23.8)	10 (47.6)
Coagulase -ve Staph	237	89 (37.6)	56 (23.6)	185 (78.1)	68 (28.7)	127 (53.6)
Corynebacterium spp	471	282 (59.9)	129 (27.4)	423 (89.8)	197 (41.8)	294 (62.4)

Table 4. An illustration of the results of major pathogen diagnoses by bacteriological test results from quarter milk samples collected on consecutive days, and a comparison with data presented by Griffin et al. in 1977.

Pathogen	% of diagnoses defined by each type of sample series							
	Quarters classified as infected				Quarters classified as uninfected			
	No. of infected qrts (%)	++	+-(+)	-+(+)	No. of uninfected qrts	--	-+(-)	+-(-)
S. aureus	41 (3.3)	82.9	7.3	9.8	1211	98.3	0.7	1.0
S. aureus (1977)	896 (16.0)	83.7	6.4	9.9	4716	97.0	1.1	1.9
S. dysgalactiae	7 (0.6)	100.0	-	-	1245	99.9	0.0	0.1
S. dysgalactiae (1977)	124 (2.2)	91.1	0.8	8.1	5488	98.8	0.7	0.5
S. uberis	41 (3.3)	73.2	12.2	14.6	1211	97.4	1.6	1.0
S. uberis (1977)	184 (3.3)	94.0	2.2	3.8	5428	99.5	0.2	0.4
All Coliforms	18 (1.4)	72.2	-	27.8	1234	97.0	1.2	1.8
All Coliforms (1977)	5 (0.1)	100.0	-	-	5607	99.6	0.2	0.2
Pseudomonas spp	0 (0.0)	-	-	-	1252	99.7	0.2	0.2
Pseudomonas spp (1977)	0 (0.0)	-	-	-	5612	99.9	0.0	0.1
Other Pathogens	29 (2.3)	51.7	13.8	34.5	1223	92.8	2.7	4.5
Others Pathogens (1977)	23 (0.4)	78.2	4.3	17.5	5589	99.8	0.1	0.1
Any Major	118 (9.4)	71.5	9.7	18.8	1087	81.0	8.4	10.6
Any Major (1977)	1446 (25.8)	87.4	4.7	7.9	4166	93.0	3.0	4.0

figures for definition of intramammary infection with *S. aureus* are remarkably similar between the two datasets.

However, there are substantial differences when one considers the environmental pathogens. The prevalence of *S. uberis* is similar in both datasets, though the more recent dataset is suggestive of a more intermittent shedding pattern of *S. uberis*. Additionally, the coliforms are significantly more prevalent in the 'modern' dataset and again would appear to be more 'intermittently' shed, though the numbers of quarters involved is small. These differences could of course be due to other differences between the studies such as laboratory technique, however it is important to remember that accredited laboratories were used throughout.

Conclusions

We have to be cautious when drawing conclusions from this dataset without substantial further analysis. Whilst many of the original definitions and descriptions hold true, this preliminary investigation of the data suggests that we may need to revisit some of our previously held 'beliefs' about how to define intramammary infection. These 'beliefs' are perhaps most challenged in the area of the environmental pathogens. This is perhaps not surprising when we consider how both the pattern of infection and prevalence of mastitis pathogens has changed over the past 30-40 years. We believe and suggest that when multiple samples are collected it is important to 'totally explore the data' to assess the impact of different diagnostic criteria on the study results; failure to do this may result in an unsafe

interpretation of the data. Further analysis and investigation of the most appropriate way to define intramammary infection with environmental pathogens is required.

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Evaluation of the Petrifilm™ culture system for the identification of mastitis bacteria as compared to standard bacteriological methods

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Abstract

Isolation and identification of mastitis pathogens is a fundamental aspect of milk quality and udder health control programs. There is an increasing demand for the development and evaluation of on-farm mastitis culture methods for rapid and accurate identification of bacterial species. Petrifilm™ plates are sample-ready selective culture media, which are used for rapid bacteriological isolation and enumeration. Petrifilm™ may be useful for farm-based culture systems, which might be used to guide clinical mastitis treatment decisions. The objective of this project was to determine the test characteristics of Petrifilm™ to characterize the causative organism in producer defined cases of intramammary infection (IMI). This study was performed using milk samples from 156 clinical mastitis cases from cows in 10 herds in southwestern Ontario. Duplicate quarter samples were taken from all cases, with one plated immediately onto sterile aerobic count, coliform count, and Staph express Petrifilm™ plates. The second sample was frozen at -20°C, and later submitted for routine bacteriological culture. The laboratory microbiological result was used as the gold standard to calculate test characteristics. The sensitivity, specificity, and predictive values were calculated for Petrifilm™ to categorize the cause of mastitis into groups of: bacterial growth, gram positive growth, or coliforms, staphylococci spp. and streptococci spp. Petrifilm™ media was very sensitive for aerobic growth (93%), but the specificity was poor (27%). The most favourable results were obtained for identification of coliforms and gram positive growth, with a sensitivity of 93% and 92%, respectively, and specificities of 86% and 73%. The Petrifilm™ media offers considerable potential for differentiating mastitis causing organisms into their respective Gram families. As such, this system could serve as a valuable tool in an on-farm clinical mastitis therapy decision-making tool, and serve as a guide towards appropriate treatment of clinical mastitis cases.

Keywords: Petrifilm™, on-farm culture, sensitivity, specificity

Introduction

Two components of the NMC recommended mastitis control program are appropriate management of clinical mastitis during lactation, and regular monitoring of udder health status (NMC, 2004). A fundamental aspect of fulfilling both of these components is regular culturing of clinical mastitis cases. Certainly, culturing all clinical cases is a useful method to monitor the profile of pathogens within a herd over time. Over the past decade, the profile

of mastitis pathogens has been shifting. Certain organisms have gained more importance, particularly the environmental streptococci (Schukken and Schulte, 2004). During this same time, blanket therapy control strategies and widespread use of core-antigen vaccines have dramatically reduced the incidence of mastitis caused by Streptococcus agalactiae and E. coli, respectively (Schukken and Schulte, 2004; Hogan and Smith, 2003). Routine milk culture programs are also very important for truly appropriate treatment decisions to be made (Sears and McCarthy, 2003). The significance of knowing the causative organism is both an economical and therapeutic consideration.

The largest cost associated with treating clinical mastitis during lactation is the discarded milk during and after therapy (Fetrow, 2000). To justify this cost, one should ensure that the present infection will respond to antibiotic therapy. Most intramammary antibiotics target Gram positive cocci organisms, and are not particularly effective against Mycoplasma spp., yeasts, and mild infections caused by gram-negative pathogens (Sears and McCarthy, 2003). Similarly, research has demonstrated that other organisms, such as *Streptococcus uberis*, are highly responsive to intramammary antibiotics (Hillerton and Kleim, 2002), or that the cure rates achieved by various intramammary treatments are not significantly better than no treatment (Wilson *et al.*, 1999). Thus, there has been an increasing demand for research towards the development of on-farm mastitis culturing media for early identification and accurate classification of bacterial species (Sears and McCarthy, 2003). To date, the time required from sample collection to submission and reporting of results back from a microbiological laboratory has been timely treatment decisions that need to be made on farm.

Petrifilm™ plates are selective culture media products, which are used for rapid bacteriological isolation and enumeration from food products. The 3M Petrifilm™ products are small, playing card size, sample-ready plates that will allow users to easily and efficiently perform on-site microbial identification (3M). Petrifilm™ plates that are potentially useful for mastitis diagnoses are the aerobic count plates, Coliform count plates, and Staph Express count plates, due to their ease of use, and the ability to reach a diagnosis in as short a time as 24 hours. The usefulness of the Petrifilm™ culture system for the diagnosis of mastitis has been previously reported (Silva *et al.*, 2004). This study evaluated the test characteristics and reader variability associated with the Staph Express count plate, and examined the use of Petrifilm™ as an on-farm culture system that was incorporated into mastitis treatment protocols.

The objective of the current project was to determine the test characteristics of Petrifilm™ to characterize the causative organism in producer defined cases of clinical mastitis.

Materials and methods

The evaluation of Petrifilm™ media was performed using milk samples from 156 cases of mastitis identified in 10 commercial herds serviced by a single veterinary clinic in southwestern Ontario. The participating producers were instructed to take duplicate milk samples from cows identified as having clinical mastitis at the time of milking. Milk samples were refrigerated and stored on farm until they were picked up regularly by a technician of the veterinary clinic. Once received at the veterinary clinic, the first milk sample was plated onto three Petrifilm™ media plates: aerobic count, coliform count, Staph express. With a single-use plastic pipette, 1.0 ml of milk of placed onto each media. Once the milk had

been completely displaced by a spreading device, it was then incubated at 37°C for 12-24 hours. If no growth occurred after 12 hours, then it was rechecked in another 12 hours. The second milk sample from each cow was frozen at -20^{0}C and later transferred to the Mastitis Research Laboratory, University of Guelph for standard microbiological culture. The laboratory used standard operating procedures for the handling of samples, culture techniques, and interpretation of results (NMC).

Classification of Petrifilm™ test results

Aerobic count plates enumerate total aerobic populations of bacteria, which could be either gram positive or gram negative, within 48 hours. Coliform Count Plates enumerate coliforms in 24 hours. The indicator dye in the plate colors all colonies red, and a top film traps gas produced by the coliforms. Confirmed coliforms produce red colonies that are associated with gas bubbles. The Staph Express Plate contains a chromogenic, modified Baird-Parker medium and a cold-water-soluble gelling agent. The test requires only one incubation temperature and is equivalent to the BAM three-plate Baird-Parker agar and single tube-coagulase method (3M). A chromogenic indicator identifies *Staphylococcus aureus* by producing red-violet colonies.

Each culture media plate was interpreted using guidelines provided by 3M Canada. The results from each different type of plate were interpreted together to record an outcome for each clinical mastitis sample. The outcome categories utilized were: bacterial growth, Gram positive growth, coliform growth, Staph. spp. growth, and Strep. spp. growth. The standard laboratory microbiology results were interpreted to provide a similar outcome.

For this study, the definition of "bacterial growth" from Petrifilm™ was based on a positive sample on the aerobic count plate. The definition of "coliform growth" was based on growth on both the aerobic count and coliform count plates and no growth on the Staph express plate. A positive result of "Staph spp". was made based on growth in the aerobic count and Staph express plates, and no growth on the coliform count. Interpretation of the indicator dye in the Staph express plate to differentiate S. aureus was not used. A "Strep spp." Petrifilm™ result was made with growth on the aerobic count plate, and no growth on either of the coliform count or Staph express plates. Finally, a result of "Gram positive growth" was recorded with a positive aerobic count plate, a negative coliform count plate coupled with either positive or negative growth on the Staph express.

Results

From the 156 samples collected, 52 (33%) had no growth recorded in the microbiology lab. For each culture result category, the sensitivity, specificity, and predictive values were calculated (Table 1). There was excellent sensitivity (> 85%) for all categories with the exception of those classified as Strep spp. There was a corresponding good specificity, indicating low numbers of false positive results, except for the category of bacterial growth. The specificity for "bacterial growth" was poor at 27%. Closer examination of the raw data revealed that of all samples evaluated, there were 38 (24%) that were positive for growth on Petrifilm™ which the mastitis lab recorded as having no bacterial growth.

The predictive values demonstrate, given a Petrifilm™ test result (either positive or negative for a particular category), the probability that test result is correct. For the coliform

Table 1. Test characteristics of Petrifilm™ culture media, compared to the gold standard of routine microbiological lab culture techniques, of 156 milk samples collected from producer defined cases of clinical mastitis.

Result	Sensitivity	Specificity	PPV[1]	NPV[2]	Apparent prevalence	
					Petrifilm™	Lab
Growth[3]	0.93	0.27	0.72	0.67	0.87	0.67
Coliforms[4]	0.93	0.86	0.59	0.98	0.28	0.18
Staph Spp.[5]	0.86	0.82	0.73	0.91	0.42	0.36
Strep. Spp.[6]	0.58	0.91	0.46	0.94	0.15	0.12
Gram positive[7]	0.92	0.73	0.77	0.91	0.59	0.49

[1]Positive Predictive Value

[2]Negative Predictive Value

[3]Microbiological growth recorded on aerobic count plate

[4]Microbiological growth recorded on both aerobic and coliform count plates

[5]Microbiological growth on aerobic count and Staph express plates, and no growth on coliform count

[6]Microbiological growth on aerobic count plate, no growth on coliform count and Staph express plates

[7]Microbiological growth recorded on aerobic count plate and no growth recorded on coliform count plate coupled with either positive or negative growth on the Staph express plate

bacteria, the specificity (and similarly the positive predictive value) was influenced by those samples which had a positive result based on Petrifilm™, but that [had] a negative result for coliforms from the mastitis lab. This occurred in a total of 18 (just over 10%) samples evaluated in this study.

Discussion

General principles for treatment of clinical mastitis include early detection of the disease, making a presumptive diagnosis of the pathogen, and formulating knowledge into the probability of a successful outcome with treatment. The key component to achieving these principles is quick and accurate diagnosis of the causative pathogen, or at least its categorization into either a Gram positive or Gram-negative group. This categorization has been the focus of on-farm culture research, and is beneficial for building mastitis treatment protocols (Sears and McCarthy, 2003). The only situation where there would not be any need to determine the causative organism of clinical mastitis would be if all treatment and prevention programs and outcomes were identical (Schukken and Schulte, 2004). Mastitis treatment protocols which incorporate therapy decisions based only on the Gram category of the causative agent, and ultimately restrict intramammary antibiotic treatment to only gram positive infections has been shown to substantially decrease, the number of days of lost production and the amount of antibiotic used, without endangering the general health, udder health or milk quality status of the herd (Hess *et al.*, 2003).

In this study Petrifilm™ was observed to have an excellent sensitivity and specificity for detecting coliforms (0.93 and 0.86, respectively). It is noteworthy that 18 of 44 Petrifilm™ plates were false positive for coliform organisms. The positive predictive value was decreased due to these samples that were positive Petrifilm™, but did not result in any coliform growth when shipped to the mastitis lab. One reason for this may be due to the freezing of the samples destined for the lab, whereas all Petrifilm™ samples were plated as

refrigerated fresh samples. Studies examining the effect of freezing in the handling of milk samples have reported that freezing is an important consideration for loss of coliforms, as well as increased recovery of S. aureus (Godden *et al.*, 2002)

Test characteristics were also calculated for identification of environmental streptococci, all gram-positive organisms, and for Staph spp. The sensitivity of the Petrifilm™ plates for environmental streptococci was 58%. This result was not totally unexpected, as there is no specific Petrifilm™ plate to differentiate streptococci. Furthermore, in this study, a very liberal definition of Strep spp was used based on interpretation of three specific Petrifilm™ plates. Still, this category was created and examined given the expected higher cure rate of streptococci relative to other organisms. The comparison between the two tests to detect the broad category of bacterial growth, revealed Petrifilm™ suffered from a low specificity (27%). 38 out of 52 positive plates were false positive for growth. This again could be due in part to the potential effect freezing had on coliforms. In addition, there is a large difference in the volume of milk used between the two tests. Finally, this difference could be due to inappropriate aseptic culturing technique and interpretation of Petrifilm™ plates in the field. The technician performing the culture techniques on Petrifilm™ was trained, and had experience based on weekly volume of samples submitted to the veterinary clinic. Reader variability in interpreting positive colonies on Petrifilm™ plates is an important consideration in interpreting results, especially for Staph express plates (Silva *et al.*, 2004).

Previous research has documented Petrifilm™ was excellent for differentiating Staphylococcus aureus (Silva *et al.*, 2004). This study did not use the full capabilities of the Staph express plate to differentiate S. aureus, but rather recorded staph spp growth. Even at this level, Petrifilm™ exhibited good test characteristics. If there was a significant effect of freezing among the samples used in this study, and if there was a high true prevalence of S. aureus, one would expect that the mastitis lab would have recovered more S. aureus form frozen samples than the Staph express plate on fresh samples (Godden *et al.*, 2002). There were 8 out of 56 Petrifilms that were false negative, but the apparent prevalence of Staph spp. was higher using PetrifilmTM (42%) than was estimated by the lab (36%).

Conclusions

In summary, on-farm culture systems that can be incorporated into mastitis treatment protocols will continue to be an active area of study. The core principle of mastitis treatment protocols begins with differentiating mastitis causing organisms into either Gram positive or Gram negative categories. User-friendly culture systems that can accurately make this distinction in a short enough time to delay commencement of therapy, will result in reduced amount of antibiotics used on farm, reduced discarded milk from clinical mastitis cases, and ultimately reduce the cost of the disease.

Petrifilm™ media plates represent a very user-friendly culture system, which can confirm growth of coliform bacteria within 24 hours. Compared to routine laboratory confirmation, Petrifilm™ had an excellent sensitivity and specificity for diagnosing coliform pathogens as the cause of producer defined cases of clinical mastitis. Petrifilm™ also performed favorably for detection of Gram positive growth and Staph spp. Growth. This on-farm diagnostic tool offers considerable potential for the implementation of therapy protocols, which could guide appropriate treatment of clinical mastitis cases.

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Testing for *Staphylococcus aureus* in herds with a low bulk milk somatic cell count

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Abstract

The validity of single quarter milk samples for culturing *Staphylococcus aureus* was quantified by comparing the results of single samples with intramammary infections (IMI) as defined by the National Mastitis Council (NMC). NMC definitions are based on consecutive samples. During a 20-month study, all lactating cows in five herds with a low bulk milk somatic cell count (BMSCC) were sampled at regular intervals to get a detailed insight in the bacteriological status of these cows. Comparison of the results of single samples with these detailed findings showed that the results of single samples culturing negative on *S. aureus* were reliable (predictive value negative 0.99). The results of single samples positive on *S. aureus*, however, were less reliable. Single samples, positive on *S. aureus*, from cows without an increased SCC, or that had had a SCC > 200,000 only once, very often did not come from quarters in which *S. aureus* was found in subsequent samples (predictive value positive (PV+) 0.54, respectively, 0.41). PV+ approximately doubled, when cows were selected that had had an increased Somatic Cell Count (SCC) during two or more consecutive milk recordings (PV+ 0.82). The consequences of these findings on selecting cows with subclinicial *S. aureus* mastitis for treatment or culling are discussed. It was concluded that in low BMSCC herds the validity of bacteriological culturing increases when the information of consecutive measured SCC is used in selecting cows for bacteriological culturing.

Introduction

Staphylococcus aureus can be the cause of chronic udder health problems in dairy herds. Generally, these herds have an increased Bulk Milk Somatic Cell Count (BMSCC). However, in herds with a low BMSCC, *S. aureus* can also be found and spreading of this pathogen should be prevented. Two factors are of major influence on the spread of *S. aureus* in a herd: the number of infected quarters (being the main source of infection) and transmission: the average number of quarters newly infected by one infected quarter (Lam *et al.*, 1996). Transmission of intramammary infections (IMI) with *S. aureus* can be influenced by management. The number of infected quarters, at any given point in time, can be influenced by treating or culling cows. Before decisions can be made on culling or treating cows because of mastitis, diagnosis of infected quarters is necessary. Early diagnosis of *S. aureus* infections is helpful in taking the correct decisions on treatment or culling (Owens *et al.*, 1997).

Udder health monitoring at the cow level is mainly done with the help of registration of clinical cases and with measuring individual cow somatic cell count (SCC). Additionally, samples can be collected for bacteriological culturing (BC). Since especially *S. aureus* has the characteristic of irregular shedding of bacteria (Sears *et al.*, 1990), consecutive samples

for BC should be taken for reliable diagnosis. In practice, however, for economical reasons usually only single samples are available. The aim of this study is to compare the results of these single samples with those of consecutive samples, to quantify reliability of single samples for BC in diagnosing *S. aureus*.

Materials and methods

During a 20-month study, 5 dairy herds with a low BMSCC were followed intensively. The herd size was on average 58.5 cows, with a production of 9604 kg FCM. Single quarter foremilk samples were collected every 5 to 6 weeks from the quarters of all lactating cows. The farmers collected samples from cows calving during the trial, within seven days of calving, from cows at drying off, and from cows showing signs of clinical mastitis. Individual cow SCC was measured during the regular milk recordings that were done every 4 weeks. Bacteriologic procedures were performed according to National Mastitis Council (NMC) standards (Harmon *et al.*, 1990).

A quarter was considered being infected with *S. aureus*, when ≥500 cfu/ml of *S. aureus* were cultured from two of three consecutive milk samples, or when ≥100 cfu/ml of *S. aureus* were cultured from three consecutive milk samples, or when ≥100 cfu/ml of *S. aureus* were cultured from a single sample from a quarter showing signs of clinical mastitis (Hogan *et al.*, 1990). The IMI was considered to be ended when *S. aureus* was not cultured from at least two consecutive samples (Roberson *et al.*, 1994). Qualifications according to these standards were considered to be as close to the truth as possible and were used as 'gold standard' in our analysis.

The results of the BC of single samples were compared with the 'gold standard'. A single quarter sample was considered positive if ≥100 cfu/ml of *S. aureus* were cultured. The prevalence of infected cows, according to the gold standard, was calculated.

	Gold standard	
Single samples	Infected with *S. aureus*	Not infected with *S. aureus*
S. aureus found	True positive	False positive
S. aureus not found	False negative	True negative

In the analysis all data were handled as independent. The sensitivity of a single sample was defined as the proportion of samples that were infected with *S. aureus* according to the gold standard, in which *S. aureus* was found. The specificity of a single sample was defined as the proportion of samples that were not infected with *S. aureus* according to the gold standard, in which *S. aureus* was not found. The predictive value positive (PV+) was defined as the portion of single samples in which *S. aureus* was found, that were infected with *S. aureus* according to the gold standard. The predictive value negative (PV-) was defined as the portion of single samples in which no *S. aureus* was found, that were not infected with *S. aureus* according to the gold standard (Martin *et al.*, 1987). Confidence intervals were calculated using an exact method (Conover, 1980).

The analysis was done at several levels. First all samples were included in the analysis. Secondly, single samples of cows with an individual SCC > 200,000 at the last available

milk recording were selected. Finally, single samples were selected of cows that had had an individual SCC > 200,000 during at least the last two consecutive milk recordings.

Results

When the data of all cows were evaluated, sensitivity and specificity of the results of BC of a single milk sample were high, 0.92 and 0.98 respectively (Table 1). Selecting cows with an increased or repeatedly increased SCC, increased the prevalence of *S. aureus* IMI among these cows, but hardly influenced sensitivity and specificity. There was no significant difference, with or without selection of cows based on SCC. The only exception was the specificity in cows with SCC> 200,000, which was significantly lower than in other situations.

With the generally low prevalence of *S. aureus* in low BMSCC herds, PV+ was low; 0.54 without selection of cows and 0.41 after selection based on a single SCC measurement. After selection of cows based on a repeatedly increased SCC values, PV+ increased to 0.82.

PV - was high in all situations. Thus, when a single BC is negative on *S. aureus*, it is unlikely that the gold standard reveals another result. Even in cows with a repeatedly increased SCC, PV - is as high as 0.98. Confidence intervals were found to be very small in all situations.

Table 1. Validity of single quarter milk samples in diagnosing intramammary Staphylococcus aureus infections

	All cows	Cows with SCC > 200,000	Cows with repeated SCC > 200,000
Prevalence	4.6% (n=513)	19.4% (n=282)	12.5% (n=200)
Sensitivity (95% CI)	0.92 (0.90; 0.95)	0.91 (0.88; 0.95)	0.90 (0.86; 0.95)
Specificity (95% CI)	0.98 (0.98; 0.98)	0.87 (0.86; 0.89)	0.97 (0.97; 0.98)
Pred. Val. Pos. (95% CI)	0.54 (0.51; 0.58)	0.41 (0.38; 0.46)	0.82 (0.78; 0.88)
Pred. Val. Neg. (95% CI)	0.99 (0.99; 1.00)	0.99 (0.99; 0.99)	0.98 (0.98; .0.99)

Discussion

Staphylococcus aureus is known for the fact that it sometimes is hard to culture this pathogen from infected quarters. High as well as low shedding cows have been described (Sears *et al.*, 1990). Methods to improve culture results have been studied (i.e. Schukken *et al.*, 1989). False-negative tests are particularly thought to be a problem in dairy practice: cows with an increased individual SCC and a negative BC often are interpreted as *S. aureus* infected. The results of this study, however, show that in herds with a low BMSCC, negative BC results for *S. aureus* in single quarter samples are reliable. A negative BC on *S. aureus* was found to be only slightly less reliable than when three or more consecutive samples were cultured, as was done in the gold standard we used. Even in cows with a repeatedly increased SCC, PV - was 0.98. Of course, the gold standard may be wrong and it may be that cows in which no *S. aureus* has been found in several consecutive samples, still are infected. However, when a single sample is found negative on *S. aureus*, increasing the number of samples from a quarter does not improve validity of results. It may be valid to question the importance of improving culture methods for *S. aureus*. In reality only cows actually shedding *S. aureus* play a role in transmission. Via liners, and probably via other

transmission routes too, cows shedding *S. aureus* are a higher risk for other cows in the herd than are cows that are not shedding (i.e. O' Shea, 1987). It may therefore be useful to discriminate between cows shedding or not shedding *S. aureus*. This, however, has to be studied further.

False positive tests for *S. aureus* are not considered an issue in daily practice. If *S. aureus* is cultured, the results are rarely questioned. From a bacteriological point of view we assume that this is a correct finding. It is not likely that bacteria that are interpreted as *S. aureus* in a certified lab, in fact are other bacteria. Samples may be false positive due to contamination, but this is unlikely to play a major role for *S. aureus* (Lam *et al.*, 1997). However, when the results of single quarter BC samples of cows with an increased SCC are compared with results of consecutive samples, only 41% of positive *S. aureus* samples in single samples are actually part of an IMI. This means that these quarters, that did not show signs of clinical mastitis, were negative on *S. aureus* on the subsequent samples. Thus, in low BMSCC herds, 6 out of 10 single milk samples in which *S. aureus* was found, were negative 5 weeks later.

If cows were selected that had had a repeatedly increased SCC, the validity of a single sample increases remarkably, PV+ was estimated at 82%. Thus, when *S. aureus* is cultured from a cow that had a SCC repeatedly > 200,000, it is likely that this infection is part of a chronic *S. aureus* IMI.

In low BMSCC herds apparently quite a number of short-term *S. aureus* infections occur. This may be due to early treatment or early culling of cows with subclinical IMI. However, this was not the case in the herds studied. The most likely explanation is that in these herds, many cows cure spontaneously. This is in accordance with, and may partly explain the results of Owens *et al*. (1997). They found cure rates in newly acquired IMI (< 2-weeks duration) that were twice as high as in chronic IMI (> 4-weeks duration). Thus, treating cows with subclinical *S. aureus* IMI in cows with a SCC that has been higher than 200,000 only once, will be very successful. This partly can be attributed to self-cure. Waiting longer probably will decrease unnecessary use of antibiotics, but will also decrease treatment success. Optimal timing of treatment of these subclinical IMI in relation to high cure rate, efficient use of antibiotics and minimal spread of infection, has to be studied further.

In our analysis we defined quarters as either infected with *S. aureus* or not infected with *S. aureus*. A quarter defined as not infected with *S. aureus* is handled as negative, but may be infected with another pathogen. Other pathogens causing an increase of SCC, like Gram-positives, played a minor role in these herds. As mentioned, the data were handled in the analysis as if fully independent, which they are not. Therefore the estimated variability in the quantitative results may not be exactly correct. The results from this study can not be extrapolated to herds with a higher BMSCC. We did not have the data to evaluate the validity of a single BC in that type of herds.

The general conclusion is that in low BMSCC herds the validity of BC increases when the information of consecutive measured SCC is used in selecting cows for BC.

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Simultaneous detection of mastitis pathogens in milk by multiplex real-time polymerase chain reaction

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Abstract

The objective was to develop a multiplex real-time polymerase chain reaction (PCR) for simultaneous detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* directly from milk. Milk samples (n=192) were analyzed by multiplex real-time PCR assay and conventional microbiological methods. An additional 57 quarter milk samples were analyzed in a separate real-time PCR assay for *Strep. agalactiae* only. The multiplex real-time PCR technique correctly identified 97.7% of all quarter milk samples, 91% of *Staph. aureus,* 98% of *Strep. agalactiae*, and 100% of *Strep. uberis*. The overall sensitivity of the multiplex real-time PCR assay to correctly identify *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk was 95.5% and the specificity was 99.6%. Results of this study indicate that multiplex real-time PCR procedure is a rapid and accurate method for simultaneous identification of *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* directly from quarter milk samples.

Keywords: multiplex real-time PCR, *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*

Introduction

Mastitis is the most common infectious disease affecting dairy cows in the world and is the most economically important disease of the dairy industry. Several bacteria are implicated including *Staph. aureus, Strep. agalactiae* and *Strep. uberis* (Oliver *et al.*, 1997). Identification of a mastitis pathogen in milk is regarded as the definitive diagnosis of intramammary infections. Identification of bacteria in most diagnostic laboratories is currently based on analysis of phenotypic characteristics utilizing biochemical tests, serotyping and enzymatic profiles. Many diagnostic and research laboratories are beginning to realize the potential advantages of DNA-based assays for identification of bacteria. The main advantage to DNA-based diagnostic assays is that these methods focus on the unique nucleic acid composition of the bacterial genome rather than phenotypic expression of products that nucleic acids encode. Therefore, DNA-based identification systems are likely to result in methods that are more accurate and targeted for a specific pathogen, allow for effective screening of a large number of pathogens simultaneously, and provide definitive confirmation of pathogens. Real-time PCR utilizes fluorescence for detection of PCR products instead of gel electrophoresis and further decreases turn-around time for bacterial identification. Real-time PCR utilizes the 5'-3' nuclease activity of *Taq* DNA polymerase to digest an internal fluorogenic probe labeled with both a fluorescent reporter dye and a

fluorescent quencher dye (Cai *et al.*, 2003). During amplification, the probe is hydrolyzed relieving the quenching of the reporter dye resulting in an increase in fluorescent intensity. This change in reporter dye fluorescence is quantitative for the amount of PCR product, and under appropriate conditions, for the amount of template. The objective of the present study was to develop a multiplex real-time PCR method to simultaneously detect common mastitis pathogens including *Staph. aureus*, *Strep. agalactiae,* and *Strep. uberis* directly from milk.

Materials and methods

Milk samples

Quarter milk samples for microbiological evaluation were collected prior to milking using standard procedures (Oliver *et al.*, 2004). Milk samples were transported on ice, frozen and maintained at -20°C until analysis. Milk samples were examined for bacteriological growth following procedures described by Oliver *et al.* (2004).

Bacterial strains

The multiplex real-time PCR assay was developed using American Type Culture Collection (ATCC, Manassas, VA) reference strains of *Staph. aureus (*ATCC 10832), *Strep. agalactiae (*ATCC 27956), and *Strep. uberis (*ATCC 27958). A total of 53 different ATCC reference strains were used in the cross-reactivity study including: 11 Staphylococci, 9 Streptococci, 4 Enterococci, 1 *Aerococcus* strain, 5 *Listeria*, 2 *Pseudomonas* strains, and 21 from the Enterobacteriaceae family. An additional 25 CAMP-positive *Strep. uberis* strains were used for cross-reactivity with *Strep. agalactiae* specific primers and dual-labeled probe that targeted the *cfb* gene encoding Christie-Atkins-Munch-Petersen (CAMP) factor.

DNA isolation

Bacterial DNA was extracted directly from reference strains and CAMP-positive *Strep. uberis* strains using the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) following manufacturer's recommendations. A 5 µl volume was used as DNA template for the multiplex real-time PCR assay. For isolation of bacterial DNA directly from milk, the method described by Allmann *et al.* (1995) was used with modifications (Hein *et al.*, 2001). Milk samples (one ml) were enriched with tryptic soy broth (one ml, Becton Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C overnight. One ml of enriched sample was mixed with 130 µl digestion buffer (100mM Tris, 100mM EDTA, 0.5% SDS, pH 8.0) and digested with 100 µl pronase (10mg/ml; Sigma-Aldrich, St. Louis, MO) at 40°C for 3 h. Bacterial cells were pelleted by centrifugation at 2,500 x *g* for 5 min at 4°C and the fat layer and aqueous phase discarded. The pellet was washed 2 to 3 times with TE buffer (10mM Tris, 1mM EDTA, pH 7.5). Lysozyme (100 µl, 2.0 mg/ml; Sigma-Aldrich) was added to the pellet, and incubated for 15 min at room temp. After incubation, 10 µl proteinase K (20 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN) was added to each tube and incubated at 60°C for 45-60 min. Sample was vortexed and incubated at 95°C for 15 min, followed by centrifugation at 16,000 x *g* for 5 min at 4°C to remove cell debris. The supernatant (5 µl) was used as DNA template for the multiplex real-time PCR assay.

Primers and dual-labeled probes

Primers and dual-labeled probes were designed using Beacon Designer 2.1 (Premier Biosoft International, Palo Alto, CA) and purchased from IDT (Coralville, IA). For detection of *Staph. aureus*, a genetic marker specific for *Staph. aureus* was designed based on primers used by Reischl *et al.* (2000). For *Strep. agalactiae*, the *cfb* gene encoding CAMP factor was the target for *Strep. agalactiae* primers and probe (Ke *et al.*, 2000). For *Strep. uberis*, the plasminogen activator gene described by Sazonova *et al.* (2001) was the target. The sequence for each primer pair and dual-labeled probe are as follows:

- SaF: TCAACG ATATTCTTCACGACTAA;
- SaR: CCAGCTTCGGTACTACTAAAG;
- SaProbe: 5`FAM-TCAAGACGGCTTTTACATACAGAACACA-3`BHQ2;
- SagF: AGCTCTATTAGAAGTA CATGCT;
- SagR: CATTTGCTGGGCTTGATTATT;
- SagProbe: 5`TexasRed ATCAAGT GACAACTCCACAAGTGGTAA-3`BHQ1;
- SubeF: AGAGGAATTCATCATGTTTTAAC A;
- SubeR: AATTGTAGAAGAACCATTTGATGT;
- SubeProbe: 5`CY5-AGCGTCTAACA ACTCGGCCTTTG-3`BHQ2.

Real-time PCR assay

The iQ Supermix (Bio-Rad, Hercules, CA) was used for the multiplex real-time PCR assay. Added to the mixture were 2 µl 50mM $MgCl_2$, and 200 nM each primer and dual-labeled probe. The iCycler iQ Real-Time PCR detection system (Bio-Rad) was programmed for 95°C for 2 min followed by 40 repeats of 95°C for 15 sec and 57°C for 45 sec. The fluorescence for each probe was measured during the 45 sec hold at 57°C. For each sample, a cycle threshold (C_T) was calculated based on baseline cycles and threshold value which is 10 times the mean standard deviation of fluorescence in all wells over the baseline cycles.

Detection limit of multiplex real-time PCR assay from pure culture

Overnight cultures of *Staph. aureus* (ATCC 10832), *Strep. agalactiae (*ATCC 27956), and *Strep. uberis* (ATCC 27958) were used to prepare 10-fold serial dilution in Ultra High Temperature (UHT) milk. Bacterial DNA was extracted directly from milk as described previously. The DNA from 10-fold dilutions was used as template for determining the sensitivity of the multiplex real-time PCR assay.

Enrichment vs. non-enrichment of milk samples

Milk samples (n=20) were evaluated to determine if enrichment of milk samples was necessary for detection of pathogens in low numbers. For enriched samples, one ml tryptic soy broth (Becton Dickinson and Company) was added to one ml of milk, mixed and incubated overnight at 37°C. After incubation, one ml of enriched sample was used for isolation of bacterial DNA directly from milk as described previously. For the non-enriched sample, one ml of milk was used for bacterial DNA isolation as described previously. Conventional bacterial methods were conducted on the 20 samples to determine bacteria identification and colony forming units (cfu)/ml. Results were compared to determine sensitivity of real-time PCR assay to identify bacteria from milk samples of enriched vs. non-enriched samples.

Results and discussion

Specificity of PCR primers and probes

For cross-reactivity evaluation, DNA extracted from 53 type strains, and 25 *Strep. uberis* CAMP-positive strains were examined. Only *Staph. aureus* strains tested positive in the real-time PCR assay with primers and probes specific for *Staph. aureus* and produced mean C_T values of 19.9 ± 0.9. For primers and probe specific for *Strep. uberis,* only *Strep. uberis* strains were positive in the real-time PCR assay and produced mean C_T values of 11.3 ± 0.5. Only *Strep. agalactiae* strains tested positive in the real-time PCR assay with primers and probes specific for *Strep. agalactiae* and produced mean C_T values of 16.1 ± 0.5. Amplification of DNA from other bacterial strains evaluated either resulted in no fluorescence or a mean C_T value of > 30. Primers and probes were specific for the target organism with real-time PCR and did not show any cross-reactivity with other bacterial species.

Sensitivity and detection limits in pure cultures

The minimum level of detection for Staph. aureus (ATCC 10832) was 103 cfu/ml in UHT. For Strep. agalactiae (ATCC 27956) and Strep. uberis (ATCC 27958), the minimum level of detection was 10^2 cfu/ml in UHT. After addition of an enrichment step, the minimum level of detection was lowered to 10^0 cfu/ml for Staph. aureus, Strep. agalactiae and Strep. uberis.

Enrichment vs. non-enrichment of milk samples

Twenty milk samples were chosen that contained *Staph. aureus* and/or *Strep. uberis* with quantities ranging from 200 to >10,000 cfu/ml. Of the 20 milk samples, 10 were *Staph. aureus*-positive and 11 were *Strep. uberis*-positive (one milk sample was positive for both *Staph. aureus* and *Strep. uberis*). The real-time PCR assay using enriched milk samples was able to detect 9 of 10 *Staph. aureus* samples with a mean C_T value of 21.5 and 11 of 11 *Strep. uberis* samples with a mean C_T value of 19.3. Using non-enriched samples, the real-time PCR assay was only able to detect 5 of 10 *Staph. aureus* samples (mean C_T value of 32.1) and 10 of 11 *Strep. uberis* samples (mean C_T value of 30.3). The one *Staph. aureus* milk sample that was negative by both enrichment and non-enrichment was positive for *Staph. aureus* by real-time PCR when DNA was isolated from the bacteria. This particular sample also contained *Strep. uberis*. Results indicate that enrichment is needed to detect low numbers of bacteria in milk and to dilute some of the inhibitory substances present in milk.

Milk samples

A single real-time PCR assay for *Strep. agalactiae cfb* gene was performed on 57 quarter milk samples previously identified as *Strep. agalactiae*-positive by conventional methods. Of these isolates, 56 were positive for the *Strep. agalactiae cfb* gene and one isolate was negative. The 57 quarter milk samples were used to develop the *Strep. agalactiae* real-time PCR assay and the remaining milk sample was not of sufficient quantity to be utilized in evaluation of the multiplex real-time PCR assay.

Milk samples (n=192) previously screened by conventional microbiological methods were evaluated by the multiplex real-time PCR method for detection of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis*. This assay correctly identified 97% (188/192) of all quarter milk samples, 91% of *Staph. aureus*, 98% of *Strep. agalactiae*, and 100% of *Strep. uberis*

(Table 1). The overall sensitivity of this procedure to correctly identify *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* directly from milk was 95.5% and the specificity was 99.6%. This assay was able to detect target pathogens even though the sample contained more than one type of bacteria. A total of 44 (Table 1) milk samples contained two different bacteria. The multiplex real-time PCR assay was able to identify all (15/15) milk samples that contained *Strep. uberis* mixed with other bacteria including coagulase-negative staphylococci (CNS), *Staph. aureus*, *Strep. dysgalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia liquefaciens* (Table 1). The multiplex real-time PCR assay identified 10 of 13 milk samples that contained *Staph. aureus* mixed with other bacteria including CNS, *Cornyebacterium bovis*, *Streptomyces* species, *Enterococcus faecalis*, and *Strep. dysgalactiae* (Table 1). The assay was also able to differentiate *Staph. aureus* from other coagulase-positive staphylococci (CPS). Five CPS isolates were negative by both real-time PCR of the bacterial isolate for *Staph. aureus* and by the multiplex real-time PCR directly from milk.

Little is known about components present in milk that might inhibit/interfere with PCR. Two *Staph. aureus* isolated in milk from cows with mastitis were repeatedly negative for *Staph. aureus* by multiplex real-time PCR, however, analysis of DNA extracted from the isolated bacteria alone produced positive results. Both of these samples were from mammary quarters with clinical mastitis, suggesting the presence of a unique PCR inhibitor(s). These

Table 1. Evaluation of quarter milk samples by multiplex real-time PCR for detection of Staphylococcus aureus, Streptococcus agalactiae, *and* Streptococcus uberis.

		S. aureus		*S. uberis*		*S. agalactiae*	
Bacteria	No.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CNS[1]	26	1[2]	25	0	6	0	26
CNS/*S. uberis*	7	0	7	7	0	0	7
CNS/*S. aureus*	4	3	1	0	4	0	4
CNS/ *S.dysgalactiae*	2	0	2	0	2	0	2
CPS[3]	5	0	5	0	5	0	5
S. aureus	59	56	3	0	59	0	59
S. aureus /other bacteria[4]	6	6	0	0	6	0	6
S. aureus /*S. uberis*	3	1	2	3	0	0	3
S. dysgalactiae /other bacteria[5]	17	0	17	0	17	0	17
Negative[6]	10	0	10	0	10	0	10
S. uberis	48	0	48	48	0	0	48
S. uberis / *S. dysgalactiae*	2	0	2	2	0	0	2
S. uberis / GNR [7]	3	0	3	3	0	0	3
Total	192	67	125	63	129	0	192

[1]Coagulase negative *Staphylococcus*.

[2]CNS positive for *Staph. aureus*, C_T= 20.6.

[3]Coagulase positive, mannitol negative, DNase negative *Staphylococcus*.

[4]*Cornyebacterium bovis*, *Streptomyces* species, *Enterococcus faecalis* and *S. dysgalactiae*.

[5]*Streptococcus bovis, Enterococcus* species and *Lactococcus* species.

[6]No growth.

[7]*Escherichia coli*, *Klebsiella pneumoniae* and *Serratia liquefaciens*.

findings suggest that PCR inhibitors were present in the original sample, but not removed by either the DNA isolation procedure or enrichment step.

Addition of an enrichment step has been reported and appears to be necessary for detecting low numbers of bacteria (<1000 cfu/ml). Meiri-Bendek *et al*. (2002) developed a PCR method for detection of *Strep*. *agalactiae* in milk that targeted the conserved areas within the 16S rRNA. The sensitivity of this assay increased from 10^4 - 10^5 cfu/ml to one cfu/ml after overnight selective enrichment. Phuektes *et al*. (2003) needed enrichment in a multiplex PCR for detection of *Staph*. *aureus*, *Strep*. *agalactiae*, *Strep*. *dysgalactiae* and *Strep*. *uberis* to detect levels of one cfu/ml.

Conclusions

The assay described here is the first to utilize a multiplex real-time PCR for detection of pathogens directly from milk. The multiplex real-time PCR technique correctly identified 97.7% of all quarter milk samples, 91% of *Staph*. *aureus,* 98% of *Strep*. *agalactiae*, and 100% of *Strep*. *uberis*. Results indicate that the multiplex real-time PCR procedure is a rapid and accurate method for the simultaneous detection of *Staph*. *aureus*, *Strep*. *agalactiae* and *Strep*. *uberis* directly from milk samples.

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Optimised sample sizes for analysing the genetic heterogeneity of mammary pathogen isolates from environmental samples

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Abstract

Modern molecular techniques and more efficient culture techniques, which may be automated in the future, allow for characterization of increasingly large numbers of micro-organisms. Very often, the number of bacterial isolates analysed per sample is determined by habit, convenience, laboratory capacity or financial resources. If too few isolates are analysed per sample, information will be missed, while analysis of too many isolates is a waste of resources. Statistical considerations and knowledge of the heterogeneity of mammary pathogens, e.g. *Streptococcus uberis*, in milk and the environment should lead to optimised sample sizes, i.e. the number of isolates from one bacterial species that needs to be characterized per milk or environmental sample. Calculation of the optimal sample size for multiple isolates originating from one clinical, food or environmental sample is a common problem in microbiology and survey design. As an example, we present data for *S. uberis* isolated from the environment and characterized by automated ribotyping with *PvuII*. Based on a Bayesian mode of inference, results show that approximately 20 isolates of *S. uberis* per sample should be ribotyped in order to find all existing ribotypes in soil samples with a probability of 95%. This high number of isolates per sample is probably never tested in real-life due to financial restrictions, showing that investigators are not gaining maximal information on strain heterogeneity from the samples analysed.

Keywords: sample size, Bayesian inference, multiple isolates, S. uberis, environment

Introduction

The idea that more than one geno- or pathotype of the same bacterial species of mammary pathogen can be isolated from one milk or environmental sample is gaining ground and co-existence of multiple strains within a sample is becoming an important factor in studies that try to elucidate the pathogenesis of bovine intramammary infections (McDougall *et al.*, 2004; Oliver *et al.*, 1998; Young *et al.*, 2001). An important mastitis pathogen that shows large genetic heterogeneity in milk samples and that is found in the environment is *Streptoccus uberis* (McDougall *et al.*, 2004; Zadoks *et al.*, 2005a)

Altekruse *et al.* (2003) and Singer *et al.* (2000) have reported solutions for calculating the number of bacterial isolates that are to be genotyped in order to find all different strains of bacteria present in one sample using a Bayesian mode of inference. This type of

"occupancy problem" can be solved for *S. uberis* isolated from the environment of dairy cows. In this paper, we present a sample size calculation that is an example of application of Bayesian methods to surveys aimed at detecting different strains of *S. uberis* in environmental samples. The same approach can be applied to similar surveys involving other mammary pathogens or food-borne pathogens occurring in the dairy environment.

Materials and methods

Data

Twenty nine samples containing soil (e.g. from doorway, lying area in pasture, gathering area around water tub or under trees) were collected from the environment of dry cows at ten monthly herd visits to one New York State dairy farm in 2003. Per sample, between 1 and 8 *S. uberis* isolates were used for automated *Pvu*II ribotyping (median = 3). For nine samples, more than four isolates were ribotyped. The sample description, the number of isolates used for ribotyping, and the number of ribotypes detected is summarized in Table 1.

This corresponds to an average of 5.89 (SD: 1.17) isolates analysed per sample and on average 3.89 (SD: 1.05) different strains per sample. The sample collection, isolation and characterization methods for *S. uberis* are described in detail elsewhere (Zadoks *et al.*, 2005a).

Table 1. Sample overview.

Sample ID	Sample description	Number of isolates	Number of ribotypes
1-3	Mud from doorway to dry-cow barn	5	4
2-6	Pasture soil from cow lying area 1	5	3
3-3	Mud around drinking trough no. 1	5	4
3-4	Mud around drinking trough no. 2	7	2
3-5	Pasture soil from cow lying area no. 2	8	5
3-6	Pasture soil from cow lying area no. 3	7	4
6-5	Mud around drinking trough no. 3	6	5
8-9	Mud around drinking trough no. 4.	5	3
9-5	Pasture soil from cow lying area no. 4	5	5

The statistical model

The problem of finding all strains of *S. uberis* that are present among multiple isolates originating from one sample involves the probability of observing j strains, when i strains are truly present in the sample. This relates to the so-called "occupancy problem" in statistics. A uniform Dirichlet distribution was specified as a prior distribution for the marginal probabilities θ_i for the true number *i* of strains (*i* = 1...*k*) of *S. uberis* in a random sample:

$$(\theta_1....\theta_k)' \sim Dirichlet(1/k...1/k) \quad (1)$$

Expert opinion was used to assume that a maximum of *k* = 5 different strains may occur in a sample. The probability of observing j strains while truly i strains are present in a sample can be obtained by simulation or direct calculation:

$$P_N(j|i) = \binom{i}{j} \sum_{r=0}^{j} (-1)^r \binom{j}{r} \left(\frac{j-r}{i} \right)^N . \quad (2)$$

Here, *N* is the number of isolates ribotyped, *i* is true number of strains in the sample and *j* is the number of strains that is actually observed. Expression (2) is based on the assumption that all strains in a sample are equally likely to be observed. The probability distribution for the data, i.e. number of strains x observed for n isolates ribotyped, follows from (1) and (2). Prior and data are combined in a Bayesian posterior analysis to evaluate the probability *p* of observing all the different strains in a sample, as a function of the number of isolates (*N)* that is ribotyped:

$$p(N) = \sum_{i=1}^{k} \theta_i P_N(i \mid i) . \quad (3)$$

The posterior mean and 95% credible interval of *p* were obtained by Markov chain Monte Carlo simulation, employing the Gibbs sampler as implemented in WinBUGS (Spiegelhalter *et al*., 2000, see also Congdon 2002). The probabilities in (2), for the numbers of isolates n in the data, were calculated with GenStat 5 (2000) and subsequently entered into the WinBUGS program. The probabilities $P_N(i \mid i)$ in (3) for a specified value *N* were evaluated within the WinBUGS program. The optimal *N,* e.g. *N* where the posterior mean of *p* from (2) equals 0.95, may be found by trial and error or may be chosen from a plot derived from a grid of values for *N*.

Results

Using the prior knowledge about the maximum number of strains truly present in one sample (k = 5) and the data from the field study, the posterior median probability of finding all of the *S. uberis* strains present per sample while analysing N isolates per sample is calculated including a 95% Bayesian confidence interval (credible interval). The results are

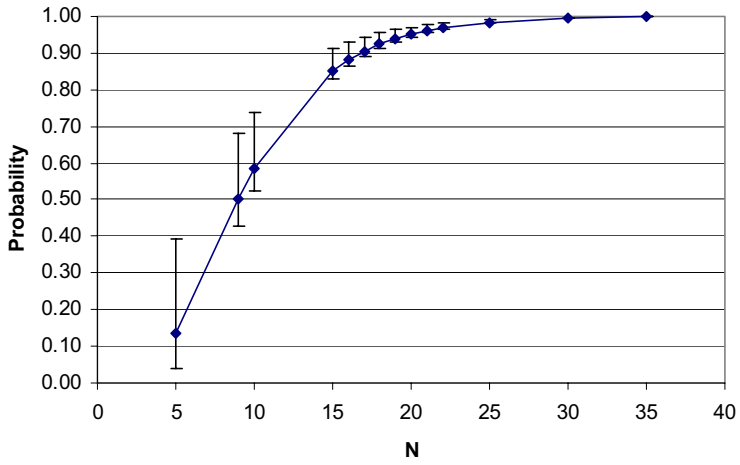

Figure 1. Probability p of finding all strains of Streptococcuccus uberis present in an environmental (soil) sample when ribotyping N isolates per strains (including 95% confidence intervals).

shown in Figure 1 where it can be seen that about 20 isolates would have to be analysed per sample in order to find all strains of *S. uberis* present with a probability of 95%.

Discussion

Ribotyping is a highly standardized but expensive procedure. Analysis of 20 isolates per sample is more than would be feasible in most surveys due to financial restrictions. Other strain typing methods exist. Random amplified polymorphic DNA (RAPD) based strain typing is less costly and more discriminatory than ribotyping for *S. uberis* (Gillespie and Oliver, 2004). In contrast to ribotyping, RAPD typing is not well-standardized so that comparison of results obtained for multiple samples in a large survey is problematic. Pulsed-Field Gel Electrophoresis is more discriminatory than ribotyping or RAPD typing (Gillespie and Oliver, 2004). With use of standardized experimental protocols and automated interpretation algorithms, results are more easily compared across studies than those of RAPD typing. Most recently, MLST has been used to characterize *S. uberis* isolates from milk and environmental samples (Zadoks *et al.*, 2005b). In contrast to the banding pattern-based methods (ribotyping, RAPD and PFGE) results are highly standardized and easily compared across studies (Enright and Spratt, 1999). As DNA-chips become more widely available and affordable, characterization of multiple bacterial isolates per sample using this method may become feasible.

If the prior had provided better information instead of a uniform Dirichlet distribution, the prior knowledge could have been more in agreement with the real-world a so-called better informed prior. A second data set could provide such a better informed prior. The current sample size calculation is a first step in streamlining the sample size of isolates analysed per sample for future surveys though. The stepwise process can optimise the allocation of resources to the ribotyping of *S. uberis* and other mammary or foodborne pathogens in microbiological surveys. This example is meant to call the attention to this type of reasoning when designing surveys aimed at finding all existing strains of mammary pathogens in a given sample. It incorporates different numbers of isolates analysed per sample, while previously reported examples of these calculations strictly used the same numbers of isolates analysed per sample (Altekruse *et al.*, 2003, Singer *et al.*, 2000).

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Somatic cell count patterns to improve udder health by genetics and management

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Abstract

Clinical mastitis (CM) is one of the major diseases in dairy herds. It induces economic costs, mainly consisting of discarded milk, increased health care costs and reduced milk quality. Mastitis also contributes to consumer concerns regarding animal welfare and regarding the impact of use of antibiotics in animals on efficacy of antibiotics for human health and the possible development of antibiotic resistant bacteria. Decreasing the incidence of CM is thus of great interest of the farmer, the cow and the consumer, and could be achieved by either designing mastitis control programs, as these provide guide-lines for udder health management, or by genetic selection. Although genetic selection is a slow process, it results in a steady change in the genetic composition of the dairy herd. This study provides insight in the use of patterns of peaks in somatic cell count (SCC) in genetic selection and mastitis control programs. Patterns of peaks in SCC were defined based on SCC recorded on consecutive test-day, and are based on biological understanding of pathogens and of the immune system of the cow. Results showed that selecting for lower lactation-average SCC caused a shift in the importance of the main mastitis-causing pathogen. Genetic selection against occurrence of SCC patterns, however, was more effective to decrease the natural susceptibility to all mastitis-causing pathogens, than selection for lower lactation-average SCC. Patterns of peaks in SCC are proven to be useful as basic tools for health management advice, as they can distinguish between cases of CM associated with either environmental or contagious pathogens, whereas the currently used primary traits were indicative for contagious, but not for environmental mastitis.

Keywords: somatic cell count patterns, genetic selection programs, farm management

Introduction

Average lactation values of somatic cell count (SCC) are generally used in mastitis control programs and for genetic improvement of udder health. However, these average values ignore variation in SCC during lactation. Curves for SCC during lactation decline to nadir before 60 days in milk, and increase during the remainder lactation (De Haas *et al.*, 2002). Clinical and subclinical mastitis can cause deviations from this typical curve of SCC, and it has been

shown that specific pathogens involved in cases of clinical mastitis (CM) affect the curve differentially (De Haas *et al.*, 2002).

Using test-day records of SCC instead of lactation-average SCC increases the possibilities to identify deviations from the typical curve of SCC during lactation. These deviations might characterise mastitis-causing pathogens. If so, analysing test-day records of SCC can be more useful in attempting to decrease the incidence of CM, than the lactation-average SCC. An effective use of SCC test-day records might be achieved by defining patterns of peaks in SCC during the lactation. If these patterns of peaks in SCC provide information on (1) the pathogen-distribution on a farm, and (2) the status of genetic resistance of the cows, these patterns might be better tools to use in mastitis control programs or for genetic selection programs. Management can then be directed specifically on lowering the incidence of pathogen-specific CM, or shortening the duration of infection. Furthermore, a decreased genetic susceptibility for the full scope of mastitis-causing pathogens might be accomplished. Eventually, this all helps to limit the losses due to CM.

Material and methods

Herds

Records on CM were available from a study carried out from December 1992 till June 1994 on 274 Dutch farms (Barkema *et al.*, 1998), resulting in 49,529 lactations that were recorded for at least one day. During the study period, farmers took milk samples from all quarters that, in their opinion, had clinical signs of mastitis. Samples were stored in a freezer at the farm (at approximately -20°C) and were collected for bacteriological examination at intervals of six to eight weeks. Two groups of pathogens were defined: 1) contagious pathogens (CONT_CM) (Fox and Gay, 1993), consisting of *Staph. aureus* and *Strep. dysgalactiae*, and 2) environmental pathogens (ENV_CM) (Smith and Hogan, 1993), consisting of *E. coli* and *Strep. uberis*. The national milk recording system (NRS, Arnhem, The Netherlands) provided information from a three- or four-weekly milk recording system. Somatic cell score (SCS = log2(SCC/100,000)+3) was averaged over the test-day records up to 150 and 305 days in lactation, respectively.

Definitions of SCC patterns

Patterns of SCC were used to distinguish lactations with short or longer periods of increased SCC, and also lactations with and without recovery from the increase in SCC. Healthy and recovered cows were assumed to have less than 200,000 somatic cells/ml (Dohoo and Leslie, 1991), and cows with intramammary infections were assumed to have more than 500,000 cells/ml (Lam *et al.*, 1997). Therefore, test-day recordings of SCC were categorised as low when <200,000 cells/ml, and when >500,000 cells/ml, the test-day recording of SCC was categorised as high.

Two patterns of SCC were defined based on these categories:

- The first SCC pattern (P1) is referred to as a "quick recovery pattern", and described consecutive test-day recordings of SCC that were low-high-low.
- The second pattern (P2) is referred to as a "no recovery pattern" and denoted a test-day with a low SCC followed by at least two test-days with high SCC.

The patterns were determined per cow (De Haas *et al.*, 2004). Only fully completed patterns were considered, and more than one pattern could have been present per cow.

For management purposes the SCC patterns will be compared to the currently used traits, which are (1) number of new infections (i.e. consecutively a low (≤250,000 cells/ml) and high (>250,000 cells/ml) test-day recording of SCC and (2) number of test-days with SCC above 250,000 cells/ml.

Statistical analyses

AS-REML (Gilmour *et al.*, 2002) was used to estimate variance components for SCC patterns, SCS150 and SCS305. The model included random effects for sire and maternal grandsire (MGS) and an effect for animal, to account for the permanent animal effects across repeated lactations. The model used was:

$$Y = \mu + \text{fixed effects} + S_{sire} + 1/2\ S_{mgs} + PERM_{animal} + e$$

The random sire effect was identified by the subscripts for sire and MGS; S_{sire} and S_{mgs}, respectively. The sire effects were linked using the relationship matrix, and were assumed to be normally distributed with $var(S_{sire\ or\ mgs}) = \sigma^2_s$. Permanent animal effects contain environmental effects common to different lactations and genetic effects not covered by sire and MGS, like a dam-component, dominance, and Mendelian sampling terms. This was assumed to be normally distributed as well, with $var(PERM_{animal}) = \sigma^2_{Ea}$. Fixed effects included were herd (with 274 levels), an interaction between year and season of calving (YS, with 43 classes), parity (with 4 classes, where the last class contains all parities ≥4), and the fraction of Holstein-Friesian genes (with 9 classes, for 0, 1/8, ..., 8/8). A polynomial of order 4 for age at calving was included.

To test if there was an association between SCC pattern frequency and the occurrence of CM, statistical analyses were carried out using logistic regression in SAS (PROC LOGISTIC; (SAS/STAT®, 2001)). Odds ratios (OR) were calculated between the 25% of herds with the lowest CM and the 25% herds with the lowest or highest ratio of SCC patterns. Similar analyses were performed with the 25% herds with the highest CM. The predictors indicated whether or not a herd was classified in the best or worst quartile of herds based on the SCC patterns.

Results

Heritabilities for patterns of peaks in SCC were 0.01 and 0.06 for P1 and P2, respectively. Heritabilities for lactation-average SCS were 0.07 and 0.10 for 150 and 305 days, respectively. Genetic selection purely on decreased lactation-average SCS will mostly decrease number of cases of *E. coli* and *Strep. uberis*, and not so much the number of cases of clinical *Strep. dysgalactiae* mastitis (Table 1). Genetic correlations between pathogen-specific CM and patterns of peaks in SCC differed per pathogen, and generally, they were generally stronger or equally strong than the correlations with lactation-average SCC (Table 1). Genetic selection on less presence of peaks in SCC would, therefore, decrease the incidence of pathogen-specific CM more effectively than selection on lower lactation-average SCC.

Table 1. Estimated genetic correlations from bivariate analyses between pathogen-specific clinical mastitis (CM), recorded in the first 450 days in lactation, lactation-average somatic cell scores, averaged over test-day records up to 150 and 305 days in milk (SCS150 and SCS305, respectively) and somatic cell count patterns[1], with their respective standard errors in subscripts.

	P1	P2	SCS150	SCS305
Clinical mastitis (CM)	$0.85_{0.18}$	$0.76_{0.13}$	$0.74_{0.09}$	$0.50_{0.13}$
Environmental CM				
Escherichia coli	$0.92_{0.25}$	$0.86_{0.14}$	$0.68_{0.17}$	$0.54_{0.20}$
Streptococcus uberis	$0.49_{0.58}$	$0.68_{0.41}$	$0.73_{0.37}$	$0.59_{0.40}$
Contagious CM				
Staphylococcus aureus	$0.66_{0.27}$	$0.50_{0.22}$	$0.53_{0.18}$	$0.26_{0.19}$
Streptococcus dysgalactiae	$0.27_{0.38}$	$0.40_{0.27}$	$0.28_{0.22}$	$0.18_{0.23}$

[1] P1:quick recovery pattern (low-high-low SCC); P2:no recovery pattern (low-high-high-high-high SCC).

The distribution of SCC patterns (relative to their maximum) varied among herds (Table 2). The distribution of these patterns was correlated with the incidence rate of CM. Herds with a relatively frequent "quick recovery pattern" had 2.5 times more chance of being classified in the upper quartile for CM. These herds also had 2.1 times more chance of being classified in the upper quartile for ENV_CM but only 0.4 times for CONT_CM. Herds with a relatively frequent "no recovery pattern" had less chance (OR = 0.5) of being classified in the lower quartile for CONT_CM. Since the distributions of SCC patterns were indicative for overall, environmental and contagious CM, the necessity to introduce pathogen-specific mastitis control programs in a herd could be determined based on occurrence of SCC patterns.

Discussion

Correlations of 0.74 and 0.50 were estimated between CM and SCS in the first 150 and 305 days, respectively. This suggests that selection for lower SCS, especially during early lactation, decreases the incidence of CM. However, genetic correlations between CM and

Table 2. Calculated odds ratios indicating the chance of being classified as one of the best (b) or worst (w) 25% herds for occurrence of clinical mastitis (CM), environmental CM (ENV_CM), and contagious CM (CONT_CM) when classified in the best (b) or worst (w) 25% of the herds with respect to the mean incidence rate of SCC patterns and currently used SCC traits[1].

	P1 (b)	P1 (w)	P2 (b)	P2 (w)	INF (b)	INF (w)	HIGH (b)	HIGH (w)
CM (b)	1.7	0.4	1.5	0.5	2.5*	0.8	1.6	1.1
CM (w)	0.8	2.5*	0.9	1.1	0.6	1.1	0.7	0.7
ENV_CM (b)	1.8*	0.6	0.8	1.0	1.3	0.9	1.0	1.1
ENV_CM (w)	0.6	2.1*	1.4	0.9	1.1	0.8	1.2	0.4*
CONT_CM (b)	1.5	0.4*	1.7	0.5*	2.5*	0.6	1.8*	0.7
CONT_CM (w)	1.4	1.2	0.6	1.8	0.7	1.4	0.4*	1.4

[1] P1: quick recovery pattern (low - high - low SCC); P2: no recovery pattern (low - high - high SCC); INF: new infections (low - high SCC); HIGH: test-day recording with SCC >250,000 cells/ml.

* Significantly different from 1, with p <0.05.

patterns of peaks in SCC were stronger than the correlations with SCS150 or SCS305, as was generally also observed for pathogen-specific CM. This suggests that genetic selection on diminishing the peaks in SCC would decrease the incidence of pathogen-specific CM more effectively than selecting purely on lower lactation-average SCS. Although standard errors are large, and caution should thus be taken, the results encourage further research in patterns of peaks in SCC for genetic selection programs. Further optimisation might be to combine the patterns of peaks in SCC with the use of the random regression test-day model. One way to implement this, is to specify the deviations from a standard curve more precisely to either the rate of increase or decrease in SCC, or the slope of increase or decrease in SCC. These two parameters are derivations of either the first or second half of the SCC patterns. Therefore, the patterns investigated here are in line with the introduction of a random regression test-day model for SCC. In fact, the random regression test-day model might prove to be a good tool to estimate breeding values for patterns.

The results show that the distribution of the current SCC parameters is mainly indicative of CONT_CM status, but not so much for ENV_CM. This is in agreement with earlier statements that the standard mastitis control program is successful in decreasing the prevalence of intramammary infections with contagious pathogens, but less successful in preventing new cases of CM with environmental pathogens (Hillerton *et al.*, 1995). However, the presence of patterns of SCC provides information on the incidence of both CONT_CM and ENV_CM. Knowledge of SCC patterns may facilitate the implementation of pathogen-specific mastitis control programs, consisting of adequate guidelines to control the predominant type of mastitis. Herd risk factors that can be linked with cases of ENV_CM are housing, nutrition and machine milking (Schukken *et al.*, 1991, Barkema *et al.*, 1999, Schukken *et al.*, 1991). Poor sanitation, use of tie stalls, and no use of individual cloths are associated with increased incidence rates of ENV_CM. Herd characteristics that are associated with higher incidence rates of CONT_CM are mainly related to the milking procedure and to the milking machine as well (Schukken *et al.*, 1991, Barkema *et al.*, 1999). Frequent cleaning of the milking system regulator, low-line milking systems and applying disinfectant solution to the teat after milking ('teat dipping') are effective in decreasing the number of cases of CONT_CM.

Conclusions

Selecting for lower lactation-average SCC causes a shift in the importance of the main mastitis-causing pathogens. Therefore, there is a growing importance for defining new traits for SCC, that are based on biological understanding of pathogens and of the immune system of the cow. These traits can be used for genetic selection and for mastitis control programs that aim to reduce the incidence of the full scope of mastitis-causing pathogens. Genetic selection against any kind of deviation from the typical lactation curve for SCC was more effective to decrease the natural susceptibility to mastitis-causing pathogens, than selection for lower lactation-average SCC.

The quick and no recovery pattern are useful as basic tools for health management advices. These patterns of peaks in SCC can namely distinguish between cases of CM associated with either environmental or contagious pathogens, whereas the currently used primary traits are indicative for CONT_CM, but not for ENV_CM. Presenting the occurrence of SCC patterns on the forms of the milk recording is of additional value. Management advices

can then be directed specifically on changing herd risk factors related to either ENV_CM or CONT_CM, and effects of these changes on the status of the udder health in a herd can be easily evaluated.

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Changes in lactate dehydrogenase, N-acetyl-β-D-glucosaminase, and somatic cell count in relation to development of mastitis in dairy cows

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Introduction

Use of endogenous enzymes like lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAGase) (EC: 3.2.1.30), as early indicators of mastitis has been previously proposed (Pyörälä and Pyörälä, 1997; Ingvartsen, 2000; Ingvartsen, 2001; Batavani *et al.*, 2003). However, comparisons of different enzymes' ability as indicators of mastitis are scarce. The objectives of the current analysis were to describe the profiles of SCC, NAGase, and LDH activity in healthy and mastitic cows, to determine factors affecting the variation in the parameters and within this the importance of milk yield as an input in the determination of SCC, NAGase and LDH activity, and to investigate the relationship between SCC, NAGase, and LDH activity.

Material and methods

Data

The data originated from a study carried out at the Danish Cattle Research Centre in Foulum, Denmark. The initial dataset had a total of 34813 test-day records from Danish Holstein (n = 11893 records), Danish Red (n=13359 records), and Jersey cows (n=9135 records). Records were from September 2003 to April 2004. In addition to milk yield, milk composition, SCC, enzyme activity of LDH and NAGase and CMT, the datas*et al*so included information on breed, cow identity, parity, days from calving, date of registration, milking date and disease records.

Variables analysed

The cows included in the study were defined as healthy or clinically infected. A healthy cow was defined as a cow with low SCC (less than 200 000 cells/ml) and no veterinary treatment (Dohoo and Leslie, 1991). A clinically infected cow was defined as a cow that received veterinary treatment after showing clinical symptoms of mastitis and high SCC (more than 800 000cells/ml) (Hillerton, 1995). A subclinically infected cow was defined as a cow with SCC of 500 000 and more but without veterinary mastitis treatment. All medical treatments were done by a veterinarian and the same protocol was consistent from cow to cow as far as possible. In the initial statistical analysis, data were divided into two subsets of healthy and mastitic cows. The days of mastitis diagnosis were identified as the recorded date of mastitis treatment. A period of 21 days before and 14 days after diagnosis was used to study the profile of LDH, NAGase, and somatic cell count in mastitic cows. In the healthy cows, the period of the first 100 days after calving was chosen to study the

profiles of LDH, NAGase, and SCC in early lactation and the period from day 290 after calving to the end of the lactation for late lactation. Three stages of early lactation were defined. These were from day 1 to day 30 after calving, day 31 to day 50 after calving and from day 51 and onwards. As there were few cows in parities 3 and 4, data from parities 2, 3 and 4 were treated as one parity group. Logarithm 10 of somatic cell count was used in the analysis.

Statistical analysis

A univariate mixed model of systematic and random factors was used to estimate mean effects of breed, parity and stage of lactation for each period analysed. A random cow effect was used to account for the co-variance between lactations of the same cow. Age at first calving and milk yield at each milking were included as covariates. A period of 21 days before to 14 days after mastitis diagnosis was included in the model for mastitic cows to study the trend of indicators before and after diagnosis/treatment. Data were analysed using REML as implemented by the MIXED procedure in SAS version 8.2 (SAS Inst. Inc., 2001) applying an autoregressive covariance structure to account for the correlations between measurements within cows. In the analysis for healthy cows, fixed effect of days after calving was included while number of days relative to mastitis diagnosis and stage of lactation were dropped from the model. The relationship between LDH activity, NAGase activity, and SCC was determined through correlation of absolute values and correlation of corresponding residuals after accounting for systematic factors.

Results

Systematic effects affecting LDH, NAGase activity and SCC

A summary of descriptive statistics for LDH, NAGase and SCC are presented in Table 1.

Of the tested factors, days before and after diagnosis, production month, lactation stage and milk yield significantly ($P < 0.001$) affected LDH, NAGase, and SCC. Age at first calving and parity did not have a significant effect on any of the parameters while breed only had a significant effect on NAGase ($P < 0.01$). In general, LDH, NAGase, and SCC in mastitic cows, tend to be higher for parities 2 and above than for primiparous cows. With respect to stage of lactation, LDH and NAGase, values are higher at the beginning and at the end of the lactation than in mid lactation. In mastitic cows all the parameters tend to increase before the day of diagnosis and drop after treatment. Relative to the day of diagnosis, SCCs tended to increase gradually from as early as 12 days before diagnosis while LDH and NAGase activity tend to increase from about day 8 before diagnosis but the enzymes increased more

Table 1. Descriptive statistics for lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAGase) and somatic cell count (SCC) (raw data).

	n	LDH		NAGase		logSCC	
		Mean	Std	Mean	Std	Mean	Std
Healthy	22929	2.92	1.49	43.47	27.85	4.84	0.37
Mastitic	11884	4.07	2.37	69.42	37.16	5.45	0.62

n = number of records

Std = standard deviation

rapidly than SCC. LDH activity had the highest percentage change while SCC had the lowest (1.89% for LDH, 0.85% for NAGase, and 0.29% for SCC). SCC and LDH tended to increase one day after diagnosis and treatment while NAGase activity dropped immediately. As with the increase, the drop of LDH and NAGase activity happened more rapidly than that for SCC. There was a general increase in all three parameters in the drying off period. Although variation was higher at the end of the lactation than at the beginning of the lactation in all the three parameters, NAGase had the highest variation during both periods. NAGase had a substantially different variation at the beginning vs. at the end of lactation (CV = 74% vs. 64%).

Relationships among parameters

The correlation of raw data indicated that in both mastitic and healthy cows, the relationship between LDH and SCC was higher than the relationship between NAGase and SCC. On the one hand, the correlation coefficient between LDH and SCC was 0.76 in mastitic cows while it was 0.48 in healthy cows. On the other hand, the correlation coefficient between NAGase and SCC was 0.58 in mastitic cows while it was 0.41 in healthy cows. Generally, the correlation coefficients of residuals were lower in healthy cows than in mastitic cows. The relationship among the three parameters was moderate at the beginning of lactation ($r = 0.40$ LDH vs. SCC, $r = 0.44$ NAGase vs. SCC, and $r = 0.37$ LDH vs. NAGase) and low at the end of lactation ($r = 0.40$ LDH vs. SCC, $r = 0.34$ NAGase vs. SCC, and $r = 0.11$ LDH vs. NAGase). The relationships were relatively high ($r = 0.61$ LDH vs. SCC, $r = 0.53$ NAGase vs. SCC, and $r = 0.43$ LDH vs. NAGase) in mastitic cows.

The changes in the activities of LDH and NAGase and the level of SCC implies that the major source of the two enzymes have a common route with somatic cells produced as a result of mastitis but not exclusively the somatic cells themselves.

Table 2. Correlation of residuals for lactate dehydrogenase (LDH) activity, N-acetyl-β-D-glucosaminidase (NAGase) activity and somatic cell count (SCC) for mastitic cows (above the diagonal) and for the first 100 days of lactation for healthy cows (below the diagonal). Correlations of residuals for healthy cows at the end of lactation are in parentheses.

	LDH	NAGase	logSCC
LDH	1.00	0.43	0.61
NAGase	0.37 (0.11)	1.00	0.53
logSCC	0.40 (0.40)	0.44 (0.34)	1.00

All the correlations showed a significant relationship ($P < 0.001$).

Conclusion

In mastitic cows LDH had a higher correlation with SCC than NAGase and also the highest relative increase of them all with respect to diagnosis time. The activity in LDH and NAGase and the level of SCC in milk increased significantly with mastitis, suggesting that they could be utilized as early indicators of mastitis.

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Prevalence of contagious mastitis pathogens in bulk tank milk from Prince Edward Island dairy farms

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Abstract

When designing both regional and herd-specific mastitis control and research programs, recent knowledge about the distribution of clinical and subclinical pathogens, as well as the frequency of adoption of mastitis prevention practices have to be considered. The objectives of this study were to estimate the prevalence of the three most important contagious pathogens on Prince Edward and to study the association between the prevalence of different mastitis pathogens and the bulk milk somatic cell count (BMSCC). Fresh bulk tank milk was obtained of all 258 PEI dairy herds. Bulk tank samples were obtained three times, with a weekly interval. BMSCC and the presence of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp was evaluated using culture media that are specific for each pathogen. The cumulative prevalence for *Staph. aureus, Strep. agalactiae* and *Mycoplasma* spp. was 74, 1.6 and 1.9%, respectively. *Staphylococcus aureus*-positive herds had a geometric mean BMSCC of 169,000 cells/ml compared to 129,000 cells/ml in herds with no contagious pathogens. The 4 *Streptococcus agalactiae*-positive herds and the 5 *Mycoplasma* spp positive herds had a geometric mean BMSCC of 177,000 cell/ml and 137,000 cells/ml, respectively. The frequency of which *Staphylococcus aureus* was isolated was positively correlated to the average BMSCC. Prevalence of *Streptococcus agalactiae* was much lower than expected and *Mycoplasma* spp in bulk milk was reported for the first time on Prince Edward Island and in Canada for the first time since 1972.

Introduction

Mastitis is the most prevalent and most expensive disease on a dairy farm. Knowledge of the prevalence and distribution of mastitis pathogens is crucial to the prevention of the disease. Bulk tank milk culture can be of great value as a monitoring tool to control and evaluate clinical and subclinical mastitis and prevent potential milk quality problems on a dairy farm (Jayarao and Wolfgang, 2003). Bulk milk culture is a cheap and convenient method compared to the collection and culturing of individual cow milk samples, and it is a useful tool to estimate herd prevalence of contagious mastitis pathogens.

Several studies have been performed to estimate the herd prevalence of *Staph. aureus*, *Strep. agalactiae* and *Mycoplasma spp*. in the US (Kirk *et al*., 1997; Khaitsa *et al*., 2000; Fox *et al*., 2003). However, few studies have been performed in Canada on the prevalence of contagious mastitis pathogens in bulk milk. *Streptococcus agalactiae* prevalence found in Canadian bulk milk ranged between 6% in Alberta and 43% in Quebec (Guillemette *et al*., 1992; Schoonderwoerd *et al*., 1993). Kelton *et al*. (1999a and 1999b) found *Staph. aureus* in 58 out of 59 bulk milk samples from Ontario, while 92% of the herds had at least

one *Staph. aureus* culture-positive cow. For Prince Edward Island, Keefe *et al.* (1997) studied herd prevalence of *Staph. aureus* and *Strep. agalactiae* and they found herd prevalences of 70% and 18%, respectively. Only one Canadian study reported *Mycoplasma spp.* in bulk milk and individual cow milk in Ontario, but this study was performed more than thirty years ago (Ruhnke *et al.*, 1976).

Bulk milk SCC (BMSCC) is used worldwide as an important measurement for milk quality. Low BMSCC has benefits for both producers and consumers (Barkema *et al.*, 1998; Schaellibaum, 2001). High BMSCC is associated with an increased prevalence of subclinical mastitis and a higher incidence of clinical mastitis caused by *Strep. agalactiae* and *Staph. aureus*. Low BMSCC is also associated with high incidence of clinical mastitis, but mainly caused by environmental pathogens (Erskine *et al.*, 1988; Schukken *et al.*, 1989). A positive association between BMSCC and the isolation of *Staph. aureus* in the bulk milk has been previously reported (Erskine *et al.*, 1988; Jayarao *et al.*, 2004), but these studies were not conducted in Canada.

The objectives of this study were to estimate the herd prevalence of contagious mastitis pathogens in bulk milk from Prince Edward Island dairy farms and to determine the association between the isolation of contagious mastitis pathogens and mean BMSCC.

Material and methods

Study population

At the start of the study, May 17th, 2004, the Prince Edward Island dairy industry consisted of 258 dairy farms. During the sampling period one farm stopped operating. From this data the average herd size was 59.6 lactating cows, mean annual milk production was 8894 kg/cow and the mean BMSCC was 234,000 cells/ml.

Sample collection

In May 2004, three sets of fresh bulk milk samples were collected from all dairy farms on Prince Edward Island with a weekly interval between collection. The milk was cultured within 24 to 36 hours after collection from the farm.

Laboratory analysis

Five different media were used for determining the presence of *Staph. aureus*, *Strep. agalactiae* and *Mycoplasma spp.*: 1) A general medium consisting of blood agar with the addition of 1 g/l esculin; 2) Vogel Johnson agar, a medium selective for staphylococci; 3) Modified Edwards' medium with the addition of colistin sulphate (5 mg/L) and oxolinic acid (2.5 mg/L), a medium selective for streptococci; 4) Modified Hayflick agar, for the culturing of *Mycoplasma spp.*, and 5) Modified Hayflick broth for *Mycoplasma spp.* enrichment. *Staph. aureus* was identified by α- and β-hemolysis on blood-esculin agar and a positive tube coagulase test. *Strep. agalactiae* was identified by growth on modified Edwards' and/or typical appearance on blood-esculin agar, a positive CAMP test and a positive latex agglutination (Remel PathoDx®, Remel Europe Ltd., Dartford, Kent, UK). *Mycoplasma spp.* were identified by the typical fried-egg appearance on Hayflick agar.

Statistical analysis

Geometric mean BMSCC per farm was calculated as the exponent of the average natural logarithm (ln) of the three BMSCC measurements. A Student's *t* test was used to test if mean ln(BMSCC) was different between pathogen positive and negative farms. Linear regression was used to calculate the association of BMSCC and the frequency of *Staph. aureus* isolation.

True herd prevalence, test sensitivity and test correlation were determined using maximum likelihood estimation based on a model where animal-level prevalence was assumed to vary between herds. This model was used assuming a perfect test specificity and constant true herd status, but allowing for conditional dependence between test results. The SAS procedure for nonlinear mixed models [PROC NLMIXED] was used.

Results

Staph. aureus was isolated in at least in one of three samples from 74% of the dairy farms (Table 1). Twenty, 21 and 33% of the herds tested positive one, two or three times, respectively. In the first, second and third week, *Staph. aureus* was isolated 52.3, 54.8 and 55.3%, respectively.

Strep. agalactiae was isolated at least once in samples from 1.6% of the farms. In the first, second and third week, *Strep. agalactiae* was isolated from 1.2, 1.2 and 0.4% of the samples, respectively.

Mycoplasma spp were isolated at least once in samples from 1.9% of the farms. In the first, second and third week, *Mycoplasma* spp were isolated from 1.9, 0.4 and 0.4% of the samples, respectively. Species determination of these 5 *Mycoplasma* cultures reveiled 2 species, *Mycoplasma bovis* and *Mycoplasma alkalescens,* both potentially pathogenic. *Mycoplasma bovis* was found on 4 farms, *Mycoplasma alkalescens* was found on one farm.

The model in the maximum likelihood procedure that fitted the data best consisted of a herd prevalence of 100% (95% CI: 80.2 - 100), a test sensitivity of 54% (54 - 66) and a rho (between test correlation) of 0.46 (0.28 - 0.46).

Farms that had at least one bulk tank sample positive for any of the contagious pathogens had a a geometric mean BMSCC which was 34,700 cells/ml higher than farms from which we did not isolate any of the pathogens in the bulk tank samples (P=0.006). No difference in BMSCC was found between the 5 *Mycoplasma*-positive and the negative herds or between the 4 *Strep. agalactiae*-positive and the negative herds (P>0.5). One of the *Strep. agalactiae*-positive farms had a BMSCC of 35,000 cells/ml and was positive only once in the three samples. Exclusion of this farm in the analyses resulted in a significant association between BMSCC and isolation of *Strep. agalactiae* (p=0.04).

Table 1. Proportion of Prince Edward Island bulk milk samples (n=258) that were culture-positive for contagious pathogens in three consecutive weeks.

Pathogen	Week 1 (%)	Week 2 (%)	Week 3 (%)	Cumulative
Staph. aureus	135 (52.3)	141 (54.8)	142 (55.3)	191 (74.0)
Strep. agalactiae	3 (1.2)	3 (1.2)	1 (0.4)	4 (1.6)
Mycoplasma spp.	5 (1.9)	1 (0.4)	1 (0.4)	5 (1.9)

BMSCC of *Staph. aureus*-positive herds was 39,700 cells/ml higher than of negative herds (P=0.001).

BMSCC increased with increasing frequency of *Staph. aureus* isolation (Table 2). *Strep. agalactiae* and *Mycoplasma spp.* were not included.

Table 2. Mean BMSCC in relation with frequency of Staph. aureus *isolation.*

Frequency of isolation	# herds	mean BMSCC	95% CI
0 (out of 3)	66	129[ac]	112-148
1 (out of 3)	51	151[a]	129-177
2 (out of 3)	53	156[d]	134-183
3 (out of 3)	87	188[bc]	167-213

[ab]Means with different superscripts were different at P<0.05

[cd]Means with different superscripts were different at 0.05<P<0.10

Discussion

Herd level prevalence of *Staph. aureus, Strep. agalactiae* and *Mycoplasma* spp, determined by culturing 3 consecutive bulk milk samples, was 74, 1.6 and 1.9%, respectively. The prevalence of *Staph. aureus* prevalence found in this study agrees with earlier studies where herd prevalence ranged from 31 to almost 100% in North America (Kelton *et al.*, 1999a; Khaitsa *et al.*, 2000; Jayarao *et al.*, 2004). The prevalence of *Strep. agalactiae* at 1.6% confirmed a trend of declining prevalence of this pathogen in North America (Keefe, 1997). Prevalence of *Mycoplasma spp.* has been reported for the first time on Prince Edward Island and in Canada for the first time since 1972.

True prevalence can only be determined if the sensitivity and specificity of this method is known, therefore these parameters have to be determined or estimated. In the statistical approach for the true prevalence, we considered the specificity of our method to be close to 100%. Allowing for a lower specificity, the estimated true prevalence would be over-estimated. Herds could go from negative to positive for *Staph. aureus* and *vice versa* in the sampling period, but the time between the first and the last sample was 14 days. The authors considered it to be unlikely that the infection status of a herd for *Staph. aureus* would have changed in that period.

Herd level prevalence of *Strep. agalactiae* has decreased considerably the last years (Keefe, 1997; Pitkala *et al.*, 2004). Herd prevalences of 89% were reported in Wisconsin in 1968 (Postle, 1968) and the most recent reported herd level prevalence is 10.3% in Pennsylvania in 2000 (Jayarao *et al.*, 2004). In this study, the *Strep. agalactiae* herd prevalence (1.6%) is approximately 10 times lower than what was reported in 1994. However, Keefe *et al.* (1997) used a more sensitive method than the standard method as recommended by the NMC for culturing *Strep. agalactiae* from bulk milk (Keefe *et al.*, 1997). The true prevalence of *Strep. agalactiae* in this study is probably higher than estimated, but even if the sensitivity of the current method would be as low as 21% (Godkin and Leslie, 1993) and assuming a specificity of 100%, the true prevalence would not be higher than 7.5%. Therefore, eradication of *Strep. agalactiae* Prince Edward Island is possible and is currently being considered.

For the last 30 years, no Canadian studies have been performed to determine herd level prevalences of *Mycoplasma* spp. Recent US studies, however, reported that 1 to 6% of the dairy herds are *Mycoplasma* culture-positive (Kirk *et al*., 1997; Fox *et al*., 2003). Since one time sampling of bulk tank milk may give an underestimation of the prevalence, due to latently infected cows possibly not shedding the organism, and subclinically infected cows may be shedding, multiple sampling and routine sampling of bulk tank milk may give more reliable results. The *Mycoplasma* species that were found in this study, *Mycoplasma bovis* and *Mycoplasma alkalescens,* are potentially pathogenic and can cause mastitis (Kirk *et al*., 1997).

In this study there was a highly significant association between the isolation of *Staph. aureus* and the mean BMSCC (P=0.001). This is in agreement with other studies (Barkema *et al*., 1998; Jayarao *et al*., 2004). From the 3 samples that were taken from the farms, the frequency of isolation of *Staph. aureus* was significantly related to BMSCC. Jayarao *et al*. (2004) found similar associations in a recent study in Pennsylvania. BMSCC and isolation of *Strep. agalactiae* were not significantly associated in this study, but only 4 farms were found to be positive for *Strep. agalactiae*. Other studies have found that isolation of *Strep. agalactiae* in bulk milk is highly correlated to high BMSCC (Keefe, 1997; Barkema *et al*., 1998). However, the presence of some strains of *Strep. agalactiae* is not correlated to high BMSCC. A possible explanation for this is that these are strains of human origin (Zadoks *et al*., 2005).

The isolation of *Mycoplasma* spp. and mean BMSCC were not significantly associated in this study, although Fox *et al*. (2003) did find an association. This may also be the result of the low number of *Mycoplasma*-positive herds in our study.

Conclusions

The prevalence of *Staph. aureus* on Prince Edward Island dairy is high as expected. The prevalence of *Strep. agalactiae* has decreased considerably and *Mycoplasma* is prevalent on Prince Edward Island and is reported for the first time in 30 years, in Canada. Reduction of *Staph. aureus* and *Strep. agalactiae* is an useful tool in the reduction of BMSCC on a dairy farm, since their presence in bulk milk is related to elevated BMSCC. The frequency of isolation of *Staph. aureus* is significantly related to the mean BMSCC.

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A Bayesian approach to interpreting *Staphylococcus aureus* diagnostic indicators for on-farm decision making

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Abstract

A Bayesian network (BN) modeled *Staphylococcus aureus* (SA) evidence on probability of mastitis in individual cows. Herd evidence was provided by bulk tank culture (BTC), antibody test (BTMAb), somatic cell count (BTSCC), and risk factors (HRF). Culture (CC), antibody test (CowAb), lactation-to-date somatic cell score (C-SCS), and risk factors (CRF) provided cow evidence. Herd evidence served as priors (P(SA)) to cow probabilities. Risk factors were processed as likelihoods based on strength of association to SA. Two herd scenarios were modeled. Herd-1 had moderate (17%) while Herd-2 had high risk of *SA* (43%). HRF had little effect on cow posterior probabilities when risk was moderate unless herd diagnostic indicators were high. Given Herd-1, P(SA) ranged from 1.7 to 91.8% depending on herd diagnostic evidence. Given Herd-2, posterior probabilities ranged from 5.8 to 97.6% with the same evidence. Two cow scenarios were modeled to evaluate effects of CRF on posteriors when combined with herd and cow diagnostic evidence. Overall likelihood was 1.33 for Cow-1 and 1.69 for Cow-2. Given Cow-2 and Herd-1, a positive BTC and high BTMAb, cow posterior probabilities exceeded 50% when C-SCS was below 3.0. Given Herd-1 and Cow-2, cow posteriors exceeded 50% when BTC was positive and C-SCS above 6.0. As strength of herd evidence increased, probability of cow infections increased, improving SA detection with less cow diagnostic evidence. Probabilities can assist dairy managers in determining relevancy of diagnostic indicators for mastitis decision-making.

Keywords: *Staphylococcus aureus*, Bayesian network, mastitis diagnosis

Introduction

Herd managers frequently use risk factors when diagnosing mastitis. Unfortunately, risk factor interpretation is not as intuitively simple as diagnostic test interpretation. Risk factor processing requires expertise to weigh evidence based on importance. Due to task complexity, single or multiple tests are often applied without considering readily available risk factor evidence. Diagnostic tests are given ultimate weight and assumed to be perfect. When both diagnostic test and risk factors are considered, the combined probability is subjectively processed and the total evidence is then used to make decisions.

Herd specific SA evidence can be determined by following the 'causal pathway' provided by unique HRF. Once the herd specific apriori probability is established, it can be combined

with herd diagnostic test result (HDT) to increase confidence in diagnosis. Tests for diagnosing SA are not perfect and may be best applied when combined with additional herd and cow evidence (Costello *et al.*,1998). Herd SA diagnostic evidence can be provided from HRF, BTSCC, BTC, and/or BTMAb. SA evidence can also be provided by cow diagnostic tests (CDT): CC, C-SCS, and/or CowAb, and CRF evidence.

Hogeveen *et al.* (1995a,b) developed a BN for diagnosing high somatic cell count (SCC) from risk factors associated with machine milking. Risk factors for specific pathogens were not considered. In addition, risk factor evidence was not combined with test probabilities, mastitis diagnosis was considered perfect, and evidence came from a single source and was considered to be readily available.

The purpose of this model was to describe relationships from readily available risk factors to determine how additional diagnostic evidence affects the posterior probability. The endpoint is a single posterior probability indicating cow probability of SA (C-P(SA)). Causal evidence is funneled from the herd level to determine herd probability of SA (H-P(SA)) and is then combined with possible cow evidence to diagnose SA. Assuming that decisions are made logically, risk preference of the decision-maker would determine when additional evidence is needed for costly decisions such as culling. This BN assumes risk factor evidence is readily available but assembly with other evidence is complex for the non-expert. Apriori risk factor evidence when combined with diagnostic evidence can be beneficial for making optimal SA decisions. The framework can be adapted to other diagnostic tests, additional risk factors, and knowledge of other mastitis-causing pathogens.

Methods and results

Methodology overview

Mechanics of BN have been reviewed in artificial intelligence and decision sciences (Pearl, 1990). BN have been used for human medical diagnosis (Sox *et al.*, 1988) and for mastitis diagnosis (Hogeveen *et al.*, 1995a,b). BN are used to understand or simulate complex situations where evidence is imperfect and requires weighted assembly for final decision-making. Mechanics of this BN are described in Costello (1998).

HRF and HDT provided evidence to calculate H-P(SA). Parameter values were derived from literature analyses (Costello, 1998). BTSCC was used to establish the initial apriori or herd index to the HRF node. P(SA) given BTSCC was set to 0.075 when BTSCC was above 150 cells/µl. HRF were then combined with BTSCC evidence. Possible HDT combinations and results provided further SA herd evidence. Finally, combined evidence from HDT and HRF served as apriori probability to cow evidence. CRF and possible probabilities from CDT were combined with herd evidence to estimate probability of SA in individual cows. Literature analyses provided background for CDT parameter estimates (Costello, 1998).

Detail on risk factor probability assembly is provided in Costello (1998). Briefly, SA-HRF and CRF parameter values were derived from literature analyses of causal relationships of herd and cow factors with SA. Parameter values were estimated based on relative importance of individual risk factor on SA. Probabilities were either set to zero if not simulated or were considered as apriori evidence if simulated (P>0) just as they would be in reality.

Causal pathway describing risk of *Staphylococcus aureus*

SA HRF were divided into two causal pathways: HRF that affect teat integrity by impairing defensive capabilities (Teat Integrity Node), or HRF that facilitate SA spread (Spread Node). SA spread is dependent on presence of HRF that facilitate transfer. The higher the SA prevalence, the more influential the evidence from this path. Importance of the Spread Node and its' descendants, and the Teat Integrity Node and its' descendants is reflected in the weights of influence of each descendant node on the root node. The root or parent node is the apriori evidence provided by HRF on H-P(SA). Risk factors affecting teat integrity are often caused by direct injury (Injury Node), teat end lesions (Teat Lesion Node), or teat chapping (Teat Chapping Node). Pathways were designed to prevent overlap between causal factors. Many studies have identified strong relationships between machine milking and milking procedures on SA probability. Simulated HRF descendants to the Teat Integrity and Spread Nodes and estimated probabilities are provided in Table 1. See Costello (1998) for a complete list of BN risk factors and estimated probabilities. Presence of short machine milking massage phase, poor vacuum regulator stability, use of a common cloth, and/or not using post-milking teat antisepsis were modeled, if present, as providing the greatest HRF evidence of SA.

Table 1. Herd risk factors and parameter values estimated to predict probability of Staphylococcus aureus *(SA).*

A. Teat Integrity Node	$\lambda(e\|SA)$[1]
Teat Lesion Descendant Node: Machine Milking Factors:	
1. Pulsator Characteristics:	
a. d-phase length (< 245 ms)	1.31
b. pulsation rate (< 55 cpm)	1.20
2. Vacuum Level:	
a. Above Standard	1.29
3. Vacuum Stability:	
a. Excess Air Admitted-Cluster	1.17
b. Poor Regulator Stability	1.31
4. Overmilking - Excessive:	1.26
Teat Chapping Descendant Node: Post-milking Teat Antisepsis Factors:	
Not included in simulation example	
Teat Chapping Descendant Node: Facility Factors:	
1. Parlor Exit	
a. Spring: Not Sheltered	1.29
Teat Injury Descendant Node: Facility Factors:	
Not included in simulation example	
B. Spread Node	
Prepping Procedure Factors:	
1. Length of Prep: Too short	1.05
2. Milking Gloves: Non-use	1.05
3. Common Cloth: Use	1.31
4. Udder Bottom: Wet/Dirty	1.23
5. Teat Dip: Non-use	1.29
6. Back Flush: Non-use	1.09

[1]Likelihood of the risk factor evidence given SA presence

Evidence from herd diagnostic tests applied singly

A threshold of 50% probability of SA was selected to present BN results. Actual decision threshold will vary with decision-maker risk preference. Based on HDT performance characteristics (Costello, 1998), less than 10% apriori evidence from additional HDT or HRF was required to diagnose herd SA when BTC was positive (Table 2). When BTC was negative, a 60% P(SA) was needed for H-P(SA) to exceed 50%. When BTMAb was high, a 20% P(SA) was necessary for the H-P(SA) to exceed 50% (Table 2).

Evidence from cow diagnostic tests applied singly and in combination

Methods for CDT probability development are provided in Costello (1998). Briefly, multiple SCC are needed to make judgments of SA and reliability improves when H-P(SA) is high. C-SCS was used instead of single SCC to provide evidence of SA. This was reflected in the model: as H-P(SA) increased, a lower C-SCS could be used to predict SA (Table 2). CC is the gold standard for SA despite its' potential to miss SA when applied without enhanced culture techniques. Little prior evidence was needed when CC was SA-positive for the posterior probability to reach 50%. When CC was SA-negative, P(SA) needed to approach 90% to reach the same posterior threshold. CowAb has exhibited lower performance to predict SA than

Table 2. Prior probabilities (P(SA)) needed to reach 50% probability of Staphylococcus aureus *in the herd (H-P(SA)) when tests were applied singly, given estimated test performance characteristics.*

Herd Level Diagnostic Tests					
Bulk Tank Culture (BTC):	Positive	Negative			
P(SA)[1]	0.10	0.60			
P(e|SA)[2]	0.34	0.66			
P(e|¬SA)[3]	0.03	0.97			
SA specific antibody test (BTMAb):	Low	Medium	High		
P(SA)	0.80	0.30	0.20		
P(e|SA)	0.27	0.74	0.87		
Cow Level Diagnostic Tests - Applied Singly					
Lactation to date somatic cell score (C-SCS):					
C-SCS Level:	< 3.0	3.0-4.0	4.0-5.0	5.0-6.0	>6.0
P(SA)	—-	0.90	0.90	0.85	0.70
λ(SA|e)[4]	0.05	0.11	0.18	0.25	0.54
Culture (CC):	Positive	Negative			
P(SA)	0.10	0.90			
λ(SA|e)	9.0	0.11			
SA specific antibody test (CowAb):	Positive	Negative			
P(SA)	0.20	0.80			
λ(SA|e)	5.91	0.39			

Model Result:

[1]Prior Probability of SA needed to reach 50% posterior probability given test result

Model Performance Characteristics:

[2] Probability that the evidence will be observed (e.g., test result) given SA is present

[3] Probability that the evidence will be observed although SA is not present

[4] Likelihood that SA is present given the evidence observed (e.g., test result)

CC (Costello, 1998). A 20% P(SA) was needed for the posterior probability to reach 50% when CowAb was positive. When CowAb was negative, prior evidence from HRF and/or HDT needed to be 80% for the SA posterior to reach 50%.

Combining C-SCS and CC with prior evidence may aid in determining importance of C-P(SA). Combined C-P(SA) was evaluated at 3 priors (Table 3). As expected, combined C-P(SA) increased as prior evidence increased. This simulates the knowledge that as more cows become infected with SA, the threat of new and chronic infections increases as H-P(SA) increases. In addition, as prior evidence decreases, the C-P(SA) when C-SCS is low and despite CC result, will be lower. When the prior was 10%, the C-P(SA) never exceeded 50% regardless of C-SCS or CC. When the prior was 50%, the C-P(SA) reached 50% when C-SCS was between 3.0 and 4.0 and CC-positive. When CC was negative and C-SCS was above 6.0, the highest C-P(SA) was 35%, even though the prior was 90%.

Table 3. Minimum lactation somatic cell score (C-SCS) needed to reach 50% posterior probability of Staphylococcus aureus *or maximum cow posterior probability (C-P(SA)) for three prior probability P(SA) scenarios when combined with cow culture (CC).*

		Cow Posterior: C-P(SA|e)	
P(SA)	Min C-SCS	CC+ [1]	CC-
0.10	> 6.0	0.35 [2]	0.007
0.50	3.0-4.0	0.50 [3]	0.012
	> 6.0	0.83	0.057
0.90	< 3.0	0.81	0.050
	> 6.0	0.98	0.350

[1]Probability when cow culture is SA positive or negative given additional evidence

[2]Probabilities *not* in bold are less than 50% at the maximum C-SCS

[3]Probabilities *in* bold reach 50% or above at the minimum C-SCS value indicated

Simulating risk factor evidence through the model

Two herd and two cow scenarios were simulated. HRF was calculated by multiplying prior odds by product of the likelihood ratios (Costello, 1998). In Herd-1, HRF_1 were as follows: short d-phase, poor regulator stability, poor milking preparation, no back-flush, and parlor exit was not sheltered (spring). Given HRF_1, P(SA) was 17%. In Herd-2, HRF_2 were as follows: d-phase was too short, pulsation rate was too low, vacuum level was too high, excess air was admitted causing vacuum instability, regulator efficiency was poor, overmilking occurred, no teat dip, milking gloves, or back-flush, common cloths were used; and udder bottoms were wet and dirty. Based on HRF_2, P(SA) was 43%. When BTC was positive, H-P(SA) exceeded 50%, given HRF_2, in all simulations except where BTSCC was less than 200 cells/µl (Table 4). In addition, when BTC was negative, H-P(SA) was above 50% if BTMAb was medium or high. When BTSCC was greater than 400 cells/µl, H-P(SA) reached 36% despite a negative BTC.

CRF is readily available to the herd manager and can be modeled in the same manner as HRF. Increasing cow age, previous SA infection, presence of teat chapping and/or lesions are risk factors for SA in individual cows. A relatively low risk (CRF_1 likelihood = 1.33) and high risk (CRF_2 likelihood = 1.69) were simulated in the BN. When herd evidence indicated high H-P(SA), probability of C-P(SA) ranged from 12% to 48%, depending on CRF, despite

Table 4. Estimated probability of Staphylococcus aureus H(P(SA)), given two herd risk scenarios (HRF) combined with bulk tank culture (BTC) or other diagnostic evidence.

Additional Evidence (e)	Herd Prior: H-P(SA|e)			
	BTC+		BTC-	
	HRF_1	HRF_2	HRF_1	HRF_2
–	0.63[1]	0.86	0.13	0.36
BTMAb-low	0.39	0.70	0.05	0.17
BTMAb-medium	0.83	0.95	0.30	0.61
BTMAb-high	0.92	0.98	0.51	0.79
BTSCC: < 200 cells/µl	0.16	0.42	0.02	0.06
BTSCC: 200-400 cells/ µl	0.48	0.77	0.08	0.23
BTSCC: >400 cells/ µl	0.63	0.86	0.13	0.36

[1]Probability of Herd SA given evidence (e.g., Herd-1 risk factors, and positive BTC)

Table 5. Estimated cow posterior probabilities of Staphylococcus aureus (C-P(SA)) given two cow risk scenarios (CRF) combined with cow diagnostic evidence and herd evidence.

Additional Herd Evidence (e)	Cow Posterior: C-P(SA|e)			
	HRF_1	HRF_2	HRF_1	HRF_2
	CC+ CRF1(CRF2)		CC- CRF1(CRF2)	
P(SA|e)	0.71[1](0.76)	0.90(0.92)	0.03(0.04)	0.10(0.12)
P(SA|BTC+)	0.95(0.96)	0.99(0.99)	0.20(0.24)	0.48(0.54)
P(SA| BTC-)	0.65(0.70)	0.87(0.89)	0.02(0.03)	0.08(0.10)
P(SA|BTC+,BTSCC 200-400)	0.92(0.93)	0.98(0.98)	0.12(0.15)	0.34(0.38)
P(SA|BTC+,BTSCC>400)	0.95(0.96)	0.99(0.99)	0.20(0.24)	0.48(0.54)
P(SA|BTC-,BTSCC 200-400)	0.50(0.56)	0.78(0.82)	0.01(0.02)	0.04(0.05)
P(SA|BTC-,BTSCC>400)	0.65(0.70)	0.87(0.89)	0.02(0.03)	0.08(0.10)

[1]Probability of Cow SA given prior evidence from herd and cow (e.g., Herd-1 risk factors, positive cow culture, Cow-1 risk factors)

a negative CC (Table 5). When herd and cow level indicators were high for P(SA), C-P(SA) only varied slightly given CRF scenarios. When BTC was negative and CC-positive, CRF altered C-P(SA) by 1 to 6% depending on other evidence. C-SCS can also be incorporated into the model to aid in interpretation (Costello, 1998).

Conclusion

BN can be used to incorporate risk factor with diagnostic test evidence to improve herd interpretation of mastitis. Perhaps its' greatest value is in processing the impact of herd and cow risk when tests are imperfect. Diagnosis of SA mastitis is unique in that tests are imperfect and within herd prevalence affects test interpretation as well as risk of new infection. BN can aid understanding of diagnostic test result by incorporating prevalence, via readily available herd and cow risk factor evidence and indicators of herd and cow inflammation (BTSCC and C-SCS tests).

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Variation in somatic cell count and milk components in fraction collected quarter milk samples

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Abstract

Milk components secretion into milk are following different routes. In the mammary gland; protein, fat and lactose are synthesised in the epithelial cells and released into the alveoli, somatic cells enter the milk by intracellular migration. Cisternal milk should be avoided when Somatic Cell count (SCC) analysis and bacteriological examination are being preformed (IDF 1984).

Dairy advisory should be based on adequate knowledge in using SCC as a management tool and thus helping farmers in controlling udder health, deciding mastitis therapy and selecting the right cows for dry cow therapy or culling.The representative of SCC in single milk fractions of quarter milk samples were therefore analysed.

The quarter milk samples were sampled from 7 Norwegian Red Cattle by catheter drainage in fractions of 120 mL, 20 samples were taken. Fraction samples were analysed for protein, fat and lactose by Milcoscan 605 and somatic cell count by Fossomatic 360.

The results showed large variations in SCC (approximately 100.000 cells/mL to 1-2.000.000 cells/mL) in different fractions. Samples from cows with low SCC (less than 50.000 - 100.000) did not show variation of great practical importance compared to cows with high SCC. Milk fractions with high SCC tended to show decreased lactose values.

The results indicated that SCC measured in one single milk fraction, from clinical healthy cows, should be carefully interpreted and inflammatory reactions in single milk fractions were observed. SCC should be measured in milk samples representative for the entire udder- or quarter milk. The Health Periodical from the Norwegian Cattle Health Services contains such information.

Introduction

The mammary secretory epithelial cells secrete milk continuously. Milk is stored in the udder compartments and removed twice a day during milking. The cisternal part of the udder consists of the teat cistern, the gland cistern and the major milk ducts. The alveolar compartment consists of small milk ducts and the lumens of the alveoli. Milk drained by catheter is called cisternal milk. Alveolar milk is drained due to an oxytocin induced response leading to contraction of the alveoli (Stelwagen 1996).

Milk samples collected before and between milking are called cisternal milk and milk samples collected immediately after milking are called alveolar milk.

It is assumed that foremilk- and residual milk samples contain the highest concentration of SCC

SCC may vary considerably among different fractions of milk (cisternal, alveolar, strippings) obtained at a single milking (Paape 1966, Gray and Schalm 1960, Schalm 1957) and it is therefore important to take composite milk samples. Composite milk samples represent samples from both cisternal and alveolar milk, thus reflecting the whole milk production from quarters or cow. In Norway, standard procedure is analysing SCC in composite cow milk samples bimonthly, Results from these analyses are presented to farmers on a Health Periodical report made by the National Cattle Health Services, giving them regular information of udder health status.

Subclinical mastitis diagnostics are done by herdsmen and veterinarians at the farms. In these cases, samples are collected at different times between milking, and alveolar milk and cisternal milk are sampled randomly. There exists no standard procedure in Norway for collecting samples for CMT testing between milking.

The aim of this trial was to investigate whether one milk fraction is representative enough to evaluate the udder health status in clinical healthy cows.

Materials and methods

Seven Norwegian Red Cattle cows in full lactation were sampled at four different days. In total 20 quarter milk samples.

The cows were kept in tie stalls and milked twice daily and the milk samples were collected during morning milking. The milk samples were fractionated and collected into 120 mL tubes.

Cisternal milk was drained from the front quarters using a catheter. When the milk flow from the drained quarter stopped, the milking machine was applied to the three remaining quarters. During milking, the alveolar milk was fractionated and collected from the catheter.

Day 1: in addition to cisternal and alveolar milk, samples were collected by hand from the catheter drained quarter two, four, six and eight hours after milking.

Day 2: in addition to cisternal and alveolar milk, oxytocin was administered intravenously (i.v.) after milking to obtain residual milk.

Day 3: only cisternal and alveolar milk was sampled.

Day 4: in addition to cisternal and alveolar milk, samples were collected three, six and nine hours after milking.

Milk samples were preserved by Bronopol® and stored at 4°C until analysis. Fat, protein and lactose were determined using Milcoscan 255 (Foss Electric, Hillerød, Denmark). Somatic cell count was measured by Fossomatic 215 (Foss Electric).

Results

Table 1 show SCC results (SCC*1000) in different milk fractions from all samples, max /min values for all fractions of cisternal,alveolar milk and residual milk.SCC is calculated for quarter composite sample.

SCC in samples manually collected 3, 6 and 9 hours after milking show a higher level than the cisternal and alveolar SCC during milking SCC > 400-500.000 pr. mL seemed to correspond with a decrease in lactose content in the same fraction. This can be explained

Table 1. SCC results.

Cow - sampling day	SCC in composite quarter sample	Cisternal milk SCC min/max	Alveolar milk SCC min/max	Residual milk SCC min/max	Hours after milking					
					2	3	4	6	8	9
388-1	10	10/10	10/10		50		30	20	20	
388-2	10	10/10	10/20	90/150						
359-1	11	10/30	10/50		50		160	140	70	
220-4	11	10/10	10/20			80		30		20
220-2	12	10/10	10/30			70		140		
359-2	21	10/40	10/70	90/170						
273-3	26	10/40	30/50							
359-4	28	10/30	10/160			180		100		80
2007-2	37	10/120	30/80	300/400				140		
359-3	37	10/80	20/130							
266-2	38	10/90	30/120					420		
273-2	38	20/50	10/100							
2007-4	45	20/130	30/90			220		240		130
266-4	65	20/130	30/170			720		590		240
388-4	182	30/380	20/820			1980		1310		530
388-3	191	20/410	60/540							
308-1	310	110/1260	230/1060		1370		2350	870	590	
308-4	390	50/1550	150/1300			2580		2150		1190
308-3	614	80/1390	300/1400							
308-2	633	530/1540	160/1360	1810/2890						

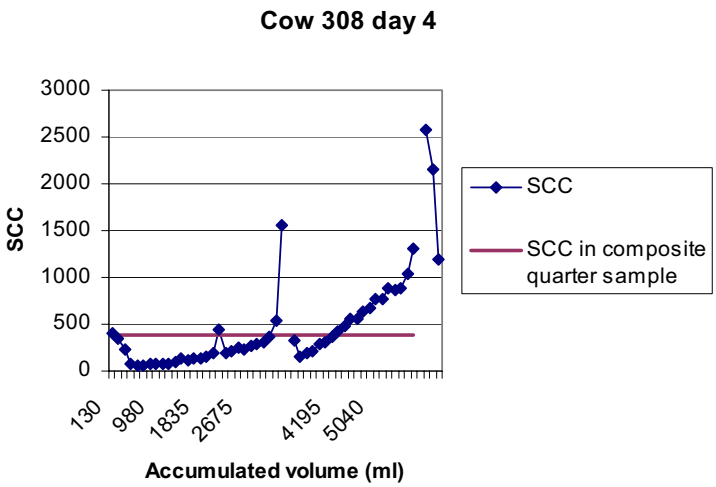

Figure 1. Variation in SCC in different fractions during milking compared to SCC calculated for the quarter composite sample. The break on the graph separates cisternal and alveolar milk i.e. the onset of the milking maschine.

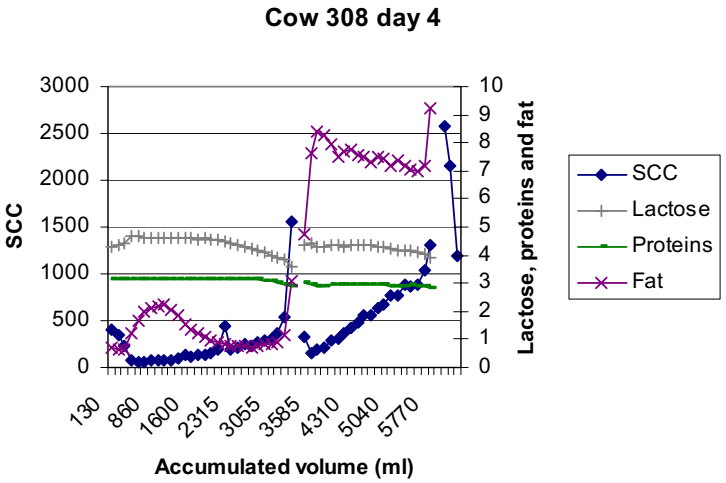

Figure 2. Variation in milk composition during milking.

by a damage in the alveolar epitelium resulting in producing less lactose. The changes in SCC , fat, protein, lactose were in accordance with earlier studies. (Vangroenweghe 2002, Ontsuoka *et al.*, 2003, Ostensen 1993, Paape and Tucker 1966).

Discussion

Our results indicate careful interpreting of SCC measured between milking, especially from healthy cows with elevated SCC, probably above 200.000 cells/mL

Paape and Tucker showed already in 1965 that SCC in strippings and recidual milk was significantly greater than in other milk fractions. They measured nine 20 mL fractions, alveolar- and residual milk. First fraction had an average of 840.000cells/mL, the ninth fraction had 590.000cells/mL, the alveolar milk had 1.020.000cells/mL and the recidual milk had 2.490.000 cells/mL.

BMSCC in Norway is low, 113.000 cells/mL inn 2003**.**The Health Periodical is sorting out cows with SCC > 100.000 cells/ml (geometric mean last three samples) This sorting of cows by udder health parameters makes it possible to do adequate decisions regarding treatment, dry cow therapy, culling etc.

Milk fraction sampled for SCC analyse is important for making right decisions.

Low SCC cows showed minor variations of SCC during milking, and the time of collecting milk samples are probably of little practical importance. From clinically healthy cows with elevated SCC, we observed fractional variations that may be of practical importance when evaluating udder health status and making decisions regarding treating, dry cow therapy, culling etc. Further investigations using milk fraction samples from cows with elevated SCC are wanted to learn more about the variation in milk from these cows, especially between milking. It is important to strictly follow the same procedures for SCC analyse to estimate correct prognosis. To control milk fraction variations sampled for analyse, the fractions has

to be specified when interpreting SCC. For optimal accuracy, SCC should be measured in representative milk samples from the entire udder- or quarter milk production.

In addition it would be interesting to investigate the mastitis pathogens distribution in relation to SCC in different milk fractions.

Sufficiently control of preanalytic variation in milk fraction sampled for analyse has to be specified when interpreting SCC. For optimal accuracy, SCC should be measured in representative milk samples from the entire udder- or quarter milk production. SCC measured in composite samples, reported in the Health Periodical from the Norwegian Cattle Health Services should always be used in mastitis advisory work in addition to clinical observations.

Conclusion

To make the most adequate advice regarding udder health, mastitis treatment, dry cow therapy, culling etc. it is important to have adequate information about SCC from composite milk samples. For cows with elevated SCC (above 200.000 cells/mL) it seems even more important because of the observed SCC increase in the hours following after milking. SCC measured in one single milk fraction from such cows should be carefully interpreted.

For sufficient advising one should always supply cow side CMT analysis with SCC measured in composite samples. This information is accessible at the farms Health Report from the Norwegian Cattle Health Services.

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Using milk L (+) lactate as a diagnostic tool to detect bovine mastitis in the early lactation

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Abstract

It is known that L (+) lactate concentrations increase markedly when a quarter becomes mastitic. To evaluate the diagnostic ability of lactate for mastitis detection, 45 German Holstein cows (n=178 quarters) were sampled weekly three times before dry-off and eight times after corresponding calving (early lactation). Quarter foremilk was used for cytobacteriology (IDF standards) while lactate was determined in quarter composite samples. The reference group contained only those animals whose udders (and all their functional quarters) displayed a somatic cell count < 100,000 /ml and were bacteriologically negative during the entire sampling period. Physiologically, lactate levels decrease from colostrum towards a minimum at the 3rd week post partum, then start to rise slightly. Significant differences ($p < 0.05$) in the early lactation were seen between the reference group and a) diseased quarters, b) healthy quarters which were diseased at dry-off and c) quarters being healthy at dry-off and early lactation, but deriving from diseased udders (i.e. at least one other quarter of the udder became mastitic), the latter phenomenon being known as quarter interdependence. Observing a diagnostic accuracy of 70 %, a threshold of 60 µmol/l was established for the early lactation (79 % sensitivity, 67 % specificity). The results suggests that lactate can be an idoneous tool in helping detect mastitis if physiological factors of influence as well the practical sampling problems (fixation in trichloroacetic acid) are taken into account.

Keywords: diagnostic ability, lactate, early lactation, udder interdependence, colostrum

Introduction

Lactate is linked closely to the energy metabolism of cells being crucial for glycolysis and glyconeogenesis. Since it also occurs in bacteria - yet in its D type rather than in the typically mammalian L conformation - lactate was used, together with pyruvate, in order to estimate the total bacterial count in tank milk. This procedure however was abandoned once it became clear that the lactate content in milk is affected by a series of both secretory and post-secretory factors (Riehl 1988).

Regarding lactate and udder health, physiological lactate levels range just above the lower limit of detection (5.0 µmol/l, Grabowski 2000), but once a mastitic condition occurs, markedly higher lactate contents are observed. This has arisen interest regarding the value of lactate for mastitis diagnosis (Davis *et al.*, 2004, Hamann and Krömker 1997, Kitchen 1981, Mackie *et al.*, 1977).

Since most mastitis cases become apparent to the practitioner in the early lactation, the present study seeks to evaluate this parameter as a tool for mastitis detection in the first eight weeks post partum (pp).

Material and methods

A total of 45 Holstein Friesian cows (n = 178 quarters) in two dairy farms was sampled on a weekly basis; sampling pattern included the three weeks prior to dry-off (D1 to D3) and the first eight weeks after the corresponding calving (E1 to E8). While foremilk samples were drawn to assess the udder health by bacteriology and somatic cell count (SCC) applying IDF standards (DVG 2000), lactate was determined in quarter composite samples. For the latter, a quarter milking machine was used. Lactate samples were fixed with trichloroacetic acid right after obtention in order to avoid post-secretory lactate increases. Lactate was determined by means of an AutoAnalyzer system (oxidation of lactate via lactate dehydrogenase into NAD^+ and pyruvate, measuring the emerging NADH photometrically [Bergmeyer 1974]).

Quarter health was assessed by combining bacteriology and SCC (threshold: 100,000 cells/ml) into four health categories (Table 1).

Only quarters with normal secretion were termed "healthy" while the others were defined as "diseased". The daily diagnoses were merged for the dry-off and the early lactation stages. A stage was defined "healthy" when all foremilk samples ranged below 100,000 cells/ml and any pathogen was found only once; otherwise, the stage was considered as diseased. With this system, the quarters were distributed as shown in Table 2.

It was intended to select a very pure group of quarters for physiological reference. Therefore, the udder health at dry-off was considered as pre-condition. As can be seen from Table 2, healthy quarters were sub grouped in those deriving from healthy udders (*i.e.* all

Table 1. Udder health categorization (Hamann and Fehlings, 2002).

Somatic cells [/ml]	Pathogens absent	Pathogens present
< 100,000	normal secretion	latent infection
> 100,000	non-specific mastitis	mastitis

Table 2. Udder health groups and sample sizes.

Group	Quarter health		Udder health	n =
	D1 - D3	E1 - E8		
1a	healthy	healthy	healthy	36
1b	healthy	healthy	diseased	33
2a	healthy	diseased	diseased	14
2b	diseased	healthy	diseased	55
2c	diseased	diseased	diseased	40
				178

Lactate values in µmol/l were skewed and had to be transformed in decade logarithms to obtain normally-distributed data.

quarters were healthy; group 1a) and in those from diseased udders (*i.e.* at least one quarter of the udder was categorized as diseased; group 1b).

SAS (version 8.2) procedures UNIVARIATE, MEANS, GLM and TTEST were applied for the statistical analyses that sought to evaluate possible influences by dairy farm (farm 1 *vs.* farm 2), lactation stage (dry-off *vs.* early lactation), udder health (1a *vs.* 1b) and quarter health (1a *vs.* 2a to 2c). Furthermore, the diagnostic ability was evaluated as diagnostic accuracy (minimum 70 %).

Results

Sample sizes are shown in Table 2.

Factors of influence

All factors but dairy farm exerted a significant influence ($p < 0.05$) on the lactate contents. Significant differences were therefore also found between healthy quarters from healthy udders and those from diseased udders, a mean difference amounting to 175 µmol/l.

Physiological reference

Since groups 1a and 1b differed, the physiological reference was calculated only from 1a (Figure 1).

Threshold determination

Applying the requirements mentioned above, a threshold of 1.7 lg of µmol/l (= 60 µmol/l) was established for the entire early lactation, yielding a sensitivity and a specificity of 78.9 % and 67.2 %, resp. The maximum diagnostic security of 82.0 % was achieved at 400 µmol/l and displayed an enhanced specificity (91.4 %), but also a markedly reduced sensitivity (53.8 %).

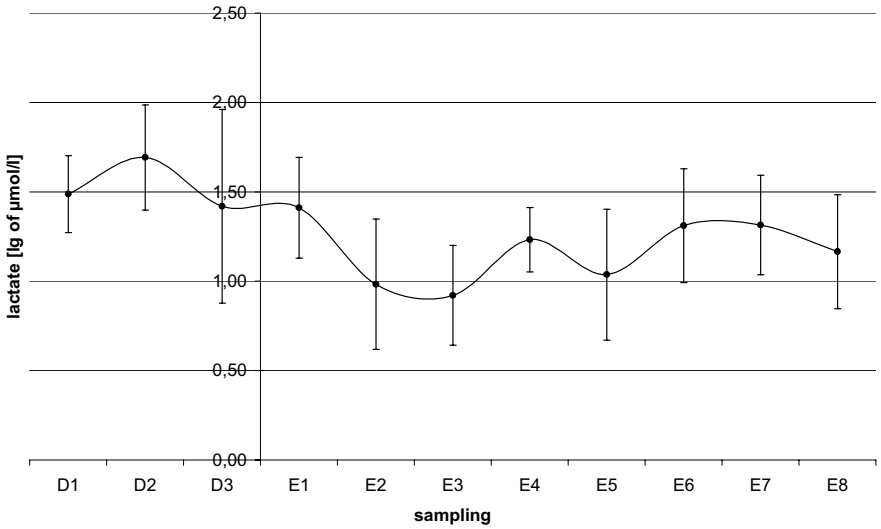

Figure 1. Lactate concentrations in the reference group 1a (n = 36).

Discussion

Factors of influence

It was expected that the farm of origin would not represent a meaningful factor of influence and that the lactation stage would do so; the results of previous works (Davis *et al.*, 2004, Mackie *et al.*, 1977) clearly suggest the latter condition. In fact it was seen (Grabowski 2000, Redetzky 2000) that lactate levels decrease from the colostral phase to a minimum in the 3rd week pp, and then increase slightly towards dry-off.

Significant differences were found among healthy quarters deriving from healthy and diseased udders, suggesting udder interdependence. This phenomenon was already observed in other milk parameters (Hamann *et al.*, 2003, Merle 2003, Woolford 1985).

With regard to udder health, it was expected that 1a would differ from 2a and 2b in the early lactation, being these the groups with an actual udder inflammation. However, all diseased groups differed significantly ($p < 0.05$) in relation to the reference group. This means that a previous inflammation, supposedly cured during dry period, still exerts a significant influence on the lactate levels of supposedly healthy quarters in the early lactation. This sensitiveness may reduce the potential of lactate as an efficient tool for mastitis detection.

Physiological reference

Comparing lactate concentrations of "healthy" quarters or udders from different authors is difficult since methods and, moreover, definitions of "healthy" vary greatly. With 2.17 lg of µmol/l, the results of Mackie *et al.* (1977) are much higher than the data obtained in this paper; however, these authors did not consider udder health at all. Davis *et al.* (2004) draw comparable conclusions like Mackie *et al.* (1977) did, but their SCC ranged about 185,000 cells/ml. This value would be considered "diseased" with the categorization scheme used here, and increased lactate levels would be expected.

Lactate and mastitis diagnosis

Several authors have described the rapid increases in lactate concentration during mastitis (Davis *et al.* 2004, Hamann and Krömker 1997, Kitchen 1981, Mackie *et al.* 1977), suggesting this parameter to be a good indicator for udder health. However, a series of problems obstaculizes the indiscriminate use of lactate in mastitis diagnosis:

- A relatively high standard variation within the physiological range (see Figure 1), despite a low coefficient of variation for the method (< 2.5 %), possibly due to a series of physiological factors influencing lactate concentrations (including the health status of the udder);
- the fact that previous mastitis cases still seem to cause lactate increases in quarters with SCC < 100,000 cell/ml, and
- the danger of false results when the sample is not fixed after obtention in order to avoid post-secretory increases.

Of those, the latter may be eliminated by proper handling and sampling, while the others must be taken into account. When it comes to establish a threshold, it is vital to set the expectation linked with it. Since it was shown that the costs produced by undetected, subclinically diseased quarters are higher than those originated from falsely classifying

healthy udders as diseased ones (and therefore treating them; Hamann and Fehlings 2002), stress must laid upon a high sensitivity. This goal was achieved with the establishment of 60 µmol/l (1.7 lg of µmol/l) for threshold in the early lactation, yielding a sensitivity of approx. 80 % and a diagnostic accuracy of 70 %. This value is below the threshold suggested by Davis *et al*. (2004). Different SCC levels as well as a different lactation stage make a direct comparison difficult. In comparison to other diagnostic tools, sensitivity of lactate ranges between that of cow SCC (72.4 %), electrical conductivity (80.6 %) and the California Mastitis Test (93.7 %; Redetzky and Hamann 2003).

Conclusions

The study sought to evaluate the diagnostic capacity of lactate. The data shown here suggests that lactate concentrations may increase for a series of secretory and post-secretory, physiological and pathological reasons. Still, lactate has proven to be a promising parameter, and a corresponding threshold for the early lactation could be established. When using lactate as a supporting parameter for mastitis diagnosis, special attention must be paid to the physiological factors of influence. On a practical level, samples should always be fixed properly, e.g. with trichloroacetic acid.

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Milk amyloid A (MAA) concentration and somatic cell count (SCC) in the diagnosis of bovine mastitis

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Abstract

One-hundred-thirty-one dairy cows from 5 herds in the Central Valley of California were selected based on somatic cell count and mastitis records, days in milk in the current lactation and lactation group to ensure an adequate spectrum of cases. All cows were clinically examined and milk samples were obtained from 2 contra-lateral quarters. From cows with clinical signs of mastitis, the affected and the contra-lateral quarter were sampled. The milk was submitted for microbiology, somatic cell count (SCC) and milk amyloid A (MAA) testing. If clinical signs were present or culturing revealed mastitis agents, the quarter was classified as mastitic. If no clinical signs were present and the culture was negative, the quarter was classified as healthy. If multiple cultures were present, the sample was classified as contaminated.

We found that the SCC and MAA-concentration were not correlated (n=254, $R^2 = 0.08$). ROC-curves demonstrated an area under the curve (AUC) of 0.80 for MAA and 0.77 for SCC (n=230). These areas were not statistically different. This indicates that despite no overall difference in diagnostic performance, the SCC and MAA may have different diagnostic potentials that could depend on factors such as causative agents or degree of tissue damage

Keywords: milk amyloid A, somatic cell count, diagnostic performance

Introduction

Diagnosing clinical mastitis on dairies usually relies on the milkers ability to detect changes in milk composition. However, some infections show only minor or no changes and the detection of these sub-clinical infections often relies on detecting increases in the number of somatic cells present in the milk. As the milk somatic cell count (SCC) can be affected by non-pathological factors such as lactation stage (Brolund 1985) and milking intervals (Hamann 2001), other potential mastitis indicators not affected by non-mastitic factors could improve udder health.

Acute phase proteins are serum proteins that undergo substantial changes in concentration following infection, inflammation and trauma (Kushner and Mackiewicz 1987). A local synthesis of a milk specific acute phase protein (milk amyloid A (MAA)) in the inflamed udder has been reported (Eckersall *et al.*, 2001; McDonald *et al.*, 2001; Jacobsen

et al., 2005) and experimental udder infections have indicated a promising potential of MAA as mastitis indicator (Grönlund *et al.*, 2003; Pedersen *et al.*, 2003; Lehtolainen *et al.*, 2004). The aim of this study was to test the diagnostic performance of MAA when applied in conventional dairies.

Materials and methods

One-hundred-thirty-one dairy cows from 5 herds in the Central Valley of California were selected based on somatic cell count and mastitis records, days in milk in the current lactation and lactation group to ensure an adequate spectrum of cases. All cows were clinically examined and milk samples were obtained from 2 contra-lateral quarters. From cows with clinical signs of mastitis, the affected and the contra-lateral quarter were sampled.

Milk was submitted for microbiology (Milk Quality Laboratory, Tulare, California) and somatic cell count (DHIA, Tulare, California). The concentration of milk MAA was determined using a commercial immunochemical assay (Tridelta ltd, Wicklow, Ireland).

If clinical signs were present or culturing revealed mastitis agents, the quarter was classified as mastitic. If no clinical signs were observed and the sample revealed a pure culture of a mastitis agent, the quarter was classified as sub-clinically infected. If no clinical signs were present and the culture was negative, the quarter was classified as healthy. If multiple cultures were present, the sample was classified as contaminated.

The milk SCC and MAA was compared at quarter level using linear regression. The diagnostic performance of SCC and MAA were assessed by Receiver-Operating-Characteristic Curve analysis after excluding contaminated samples.

Results

Mastitis was diagnosed in 42 (30 clinical and 12 sub-clinical) of 254 quarters. The SCC and MAA-concentration at quarter level was not correlated ($r^2 = 0.08$).

ROC-curves demonstrated an area under the curve (AUC) of 0.80 for MAA and 0.77 for SCC (Table 1). The AUCs were not statistically different. MAA was more sensitive and less specific than the SCC. The optimal cut-off values calculated in this study were 5854 mg/mL (MAA) and $957x10^3$ cells/mL (SCC).

Table 1. Performance of milk MAA and SCC in diagnosing mastitis (n=254).

	MAA	SCC
Mastitic samples	42	42
Negative samples	210	210
AUC	0.80	0.77
95% CI of AUC	0.75-0.85	0.74-0.84
Cut-off	5854 mg/ml	$957x10^3$ cells/ml
Sensitivity	86.0	69.0
Specificity	74.5	84.8

Discussion

The overall diagnostic performance of MAA and SCC expressed using the AUC was not different. However, as we found no correlation between milk SCC and milk MAA at quarter level in agreement with previous field studies (Eckersall *et al.*, 2001, Nielsen *et al.*, 2004), the SCC and MAA are not identical mastitis parameters. The diagnostic potential of the parameters may depend on factors such as causative agents, milk kinetics during inflammation or degree of tissue damage. However, the present study does not provide data sufficient for such conclusions.

Only mild to moderate cases were diagnosed among the cows included in the study. Therefore spectrum bias may reduce the calculated sensitivity and specificity as severe cases are easier to diagnose correctly. Despite this possible bias, the sensitivity and specificity of MAA was acceptable with MAA as the most sensitive and SCC the most specific mastitis parameter.

The cut-off values of both SCC and MAA reported here are higher than expected (Dohoo and Leslie 1991; Grønlund *et al.*, 2003). However, the selection criterias may bias the cut-off value as cows were not selected at random. A relatively high number of cows with high SCC were included. Also, only one milk sample per quarter was submitted for microbiology, and therefore sub-clinical cases with possible high SCC and MAA concentration may have been misclassified as healthy.

Conclusion

Our results indicate that despite no overall difference in diagnostic performance, the SCC and MAA have different diagnostic potentials that could depend on factors such as the nature of the causative agents, milk kinetics during inflammation or degree of tissue damage.

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Diagnosis of mastitis by benzoic acid (benzene sulfonic acid)and sodium carbonate on pregnant and lactating mastitic animals

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Abstract

Mastitis diagnosis in sub clinical cases of cattle and buffalo especially in the last trimester of the pregnancy has a prime importance and has great value regarding the control and treatment of the mastitic animals in time. For the diagnosis of mastitis 130 animals cows and buffalo cows were divided in to three groups, Group A, Group B and group C randomly and for sub clinical mastitis in cows and buffalo cows 20 animals of group B in last trimester of pregnancy specially in the early 8^{th} month were selected randomly. The animals were physically normal and their udder was normal and developing. Their 1 ml of colostrum was squeezed and mixed in the Petri-dish with equal volume of solution containing sodium carbonate and benzene sulfonic acid dissolved in distilled water. The test could compete the California Mastitis Test (CMT). This test could be the cheapest and easily made by the farmers and field veterinarians for the early diagnosis of the mastitis in pregnant and lactating mastitic animals.

Keywords: diagnosis, sodium carbonate, benzene sulfonic acid, somatic cell count

Introduction

In scientific terms subclinical mastitis is defined as "increase in number of somatic cells in milk" (Khan, 1997; Schukken *et al.*, 1989). This increase in somatic cells can be used for the diagnosis of subclinical mastitis. In Pakistani cattle sub clinical mastitis is prevalent (Khan, 1997). Various tests are performed to diagnose sub clinical mastitis like California Mastitis Test (CMT) and bacterial count (Radostitis *et al.*, 1994). Some tests are complicated and not easy to adopt in routines and at farm level like the Electric Conductivity meter (EC) (Musser *et al.*, 1998), while some are very simple that give no more information like white side test. Keeping in the view these all hurdles some chemicals were tested on milk for mastitis that could help to guide us during the increase in somatic cells and indicate mastitis subclinical or clinical mastitis. For these two easily available chemicals sodium carbonate ($Na_2 CO_3$) and benzene sulfonic acid ($C_6H_5SO_3H$) were found helpful in combination for the diagnosis test that could be utilized in mastitis. The objective of this study was to know other chemicals that could work on increased somatic cells of milk other than sodium-alkyl-aryl-sulfonate, potassium hydroxide and sodium hydroxide.

Materials and methods

Preparation of solution

Five grams of sodium carbonate (Na_2CO_3) was added to one liter of non ionized distilled water at pH 11(Solution A). In the same manner commercially available benzene sulfonic acid at pH 1 and was mixed with non ionized distilled water at the ratio of 1:26 and the pH was adjusted at 2 (Solution B).These two solutions were taken equal in volume and mixed in a container (Solution C).

Mastitic experimental animals

One hundred and thirty animals including cows and buffalo cows were included in the study. Animals were divided in to three groups, Group A, Group B and group C. In group A a total of hundred animals were kept, among them 50 lactating cows and 50 lactating buffalo cows were divided and their milk was used while in Group B twenty animals were kept, among them 10 pregnant cows and 10 pregnant buffalo cows were used and they were in the last trimester of their pregnancy. The group C contained 10 healthy female cows and buffalos and was kept as control. One ml of milk from animals of group A, showing clinical mastitis and showing symptoms of inflammation of udder and One ml of colostrum like fluid from the teats of group B was taken in sterilized test tubes. The teats were washed aseptically before and after milking in all the groups.

Test procedure

One ml of milk/colostrum like material was taken in Petri dish and added with one ml of solution C, mixed thoroughly and tilted gently for one minute. A jelly like material was obtained in the Petri-dish and the results were recorded and analysed statistically by average and percentage method. (Steel and Torrie, 1982).

Results

When the milk from mastic animals and pregnant healthy animals was tested for the mastitis the results of various degrees of mastitis were found that were noted and explained. In group A 50 cows and 50 buffalo cows were placed and these animals were showing clinical mastitis and symptoms like inflammation of udder, shreds and pus like material was oozing from the udder were observed. The mastitis test also exhibited the various degrees of mastitis by forming jelly as presented in table 2 and 70 % of the animals of group A and 50 % of group B were found mastitis positive while Group C, control did not show any symptoms and were absolutely normal as in Table 1.

The positive milk was denoted sign to show the severity of the disease in various animals. The (+) was less severe or minute mastitis and showing less numbers of somatic cells in

Table 1. Animals of group A and group B

Groups	Positive cases	Percentage
Group A (n=100)	70	70
Group B (n=20)	10	50
Group C (n=10)	0	0

Table 2. Test differentiating various degrees of mastitis.

Milk sample of Group	Differentiation of various degrees of mastitis				
	No. of animals	Normal	+	++	+++
Group A	100	30	40	20	10
Group B	20	10	10	-	-
Group C	10	10	-	-	-

milk or sub clinical mastitis, (++) was moderate and exhibiting more numbers of somatic cells in milk or clinical mastitis and (+++) was more severe or pus containing and showing loss of udder.

Discussion

Infection from pathogens or mastitis causing factors causes irritation in the udder of female animals that results in the production of higher somatic cells (neutrophils). The active principle in the mastitis milk is deoxy ribonucleic acid (DNA) of somatic cells. The presence of sodium carbonate in the solution liquefy or dissolve DNA of somatic cells and release it in the solution for gel formation with benzene sulfonic acid (A foaming agent). By the number of somatic cells various degrees of infection are detected (Schalm *et al.*, 1971). Diagnosis of mastitis remained always a complicated and professional job in the field for the farmers (Musser *et al.*, 1998). The tests working presently do not give the detail about the severity of the mastitis. The most serious but mostly un noticed form is sub clinical form of mastitis in pregnant cows and buffalo cows in their last trimester of pregnancy that is a serious problem and noticed commonly in Pakistan (Khan, 1997) and results in the complete loss of the udder after the termination of the pregnancy. Sodium carbonate is strong alkali (Base) that commonly used in detergents while benzene sulfonic acid is used as intermediate in manufacturing of certain dyes and drugs as well as foaming agent (Bahl, 1964 and Mark *et al.*, 1983). The working of this test is in accordance with the test of Muhammad *et al.*, 1995 who compared their test with California Mastitis Test (CMT). These types of cheap and reliable tests could be an adjunct for the diagnosis as well as differentiation of the various stages of the sub clinical or clinical mastitis in lactating or pregnant cows or buffalo cows of developing or developed countries and could be substitute for expensive and complicated test for the farmers.

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Use of on-farm testing of somatic cell count for selection of udder quarters for bacteriological culturing

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Abstract

In this study the usefulness of direct (Delaval cell counter DCC) and indirect (California mastitis test CMT) measurement of somatic cell count (SCC) to detect an intramammary infection (IMI) caused by a major pathogen was evaluated and compared. Bacteriological culture was the gold standard. The DCC was recently introduced on the market and is an on-farm test to measure directly the SCC in milk. It measures the number of somatic cells in milk in a fast (40 seconds), reliable and precise way. Samples were taken from 131 cows from six herds. Selected cows had two or more consecutive test days with a cow composite SCC higher than 250,000 cells/ml, measured during milk production recording. Quarter milk samples were taken for SCC measurement and bacteriological culturing. Bacteriological culturing of quarter milk samples identified IMI in 19.8% of the quarters. The usefulness of direct and indirect measurement of quarter SCC for screening was evaluated by calculating the sensitivity and specificity for various threshold values. Both methods, direct and indirect measurement of quarter milk SCC showed decreasing sensitivity and increasing specificity at increasing threshold levels. Sensitivity of the direct measurement of SCC (DCC) at the threshold levels of 100.000 and 250.000 cell/ml were higher than the sensitivity of indirect measurement of SCC. Using a threshold of 250,000 cells/ml for direct measurement of SCC in quarter milk, resulted in a sensitivity and specificity for detecting positive bacteriological culturing of 74.4 and 58.1 respectively. Evaluation of the indirect measurement of SCC (using CMT) with a threshold > ++ showed a sensitivity and specificity of 46.3 and 81.9 respectively.

On-farm measurement of SCC in quarter milk is useful to select udder quarters for bacteriological culturing in cows with an increased composite SCC (higher than 250.000 cells/ml) for two consecutive test days. The performance of direct measurement of SCC (using DCC) for this purpose was better than the use of indirect measurement of SCC (using CMT).

Introduction

Mastitis still is the most common and costly disease of cattle. In the Netherlands many control measures are implicated in order to prevent new intramammary infections (IMI) and/or to diagnose and cure new infections as soon as needed. One of the control strategies is taking quarter milk samples from cows with a SCC above 250,000 cells/ml for bacteriological culture. A scheme for doing so is running in the Netherlands for many years and gives interesting insight in the pathogens causing subclinical mastitis (Poelarends *et al.*, 2001). Knowing the causing pathogen might be beneficial for the choice of treatment or might help in making other management decisions like segregated milking or even culling.

The most reliable results will be achieved when from each high SCC cow four quarter samples are taken for bacteriological culture. Since many cows with high SCC only have one or two quarters infected it is expensive and time consuming to take four samples of each cow. Many studies have showed the advantage of using the California Mastitis Test (CMT) in selecting the infected quarters (Middleton *et al.*, 2000; Sargeant *et al.*, 2001; Dingwell *et al.*, 2003; Poutrel and Rainard, 1981). The CMT is a qualitative method that differentiates between cell counts lower or higher than 500.000 cells/ml. The CMT needs experienced technicians and interpretation is more or less subjective.

Recently the DeLaval cell counter DCC (DeLaval, Tumba, Sweden) was introduced on the market, making it possible to measure milk somatic cells directly on the farm. Each single measurement takes approximately 40 sec. The measuring performance of the DCC is at the same level as of other methods using similar staining of the DNA of the cells. The principle of the DCC is based upon counting the stained DNA by means of a CCD camera. Results of the test are given in number of cells/ml and are therefore easy to interpret. Therefore, DCC might be a good alternative for CMT in selecting infected quarters for bacteriological culturing.

The aim of this study was to evaluate the usefulness of measuring somatic cells to identify udder quarters for which bacteriological culture is appropriate by direct counting with the DCC in comparison with indirect counting by CMT.

Material and methods

Farms and animals

Animals were selected from six different farms belonging to the Ambulatory Clinic of the Faculty of Veterinary Medicine, Utrecht, the Netherlands. The farms were selected because of a high incidence of new IMI within the herd. Cows were selected based on the results of the Milk Production Recording (MPR). Cows were sampled when cow composite SCC was higher than 250.000 cell/ml on at least two consecutive test days.

Sampling strategy

Quarter foremilk samples were collected aseptically by two trained students for CMT and SCC analysis. All samples were taken in the milking parlour before milking, at the morning or the evening milking. Before collection the first five squirts of milk were discarded and the teat ends were disinfected. Per quarter, two samples were taken directly after each other. One of the samples was used to test the somatic cell count when the milking was finished. First the sample was used to determine the CMT score, then the remaining milk in this sample was used for the DCC test. Before measurements, the milk in the sample was thoroughly mixed. CMT was always performed before the DCC in order to avoid interpretation bias of the CMT by already knowing the SCC results. Both measurements were single and performed on the farm. Each second sample per quarter was transported to the Veterinary Medicine Diagnostic Center (VMDC) from the Faculty of Veterinary Medicine within five hours after sampling.

Analysis of samples

The CMT was performed on quarter foremilk samples and the test results were read and recorded by two trained students on all the six herds. The CMT reaction was recorded as 0 (negative), 1 (trace or +), 2 (++), or 3 (+++). Immediately after the CMT measurement, the

DCC measurements were carried out on the same quarter foremilk samples as used for the CMT measurements. Handling of milk and cassettes were done in accordance with the guidelines in the instruction book of the DCC. Since the milk samples were taken during routine milking, the time between milk sampling and somatic cell count measurement depended on the milking time and the position of the cow in the milking order. The time between sampling and somatic cell count measurement was never longer than 1.5 hours.

The milk, sampled for bacteriological analysis was handled, cultured and interpreted following the procedures of the National Mastitis Council (1987). An IMI was defined as the presence of a major mastitic organism, including *Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis,* nonspeciated streptococci, *Escherichia coli*, Klebsiella spp., and Arcanobacterium pyogenes. *Corynebacterium bovis* and coagulase- negative staphylococci were considered minor pathogens. Samples with growth of 3 or more pathogens which means contaminated were excluded.

Results

Data were collected form November 2003 to December 2004. From 131 cows a total of 414 quarter measurements (indirect SCC with CMT and direct SCC with DCC) and culture results were available for analysis as summarized in Table 1.

In 88 quarter samples growth of a major pathogen was shown, which means that IMI was identified in 19.8 % of all quarters.

Sensitivity, specificity, positive and negative predictive value for both indirect (CMT) and direct (DCC) measurement of SCC were calculated for different thresholds. Table 2 shows the results for indirect and direct measurement of SCC with bacteriological culture as the gold standard.

Table 1. Comparison of different methods to measure SCC in quarter samples from cows which had a cow composite SCC > 250.000 cells/ml in two subsequent test day records

Indirect measurement (CMT)	Number of samples	Range (mean) of direct measurements (DCC)	# major pathogens (%)
CMT 0	199	0 - 958 (147)	27 (13.6)
CMT +	117	8 - 4,205 (386)	17 (14.5)
CMT ++	65	135 - 5,260 (1,148)	22 (33.8)
CMT +++	34	437 - 6,858 (2,590)	16 (47.0)

Table 2. Test characteristics of indirect (CMT) and direct (DCC) measurement of SCC at various threshold levels calculated for quarter samples from cows with twice or more SCC > 250.000 cell/ml measured at the MPR

	Indirect measurement			Direct measurement (* 1,000 cells/ml)				
	≥ +	≥ ++	≥ +++	≥ 100	≥ 250	≥ 500	≥ 750	≥ 1,000
Sensitivity (%)	67.0	46.3	19.5	90.2	74.4	52.4	40.2	28.0
Specificity (%)	51.8	81.9	94.5	34.7	58.1	77.4	86.1	91.2
Pos predictive value (%)	25.6	38.7	47.0	25.4	30.5	36.4	41.7	44.2
Neg predictive value (%)	86.4	86.0	82.6	93.5	90.2	86.6	85.3	83.7

As can be expected, for both indirect and direct measurement of SCC, the sensitivity decreased and the specificity increased with increasing threshold values (Figure 1). If we consider sensitivity and specificity equally important (which is in many practical situations not the case), results indicate that the optimal sensitivity and specificity for this population of cows was seen at a threshold of ≥ 250.000 cells/ml for direct measurement of SCC and at a threshold of ≥ ++ for indirect measurement of SCC using CMT.

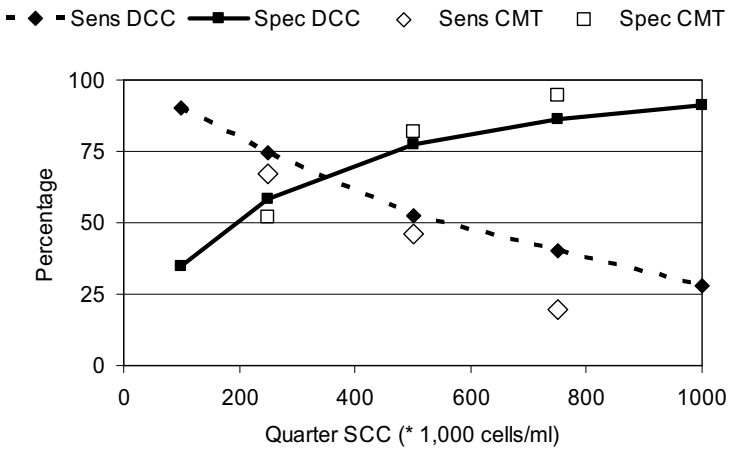

Figure 1. Sensitivity and specificity of direct (DCC) and indirect (CMT) measurement of SCC for various thresholds. The thresholds for CMT (≥ +, ≥ ++ and ≥ +++) are given as 250,000 cells/ml, 500,000 cells/ml and 750,000 cells/ml.

Discussion

Several studies showed the usefulness of indirect measurement of SCC using CMT in identifying infected quarters for bacteriological culture (Dingwell *et al.*, 2003, Sargeant *et al.*, 2001). However CMT measures SCC in milk indirectly. Moreover, the results are given in a qualitative way and therefore needs experienced technicians or farmers to interpret the test. The advantage of the CMT is that it is inexpensive and that the test provides real time results (Sergeant *et al.,* 2001). Compared to SCC measurements at central laboratories, results from CMT are directly available which means no time lack between sampling and results. Recently the DCC was developed as an on-farm test to measure SCC in milk (either bulk milk, cow composite milk or quarter milk). Results are given in the exact number of cells/ml and there is no time lack between sampling and results. Direct measurement of SCC by the use of DCC has all the advantages of CMT, but is more objective to perform and to interpret. In this study direct measurement of quarter milk SCC showed to be a useful test in identifying infected quarters for bacteriological examination, when a cow has an elevated composite SCC.

In this study the used golden standard was bacteriological examination. It is known that with this methodology not all existing pathogens can be detected (the sensitivity of bacteriological examination is not 100 %). Although this lack of diagnostic accuracy has consequences for the usefulness of bacteriological examination for problem diagnosis at the dairy farm, it has not a problem in the interpretation of the results found in this study.

In this study, bacteriological culturing and SCC measurements were carried out in different milk samples. There might be variation caused by the use of the different samples. However, because the samples were taken one squirt from each other after removal of the first five squirts of milk before milking, the variation will be relatively small. The alternative, doing SCC measurements and bacteriological culturing from one sample would incur the risk of contamination which makes the sample useless for bacteriological culturing.

Both, direct and indirect on-farm measurement of SCC can be used to select udder quarters for bacteriological sampling. When comparing sensitivity and specificity of direct and CMT at selected threshold levels, direct measurement showed to have a higher sensitivity than the indirect method, whereas the specificity was roughly the same. With both methods, selecting quarters for bacteriological culturing will lead to a lower level of identified quarters with major pathogens; the sensitivity is not 100 %. However, when screening, a lower number of samples has to be sent in for bacteriological culturing, which saves money. Whether screening is beneficial depends on the price of bacteriological culturing and the economic damage caused when missing a quarter infected with a major pathogen. There are hardly any studies on the cost-effectiveness of management associated with subclinical mastitis at the cow level. Recently few studies on treatment of subclinical mastitis caused by *Streptococcus uberis* and/or *Streptococcus dysgalactiae* (Swinkels *et al*., 2005; Hogeveen *et al*., 2005).

From this study it can be concluded that both direct (using the DCC) and indirect (using the CMT) on-farm measurement of SCC can very well be used to select udder quarters of cows with a high somatic cell count for bacteriological culturing. The performance of direct measurement is better than the performance of indirect measurement.

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Diagnostic value of the California Mastitis Test in comparison to electronically-counted somatic cells in bovine milk

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Abstract

There is still a strong need for suitable, quick - and preferably inexpensive - diagnostic tests to screen the herd at quarter level and at shorter intervals for rapid assessment of udder health status that will allow immediate therapeutic or management-related action. The California Mastitis Test makes it possible to detect subclinically inflamed udder quarters, even in subclinical mastitis cases with somatic cell counts only slightly above the physiological threshold of 100,000 cells/ml.

Keywords: California mastitis test (CMT), cow-side test, somatic cell count (SCC), intramammary infections (IMI), cut-off level

Introduction

Determination of udder quarter health status requires measurements to be made of the presence or absence of microbiological pathogens in the milk and of inflammation-related changes. The somatic cell count (SCC) has been chosen as the best indicator for the inflammatory response (Griffin *et al.*, 1987). The methods used depend on the objectives of the work: While surveys and control programmes can be carried out with indirect methods, experimental and observational studies require direct measurements of the SCC in foremilk samples, preferably using electronic methods (IDF, 1987). However, with the exception of the Direct Cell Counter DCC (DeLaval International AB, Tumba, Sweden) electronic counting systems for somatic cells are laboratory-based and therefore time-consuming. Furthermore, all direct cell counting methods are costly in comparison to indirect tests. In light of these disadvantages, the cow-side California Mastitis Test (CMT) appears to be an attractive alternative which could enable bovine practitioners to detect inflamed udder quarters promptly for immediate therapeutic or management-related action. Since the application of external or internal teat sealants has made prevention of mastitis more important, a paradigm shift has taken place: diagnostic procedures, methods or tests (e.g. CMT) are now used with the intent to detect "healthy" cows (Woolford *et al.*,1998; Huxley *et al.*, 2002), i.e. those with four normally secreting udder quarters. Concerning the CMT, already in 1957 Schalm and Noorlander pointed out: "In normal foremilk, that is milk negative to the test, the mean total count did not exceed 100,000 cells per millilitre of milk [...]." This observation has since been confirmed by numerous authors for various aspects of the characterisation of the physiological pattern of milk secretions including the concentration

of cells (Doggweiler and Hess, 1983), milk composition (Tolle, 1970; Hamann, 2002), milk processability (Barbano *et al.*, 1991), and yield (Raubertas and Shook, 1982). Latest information on the basis of 178,374 cow composite milk samples (CCM) clearly demonstrated that even an increase in SCC from below 50,000 cells up to 100,000 cells/ml CCM resulted in milk losses of three percent (Jahnke, 2004).

The CMT is based on a precipitate-forming reaction between a reagent and the somatic cells (i.e. the amount of DNA) present in the milk. After mixing the test reagent with an equal quantity of milk, the degree of visible reaction is scored as "Negative" (0), "Trace" (T), or "Positive" (1, 2 or 3). Considering several reports (Schalm, 1960; Marshall *et al.*, 1993; NMC, 1999), the CMT scores seem not satisfactorily differentiate between inflamed and uninflamed udder quarters on the basis of an SCC threshold of 100,000 cells/ml. In that case, the CMT would not be an appropriate diagnostic test for the determination of udder quarter health. Therefore, the present study is concerned with an evaluation of the diagnostic potential of the California Mastitis Test performed under defined laboratory conditions.

Material and methods

This study included a total of 107 German Holstein cows at different lactation stages and lactation numbers and 1,426 samples of quarter foremilk (QFM) from clinically inconspicuous quarters and 25 QFM samples from clinically affected ones. Samplings were always performed at morning milking time. The first milk jets were discarded, the cow's teats were wiped clean with dry paper tissues and the teat apices were disinfected with ethyl alcohol (70% vol.). After complete milk ejection, approx. 10 ml foremilk per lactating quarter were milked separately into a glass tube. Within two hours, samples were brought to the Institute and stored in a cooling chamber (+4 °C). Microbiological examinations were performed according to the German Veterinary Medicine Society guidelines for isolating and identifying mastitis pathogens (DVG, 2000). The somatic cells were counted with a Fossomatic 360 (Foss, Hillerod, Denmark). According to IDF, the udder health status was categorised as "Normal secretion", "Latent infection", "Unspecific mastitis" or "Mastitis" (IDF, 1967) with an SCC threshold of 100,000 cells/ml QFM. Each diagnosis at quarter level was based on the cytological and microbiological findings of one sampling day.

Under laboratory conditions, the mixing ratio between CMT reagent and test milk was precisely 1:1. Equal volumes of 2 ml of CMT reagent and test milk were transferred with pipettors (Eppendorf AG, Hamburg, Germany) into the cups of the testing paddle (definite volume of mixture per cup: 4 ml). Milk and reagent were mixed by gently moving the paddle in a swaying motion. The testing paddle was tilted and one-half of the mixture was decanted to make it easier to detect minor changes. Readings were made while tilting the paddle for 20 seconds under good lighting conditions. A more detailed CMT scoring was defined in order to take into account the internationally accepted physiological threshold of 100,000 cells/ml for the SCC. For the determination of the most appropriate cut-off level for the interpretation of CMT results, these scores were grouped into two classes, "Negative" and "Positive" (Table 1).

Data recording and processing were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Data was evaluated statistically applying the scientific software SAS 8e (SAS Institute Inc., Cary, NC, USA) and BiAS 7.07 (Ackermann, 1999; University of Frankfurt am Main, Germany). The specific statistical procedures will be mentioned in the text.

Table 1. Comparison of different CMT scorings.

Conventional CMT scoring (Schalm and Noorlander, 1957)			Modified CMT scoring			Cut-off
Score		Observation	Score		Observation	Determination
Negative	(0)	Mixture remains liquid with no evidence of formation of a precipitate	Negative	(0)	No smears	Negative (0)
Trace	(T)	A slight precipitate which tends to disappear [...]	Questionable	(Q)	Traces of smears seem to appear	
			Positive I	(1)	Slight smears	
Weak positive	(1)	A distinct precipitate but no tendency toward gel formation	Positive II	(2)	Moderate smears	
			Positive III	(3)	Strong smears	
Distinct positive	(2)	The mixture thickens immediately with some suggestion of gel formation [...]	Positive IV	(4)	Incipient gel formation	
Strong positive	(3)	A distinct gel forms which tends to adhere the bottom of the paddle [...]	Positive V	(5)	Gelatin	Positive (1)
			Positive VI	(6)	Visibly abnormal milk	

Results

The evaluation of the diagnostic potential of the California Mastitis Test included the findings for 1,426 QFM samples of clinically inconspicuous quarters. In order to obtain more precise results, conventional CMT scores were augmented as detailed in Table 1. Table 2 shows these modified scores and the corresponding cell counts. A linear correlation between these CMT results and SCC was calculated using Spearman's coefficient of rank correlation ($r_s = 0.801$; $CI_{P=0.99} = 0.756, 0.846$).

It was decided that the occurrence of even the slightest precipitates would be scored as a positive result (Cut-off: ≥ 1). To verify this decision, the CMT results were contrasted with the udder health categories (Table 3) which showed the chosen cut-off level led to the detection of 95% of all subclinically inflamed quarters (93.2% of unspecific mastitis cases and 96.3% of mastitis cases, i.e. 538 of 565 cases). It therefore appears to be

Table 2. Modified CMT scores and corresponding cell counts.

CMT Scoring		Number of samples			$\log_{10}$ SCC/ml		SCC/ml
		Total (100%)	≤100,000 (%)	>100,000 (%)	X_a	±sd	X_g
Negative	(0)	222	218 (98.2)	4 (1.8)	4.084	0.417	12.134
	(Q)	394	371 (94.1)	23 (5.8)	4.264	0.443	18.365
Cut-off							
	(1)	301	207 (68.8)	94 (31.2)	4.761	0.462	57.677
	(2)	245	61 (24.9)	184 (75.1)	5.234	0.484	171.396
Positive	(3)	171	4 (2.3)	167 (97.7)	5.600	0.327	398.107
	(4)	63	0 (0.0)	63 (100.0)	5.982	0.335	959.401
	(5)	30	0 (0.0)	30 (100.0)	6.485	0.406	3,054,921
	(6)	25	2 (8.0)	23 (92.0)	Visibly abnormal milk		

Table 3. Comparison of CMT results with udder health categories.*

Somatic cell count [cells/ml milk]			Udder pathogens	
			Not detected	detected
			Normal secretion	latent infection
	N-diagnosis		642	219
	$Log_{10} X_a \pm sd$		4.189 ± 0.397	4.547 ± 0.374
?100,000	X_g		15,453	35,237
	CMT	score?1	24.95%	51.1%
		Score<1	75.1%	48.9%
			Unspecific mastitis	Mastitis
	N-diagnosis		192	373
	$Log_{10} X_a \pm sd$		5.582 ± 0.496	5.562 ± 0.390
>100,000	X_g		381,944	364,754
	CMT	score?1	93.2%	96.3%
		Score<1	6.8%	3.7%

*n = 1,426 QFM samples of clinically inconspicuous quarters

Table 4. Comparison of CMT results and SCC values by means of the two-way table.*

SCC [cells/ml milk]	CMT		
	Positive (≥1)	Negative (<1)	n Quarters
	false positive	true negative	"Healthy"
≤100,000	31.6%	67.4%	861
	True positive	False negative	Diseased
>100,000	95.2% n=538	4.8% n=27	565
Of those			
Up to 200,000	90.1% n=164	9.9% n=18	182
Up to 300,000	96.2% n=100	38% n=4	104
Up to 400,000	98.2% n=54	1.8% n=1	55
>400,00	98.2% n=220	1.8% n=4	224

*n = 1,426 QFM samples of clinically inconspicuous quarters

appropriate to include even the slightest smears in the positive CMT results. The most frequent CMT failures were seen for "latent infections".

Results of the CMT were compared with corresponding SCC values based on the two-way table. The cut-off levels for CMT and SCC were set as scores of 1 and 100,000 cells/ml, respectively. Table 4 shows the two-way table. The corresponding basic statistics can be specified as follows: prevalence (0.396), sensitivity (0.952; $CI_{P=0.99}$ = 0.924, 0.972), specificity (0.684; $CI_{P=0.99}$ = 0.642, 0.724), predictive values (PV^+: 0.664; $CI_{P=0.99}$ = 0.620, 0.707; PV^-: 0.956; $CI_{P=0.99}$ = 0.930, 0.975), likelihood ratios (LR^+: 3.014; LR^-: 0.070), diagnostic accuracy (0.790; $CI_{P=0.99}$ = 0.761, 0.817), Youden's Index (0.636; $CI_{P=0.99}$ = 0.584, 0.688). The agreement between SCC and CMT was estimated by applying Cohen's coefficient Kappa (Ackermann, 2005: personal communication). With a coefficient k = 0.592, the

agreement between the two diagnostic tests can be rated as "definite" (Fleiss, 1981). Under field conditions, the very low number of false negative CMT results (27 of 565 diseased cases) is of great importance for the practicability of CMT because these cases represent undetected diseased quarters. Considering SCC ranges (Table 4), the percentage of false negatives is extremely low, even in a SCC range between 100,000 and 200,000 cells/ml milk (9.9%).

In order to determine the highest power of the CMT, the Youden's Index was used as a measure of quality for the definition of the cut-off levels for both SCC and CMT (Table 5).

As detailed there, the highest Youden's Index was found for a CMT score of 2 (moderate smears, see Table 1) and for the corresponding SCC threshold of 150,000 cells/ml. The following statistical values were calculated for these cut-off levels: prevalence (0.321), sensitivity (0.871; $CI_{P=0.99}$ = 0.826, 0.908); specificity (0.885; $CI_{P=0.99}$ = 0.857, 0.910); predictive values (PV^+: 0.782; $CI_{P=0.99}$ = 0.731, 0.827; PV^-: 0.936; $CI_{P=0.99}$ = 0.912, 0.955), likelihood ratios (LR^+: 7.603; LR^-: 0.146), diagnostic accuracy (0.881; $CI_{P=0.99}$ = 0.857, 0.902), Youden's Index (0.756; $CI_{P=0.99}$ = 0.709, 0.803). With a Cohen's coefficient Kappa k = 0.734 the agreement between the two diagnostic tests can be rated as "strong" (Fleiss, 1981).

Table 5. Youden's Indices depending on SCC thresholds and CMT scores.

		CMT scores					
	Cut-off	Q	1	2	3	4	5
Scc threshold	50,000	0.291	0.692	0.608	0.347	0.123	0.040
	100,000	0.246	0.636	0.710	0.456	0.465	0.053
	150,000	0.219	0.574	0.756	0.529	0.204	0.066
	200,000	0.202	0.559	0.736	0.575	0.232	0.075
	300,000	0.185	0.515	0.728	0.639	0.307	0.103
	400,000	0.174	0.491	0.710	0.649	0.373	0.129

Youden's Index = Sensitivity + specificity -1

Discussion

As demonstrated here, the California Mastitis Test allows reliable detection of subclinically inflamed udder quarters, even in subclinical mastitis cases only slightly above the physiological threshold of 100,000 somatic cells/ml. This is in contrast to commonly reported cell count levels in the range of up to 200,000 cells/ml for a negative CMT and of up to 500,000 cells/ml for trace results of the CMT (Schalm, 1960; Marshall *et al.*, 1993; NMC, 1999). Comparisons with the results of numerous other scientific works is difficult since SCC was not used as the reference parameter in these studies (Sargeant *et al.*, 2001; Dingwell *et al.*, 2003). Although generally applied as an indirect cell count method, the CMT is often compared with intramammary infections (IMI). Based on the results of 1,426 QFM samples of clinically inconspicuous quarters, the best agreements (Youden's Index) were determined for comparisons of SCC with the corresponding CMT results (Figure 1). It is therefore preferable to evaluate the CMT on the basis of comparisons with SCC as the gold standard.

Ideally, a diagnostic test would provide both 100% sensitivity and 100% specificity. For the detection of subclinical mastitis, lower specificity means that false-positive quarters will occur, i.e. "healthy" quarters will be erroneously regarded as inflamed. Such quarters may cause economic losses due to unnecessary treatment-related costs. On the other hand, lower sensitivity will result in false-negative quarters and these subclinically inflamed quarters remain undetected. As far as contagious udder pathogens are concerned, such quarters seriously increase the risk of new infections within a herd. With regard to this infection-related implication, high sensitivity is a precondition for successful herd health management. Despite the fact that the CMT can be influenced by several factors (Hamann *et al.*, 2005), the precision of this cow-side test can be improved if the following procedures are strictly observed (Table 6).

More detailed CMT scores will not result in more information. On the contrary, more detailed scores involve the risk of herd management failures when treatment procedures only include cows with the highest scores.

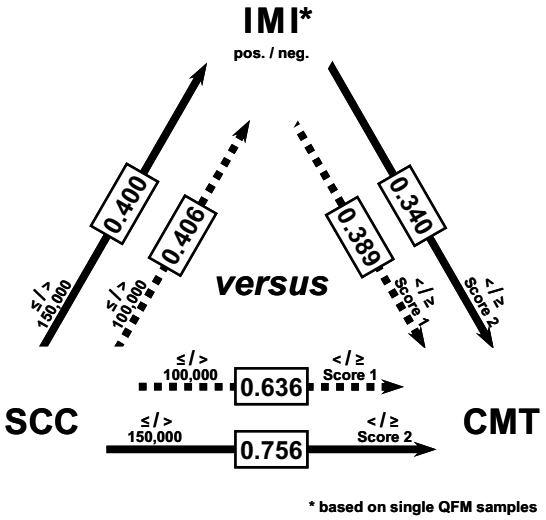

Figure 1. Youden's Indices of comparisons between SCC, IMI and CMT, as to the different cut-off levels (n = 1,426 QFM samples).

Conclusions

Under standardised conditions, it is possible to detect subclinically inflamed udder quarters with the California Mastitis Test, even in those quarters only slightly above the physiological threshold of 100,000 cells/ml (sensitivity: 95.2%; specificity: 68.4%; diagnostic accuracy: 79.0 %). The most appropriate cut-off levels were found to be an SCC threshold of 150,000 cells/ml and a CMT score of 2 (comparable to a conventional CMT score of 1), which gave the highest diagnostic accuracy (88.1%).

Table 6. Recommended procedures for the CMT (Hamann et al., 2005).

1	Discard the first jets of milk → strip cup
2	Use only foremilk obtained after milk is completely ejected
3	Add an equal amount of CMT reagent to the test milk in the cup → Avoid underdoses!
4	Mix milk with the reagent by gently swaying the paddle
5	Decant supernatant reagent-milk mix carefully to a volume of 2 ml, as it is difficult to detect minor changes in a larger volume
6	Take readings within 20 seconds under good lighting conditions
7	Use only sufficiently experienced operators for CMT
8	Consider only two CMT scores: → Negative = no precipitates → Positive = precipitates: even the slightest smears

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Mastitis in small ruminants

Aetiological, clinical and epidemiological characterization of clinical mastitis in dairy sheep

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Abstract

This work synthetises data concerning the aetiology of different forms of dairy ewe clinical mastitis. Results were collected in all French dairy sheep areas from 1991 to 2005 and originated from epidemiological survey, field veterinary laboratories diagnosis and Veterinary School epidemiological and bacteriological investigations. A total of 1,615 specimen from 335 flocks were submitted to conventional bacteriological examination. The average incidence of acute mastitis was 3.1% of ewes per lactation in flocks exhibiting sporadic cases (stratified-random sample). Overall negative results were obtained in 7.7 to 19.5% of specimens. The most common isolates for acute clinical mastitis were, according to the data source and the lactation stage: *S. aureus* (49-62%), coagulase-negative Staphylococci (13.7-18%), *Streptococcaceae* (6.3-7.6%), *M. haemolytica* (2.8-7.5%), Enterobacteria (2.8-7.3%) and other pathogens (3.4-9.1%). In case of clinical chronic mastitis, coagulase-negative Staphylococci were the most prevalent organisms (38%). The overall frequency of non-environmental bacteria was 75 to 85% of cases. During the suckling period of lactation, *M. haemolytica* mastitis appeared to be more frequent in certain flocks and certain area. Detailed bacteriological investigations were performed for 27 specific outbreaks due to *S. suis* (or *S. ovis*), *S. uberis*, *M. haemolytica, A. fumigatus* and *P. aeruginosa*. The two latter ones may be responsible for epizootic mastitis with mortality, mainly in the peri-partum period, in flocks performing drying-off intrammamary antibiotherapy in contaminated environment without the necessary hygienic and atraumatic precautions.

Keywords: dairy ewe, clinical mastitis, aetiology**,** epidemiology

Introduction

During the last decade, the need for improvement of knowledge about clinical mastitis has become more important as they are related to important evolutions of the dairy sheep husbandry: development of milk bacteriological safety control, improvement of genetic mastitis resistance, detection of antibiotic residues, expansion of drying-off intramammary antibiotherapy with possible selection of opportunistic pathogens. In the same time, many

investigations have been conducted to identify the causative agents of bovine mastitis and to a lower extent of ovine subclinical mastitis. There has been less work published about dairy ewe clinical mastitis. The present report synthetises French data collected in the 3 dairy sheep areas (1991-2005) and originating from epidemiological survey, field veterinary laboratories results and universitary on-farm audits or laboratory diagnosis.

Materials and methods

Studies design

Epidemiological survey

This work was performed during the lactation 2003 in the Roquefort area (Lacaune breed) and Pyrénées-Atlantiques (Manech and Basco-béarnaise breeds). A stratified-random sample of 72 flocks was selected according to the district and the recording organism of the flocks. Farmers were asked to sample each case of clinical mastitis by collecting aseptically the mammary secretion immediately after the detection of the clinical signs (n=302). Farmers also agreed to notice the clinical type of mastitis and the date. These flocks were maintained under a high standard of technical and sanitary management including immediate culling of acute mastitic animals and annual culling of subclinical mastitic ewes. Approximately 70% of these flocks in Roquefort area performed drying-off treatment (intramammary route), but only 30 to 40% in Pyrénées-Atlantiques (systemic more often than intrammary treatment).

Field veterinary laboratories aetiological diagnosis

This part synthetises results obtained from mastitis specimens (1998 or 1999 to 2003) submitted to diagnostic laboratories in the Roquefort area (Aveyron and Tarn, 180 mastitic milks from 94 flocks) and Pyrénées-Atlantiques (168 milks from 85 flocks). Veterinarians (or farmers) submitted specimens in case of high incidence, high overall prevalence or duration of the outbreak, « atypic » forms of mastitis,...

Veterinary School epidemiological and laboratory diagnosis

Clinical, epidemiological and bacteriological examinations were conducted within the frame of the teaching programme. The clinical status of glands and mammary secretions was recorded. Animals to be sampled were selected according to the symptomatology, the absence of treatment, the lactation stage or parity,...These investigations were requested by veterinarians generally in the same cases than above (section 212). Among the 84 flocks investigated between 1991 to 2005, certain « specific » cases were considered as rare or particularly interesting : *A. fumigatus, P. aeruginosa, M. haemolytica, S. suis* (or *S. ovis*) *or S. uberis* outbreaks. Thus a more important number of bacteriological examinations were performed (estimation of prevalence). These cases were differenciated from the routine ones, in order not to introduce a bias in the estimation of the causative organisms frequence. Regarding the « non specific » cases, 965 clinical specimens were bacteriologically analysed.

Definition of clinical cases

During acute mastitis, the gland becomes hot, swollen, reddish and the secretion is modified. Peracute mastitis is characterized by general signs. A modification of the secretion alone is considered as a subacute mastitis. During chronic clinical mastitis, one can observe

induration of the gland, abscesses or unilateral volume reduction or atrophy, generally without abnormal secretion. The epidemiological survey deals with acute and peracute forms (and a few subacute ones). Data from the field laboratories concern peracute to subacute forms. Results from the Veterinary School investigations are differenciated between the acute group and the chronic mastitis.

Sampling procedure

Milk samples were collected from affected halves. The first streams were discarded and the teats disinfected with alcohol prior to collection into sterile vials. The samples were kept cool and generally assayed within 24h. In the epidemiological survey, specimens were frozen at the farm and were kept at -20°C until assayed.

Isolation and identification of mastitis-causing organisms

A loopful (0.01 ml) was plated onto tryptic soy agar base with 5% sheep blood. The inoculated plates were incubated aerobically and examined at 24 and 48h. If there was no growth, the plates were re-incubated anaerobically up to a further 48h. Micro-organisms were identified according to routine techniques (API system). Mycoplasma specific broth were used for samples originating from Contagious Agalactia positive areas, but Mycoplasma infected flocks were excluded.

Results

Epidemiological survey

The average incidence of clinical mastitis was 3.1% of ewes per flock-lactation. Of the 302 specimens assayed, 13.2% were considered as negative. Co-infections occured in 4.5% of the cases. Global results of pure cultures identifications or contaminated ones (more than 2 types) are given in figure 1. Among the 10 identified species of coagulase negative

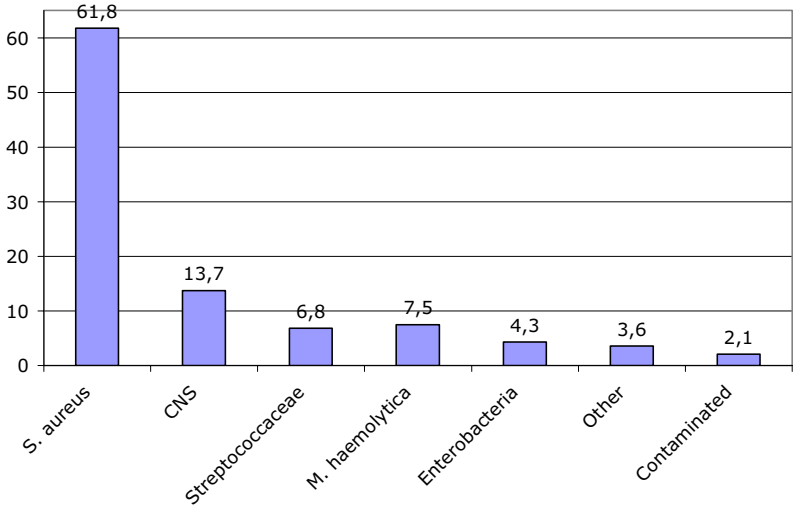

Figure 1. Frequency (percentages) of isolation of bacteria from acute to peracute mastitis (epidemiological survey, n=302 cases). CNS : coagulase negative Staphylococci.

Staphylococci, *S. chromogenes* was the most common isolate (29% of the CNS), then *S. simulans* and *S. xylosus* accounted for 16% each. Eight species of *Streptococcaceae* were identified : *S. bovis* and *S. suis* constituted the most frequent ones (26.3% each). Between breeding areas, there was evidence of a difference only for *M. haemolytica*: it is responsible of 10% of cases in Roquefort area, and no isolation was made in Pyrénées-Atlantiques.

Interestingly, percentages of identified bacteria varied according to the lactation stage (figure 2). During the first month (suckling and milking), *M. haemolytica* and coagulase negative Staphylococci had the first and then the second place in the order of frequency. The role of *S. aureus* increased from day 0 to day 60. No effect of the parity was noticed.

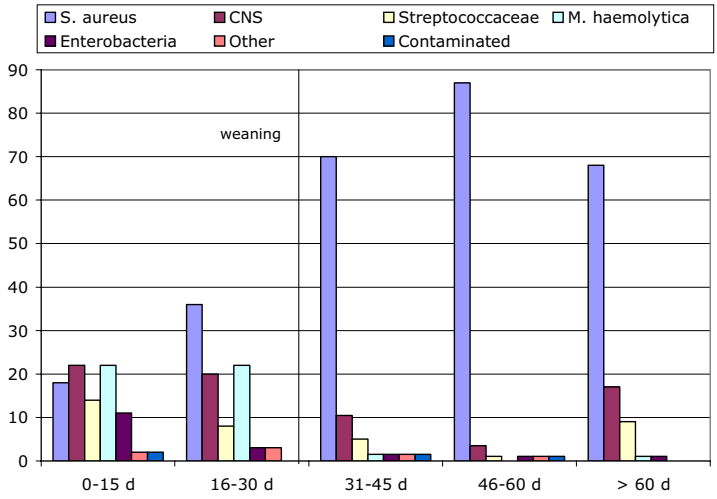

Figure 2. Effect of the lactation stage (days after lambing) on the percentages of bacterial isolation. CNS : coagulase negative Staphylococci. d : days.

Field veterinary laboratories aetiological results

When comparing the two breeding areas, 7.7 and 9.6% of the specimens were considered as negative respectively in the Roquefort area and in the Pyrénées-Atlantiques. Co-infections (2 types of micro-organisms) were found respectively in 31.7 and 8% in the same areas. Those Roquefort area co-infections (31.7%) included *S. aureus* in 8 cases out of 78. Global results of bacteriological identifications from pure cultures or contaminated ones are given in figure 3. In the Roquefort area, « other » micro-organisms included 17 *A. fumigatus* and 6 *P. aeruginosa* out of 27 pures cultures, whereas homologous number of isolation in Pyrénées-Atlantiques were 0 and 1. Among the 12 identified species of coagulase negative Staphylococci in the two areas, *S. xylosus* (27%) and *S. epidermidis* (22%) were the most common (n=62). *Streptococcaceae* were unfrequently isolated (n=29), group D ones (48%) and *S. uberis* (20%) being the most frequent.

Veterinary School aetiological results

'Non specific' cases

Negative results were obtained in 19.5% of the cases. Co-infections occured in 4.5% of the halves. Other positive results or contaminated specimens are presented in figure 4. Ten

species of coagulase negative Staphylococci were isolated (n=75), the most frequent being *S. epidermidis* (36%), *S. simulans* (17.3%), *S. chromogenes* (12%) and *S. xylosus* (10.7%). The first two species were also the most common organisms isolated from the acute group of mastitis (resp. 33 and 20%). Twelve species of *Streptococcaceae* were isolated (n=40) : *S. suis* (30%), *A. viridans* (15%), *E. faecalis* (10%),...The most frequent in the acute group was *S. suis* (40%). « Other » organisms (n=83) grouped 20 species in the following order of frequency : *Corynebacterium* spp. (24%), *A. pyogenes* (20.5%), *P. aeruginosa* (16.8%), *Candida* spp. (13%),...*A. pyogenes* was the most common in the acute group (32.5%, n=43). The comparison of the two breeding areas did not show any difference.

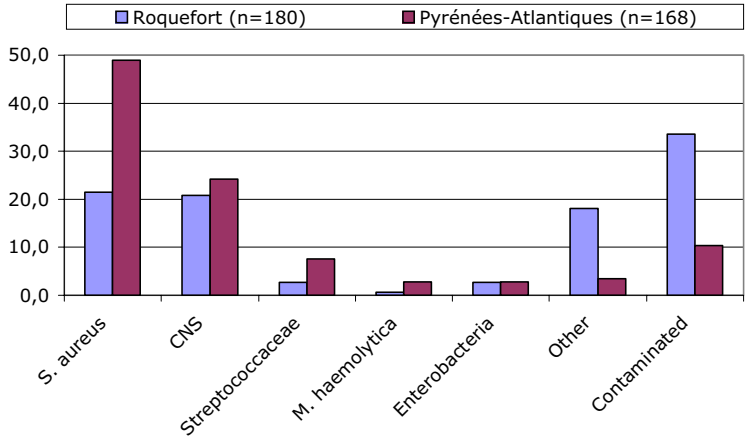

Figure 3. Frequency (percentages) of isolation of micro-organisms from clinical mastitis in Roquefort and Pyrénées-Atlantiques areas by the veterinary laboratories. CNS : coagulase negative Staphylococci.

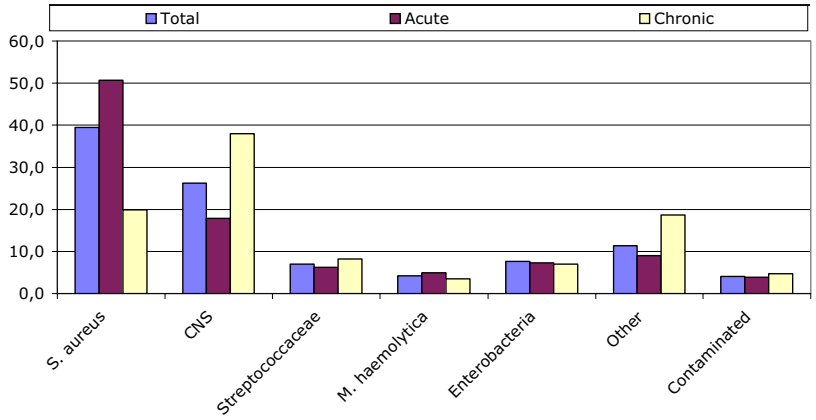

Figure 4. Frequency (percentages) of isolation of bacteria from clinical mastitis (National veterinary School results, n=965 cases). Acute group : peracute to subacute mastitis. CNS : coagulase negative Staphylococci.

'Specific' cases

These cases are generally epizootic or enzootic.

During the dry and the peri-partum periods, outbreaks due to *A. fumigatus* or *P. aeruginosa* occured in a few flocks performing exhaustive drying-off intrammary antibiotherapy. Peracute to chronic mastitis, with possible mortality, mainly appeared during the peri-partum period, and sometimes in the days following the intrammary treatments when *P. aeruginosa* was involved. In the case of mammary aspergillosis, other opportunistic pathogens were sometimes isolated in the same flock: *A. nidulans, A. terreus, A. flavus* and various species of *Candida*. Average aspergillosis morbidity and mortality were respectively 9 +/- 5% and 4.7 +/- 3.7% of the whole flock (n=14). Regarding *P. aeruginosa* outbreaks, morbidity and mortality were 17.5 +/- 13.1% and 4.1 +/- 6% (n=7). In these two kind of cases, the udder contamination probably occured at the end of lactation and above all at the time of intrammamary injections. The main sources for *A. fumigatus* were wet bedding or mouldy forage and, for *P. aeruginosa*, clusters residual water, where the organism was frequently isolated. Moreover, in the two cases, precautions for hygienic and atraumatic injections were not stricly respected.

During the suckling period (1 month) and sometimes the following weeks, outbreaks of acute to chronic mastitis due to *M. haemolytica* occured with a low frequence. The most common serotypes seem to be A1 and A11. Ewes were generally free from respiratory symptomatology, whereas a moderate to null incidence was observed on lambs. Bacterial source is probably the lambs mouth and nasopharynx.

Mainly during the milking period, outbreaks due to *S. suis* (n=2) or *S. uberis* (n=2) were recorded. In the two cases, affected ewes generally exhibited unilateral, non gangrenous, acute to chronic mastitis, without general signs (no mortality). The prevalence may be important : 40 to 75% of the flock.

Discussion

Methodologically, the three parts of this report rely on different samples of flocks and animals. The epidemiological survey is the most representative one, as nearly all the acute to peracute mastitis cases, and some subacute ones, were sampled by the farmers in 72 flocks. These flocks were not selected for high mastitis incidence. Thus these results seem to be the most suitable ones to establish the frequency of the various mastitis-causing organisms. Other results must be considered cautiously from the representativity point of view, as investigated flocks were selected by the veterinarian and/or the breeder. Variations in results between field veterinary laboratories may reflect true differences in husbandry or technical level, as well as variations in sampling aseptic precautions or microbiological procedures. An additional factor may be the number of bacteria (resp. different bacteria) which individual technicians consider necessary for a specimen to be classed as positive (resp. contaminated). A proportion (7.7 to 19.5%) of the samples did not yield micro-organism in conventional bacteriological examination. There is no evidence of deleterious effect of freezing (epidemiological survey : 13.2%). Some mastitis, particularly with chronic lesions, may be associated with intermittent shedding or encapsulation of bacteria (Fthenakis and Jones, 1990).

Literature pertaining to dairy ewe clinical mastitis incidence is limited. Our results (3.1%) seem to be lower than those reported by Al-Samarrae *et al.* (1985), Watson *et al.*(1990) or

Lafi *et al.* (1998). This is probably due to differences in detection techniques, implementation of control programmes or existence of milk quality payement (Roquefort area). This incidence may also reflect the routine use of drying-off antibiotherapy. Another important difference with homologous results in dairy cattle is the frequence of the different causative agents. In the three parts of this work, *S. aureus* is the principal isolated organism (49 to 62%, independantly of Roquefort area laboratory). Coagulase-negative Staphylococci had been considered as non-pathogenic or as minor pathogens, mainly in dairy cattle (Saratsis *et al.*, 1998). Our results show that they are associated with acute mastitis, particularly *S. epidermidis*, *S. xylosus*, *S. simulans* and *S. chromogenes*. Equivalent results had been reported by Saratsis *et al.* (1998) about chronic mastitis during the dry period. The overall frequence of Staphylococci for acute mastitis ranges from 69 to 75.5% in this work. Moreover, the non-environmental pathogens (Staphylococci, animal Streptococci, *M. haemolytica*,...) range from 75 to 85%, which is an important difference compared to dairy cattle. The incidence of the mastitis caused by these bacteria, particularly *S. aureus*, increases during the milking phase of lactation, as they are transmitted by machine milking. On the other hand, the most common *Streptococcaceae* seem to be D group ones. Mastitis due to *A. fumigatus* or *P. aeruginosa* raise questions regarding the hygiene of injections and environment, and the possibility to perform selective drying-off treatments.

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Detection of enterotoxins and virulence genes in *Staphylococcus aureus* strains isolated from sheep with subclinical mastitis

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Abstract

Thirty-two *S. aureus* strains isolated from sheep subclinical mastitis (SM) were analysed by multiplex PCR for staphylococcal enterotoxins (SEs) and virulence factor encoding genes. All strains were tested for SEs genes (*sea-e*, *seh*, *sek*, *sel*, *sem*, *seo*) and TSST-1 (*tst*). SEA-D production was tested using RPLA. All strains were also analysed for the following genes: *gyrA* (gyrase), *agr* I-IV alleles (accessory gene regulator), virulence factors *eta*, *etb*, *etd* (exfoliatins), *lukF-PV* and *lukS-PV* (Panton-Valentine leukocidin), *lukE* and *lukD* (LukE-LukD leukocidin), *lukM* (LukM leukocidin), *hlg* (hemolysin γ), *hlgv* (hemolysin γ variant) and *hlb* (hemolysin β), *edin* (epidermal cell differentiation inhibitor), *mecA* (methicillin resistance determinant). In eight out of thirty-two strains *sec*, *sel* and *tst* were detected, while three isolates harboured the *seh*. Genes *sea*, *seb*, *sed*, *see*, *sek*, *sem* and *seo* were not found. RPLA also detected SEC in ten strains. RPLA and PCR results in four cases did not agree. Further test conducted on these strains by ELISA given concordances with PCR. *agr*, *hlgv* and *hlb* were found in all strains. Twenty-three isolates harboured *edin*, nineteen *luk-E* and *luk-D*, and eight *lukM*. *mecA, eta*, *etb*, *etd*, *hlg*, *lukF-PV* and *lukS-PV* were not detected. *S. aureus* enterotoxigenic strains are less frequently found in sheep with SM than as has been reported for sheep with clinical mastitis. Six different profiles were observed based on the association of SEs and virulence genes.

Introduction

S. aureus is one of the most important pathogens for the dairy chain and affects animal health and food safety. In large and small ruminants it is well known as a mastitis causing agent. Staphylococcal enterotoxins (SEs) may contaminate milk and milk products and cause foodborne diseases. In dairy milk sheep *S. aureus* is the cause of mastitis in approximately <5% of cases and makes up 17-57% of the isolated pathogens, with the exception of *Mycoplasma spp*. Subclinical mastitis (SM) is found in nearly 30% of sheep in the Mediterranean countries, and in certain areas reaches 40%. Sheep SM is principally caused by Coagulase Negative Staphylococci (CNS), while *S. aureus* usually account for 5-10% of isolated pathogens (Cosseddu *et al.*,1996; Ariznabarreta *et al.*, 2002; Bergonier and Berthelot, 2003). Despite its low incidence SM is one of the main sources of *S. aureus* raw bulk milk contamination. The animals' body sites, the farmworkers, the environment and the farm equipments may also contribute to the latter (Vautor *et al.*, 2003). *S. aureus* in bulk sheep milk, or more frequently during cheese production due to technological failures,

may reach 10^5-10^6 cfu/g. Such levels are associated with production of SE in concentrations capable of causing foodborne disease. Technological and production variables influence the risk rate. These include the type of milk used (raw milk, thermized or pasteurised milk), whether or not starters are used, the type of starter lactic microflora, and the speed and final level of acidification (Meyrand and Vernozy-Rozand, 1999). Cases of food poisoning from contaminated sheep cheese have been reported. In foodborne disease outbreaks the SE involved was principally SEA, as a result of human strain contamination of the cheese, and less frequently SEC (Cosseddu *et al.*, 1991; De Buyser *et al.*, 2001). Characterization of *S. aureus* virulence factors profiles are needed to understand their epidemiological implications, but little data are available on the strains isolated from sheep (Scherrer *et al.*, 2004). *S. aureus* has a wide pattern of virulence factors including hemolysins, nucleases, proteases, lipases, hyaluronidase and collagenase. Some strains produce other specific factors such as toxic shock syndrome toxin-1 (TSST-1) and SEs, exfoliative toxins and leukocidins. The virulence of the strains is controlled by complex networks of two component regulatory factors which allow the bacteria to adapt to environmental conditions and to develop infections (Jarraud *et al.*, 2002). The *agr* locus regulates certain extracellular toxins and enzymes expressed postexponentially and represses some exponential-phase surface components. The *agr* locus has been shown to be polymorphic and can be divided into four (I-IV) distinct genetic groups (Jarraud *et al.*, 2002). *S. aureu*s produces a number of cytotoxic molecules. These include the four hemolysins (α, β, γ and δ), and Panton-Valentine leukocidin (PVL), leukocidin E/D (LukE/D) and leukocidin M (LukM). β-hemolysin is strongly associated with animal isolates (Dinges, 2000). Isolates from ruminant mastitis have γ-hemolysin, LukE/D and LukM genes in different arrangements, while the PVL locus was not detectable (Rainard *et al.*, 2003). Five classical SEs (SEA-SEE) have been recognised, and these are responsible for 95% of foodborne outbreaks. New SEs (SEG-Q and SEU) have been identified more recently, although their role in food poisoning has not yet been clarified (Rosec and Gigaud, 2002; Scherrer *et al.*, 2004). TSST-1, which is responsible for human toxic shock syndrome, has been detected in 20-40% of cow and sheep mastitis strains, and is strongly (90%) associated with SEC (Zschock *et al.*, 2004). Exfoliatins encoding genes (*eta* and *etb*) were detected at low rates (1.2%-0.6%) in isolates from bovine mastitis and from farm bulk milk (Endo *et al.*, 2003). The epidermal cell differentiation inhibitor (EDIN) has been described in experimental studies but its precise role has yet to be identified (Gravet, 2001). TSST-1, SEs and exfoliatins are defined as superantigens (SAgs) and their primary function in vivo may be to inhibit host immune responses (Schuberth *et al.*, 2001). Methicillin resistance is more commonly found in human rather than in animal strains and only a limited number of publications have reported its spread to dairy herds (Lee, 2003). Methicillin resistance and PVL are associated with community-acquired *S. aureus* strains (CA-MRSA) which harbour *mecA* and PVL genes (Vandenesch *et al.*, 2003). The purpose of the paper is to perform an in depth characterization of the virulence factor profile of *S. aureus* isolated from sheep SM. An exhaustive pheno- and genotype characterization of SEs should provide data which can be used in dairy product risk assessment procedures.

Materials and methods

Between january and may 2001 thirty-two strains were collected from half-udder SM affected sheep milk in four commercial flocks in Sardinia. Bacteriological analysis was

performed on 10 µl milk samples striked on 5% sheep blood agar. Half-udders were considered to be affected by SM when: a) no clinical findings were detectable; b) on milk samples 5 or more identical colonies (by means of bacteriological analysis); c) somatic cell count (SCC) ≥300,000/ml were found. All strains were tested for hemolysis on 5% sheep blood agar incubated at 37°C for 48hrs. *Staphylococcus spp* were identified on the basis of conventional phenotypic characteristics: Gram staining, cell morphology and cell arrangement, colony morphology, catalase activity, coagulase production in rabbit plasma (bioMérieux, France), and production of clumping factor (Staphytect Plus, Oxoid, England) and thermonuclease. For the overnight broth cultures, a tube coagulase plasma-EDTA test (bioMérieux, France) was performed on all suspected Staphylococcal colonies (G+, catalase +). The isolates were identified by ID 32 Staph (bioMérieux, France). SEA-SED production was tested by SET-RPLA (Oxoid, England), according to manufacturing instructions. Genomic DNA was used as PCR target after extraction with a standard procedure: strains grown in brain-heart infusion broth at 37°C overnight were centrifugated (3000 rpm 10 min) and resuspended in 500 µL of Tris -EDTA buffer saline (Tris base 10 mM + EDTA 1 mM). The suspension was added with 10 µL of 1,5 mg/ml lysostaphin (Sigma) and incubated 1 hr at 37°C. Then, 5 µl of 20 mg/ml proteinase K (Eurobio, France) were added and incubation was continued at 50°C for 1 hr. An equal volume of phenol/chloroform/isoamylic alcohol (50:48:2) was added and mixed by inverting the tube until the phases are completely mixed. After centrifugation (10 000 rpm, 15 min) the upper layer was collected and 500 µL chloroform-isoamyl alcohol (24:1) solution was added. The mixture was centrifugated (10 000 rpm, 15 min) and the upper aqueous phase was transferred to a new tube. A 800-1000 µl of absolute ethanol was added and gently mixed until the DNA precipitated. DNA was resuspended in 100 µl distilled water. DNA concentration was estimated spectrophotometrically. Sequences specific for accessory genes regulator allele (*agr* I-IV), SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seh*, *sek*, *sel*, *sem*, *seo*), TSST-1 (*tst*), haemolysin γ (*hlg* and *hlgv*), haemolysin β (*hlb*), leukocidins LukED (*lukE* and *lukD*), PVL (*lukF-PV* and *lukS-PV*), leukocidin LukM (*lukM*), exfoliative toxin genes (*eta*, *etb*, *etd*), epidermal cell differentiation inhibitor gene (*edin*) were detected by PCR as previously described (Jarraud *et al*., 2002; Vandenesch *et al*., 2003). The *mecA* gene coding for methicillin resistance was detected by PCR as described by Murakami *et al*. (1991). Amplification of *gyrA* was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. *S. aureus* reference strains were used as positive controls as in Vandenesch *et al*. (2003). All PCR products were analyzed by electrophoresis through 1.5 % agarose gels (Sigma, Saint Quentin Fallavier, France).

Results

In the half-udder milk samples SCC geometric mean was $1{,}828 \times 10^3$/ml (ranging 329 - 12,771 $\times 10^3$/ml). The number of homogenous colonies in blood agar plates was $<10^4$ cfu in 3 samples, between 10^4 and 2×10^4 cfu in 27 samples, between 2×10^4 and 3×10^4 cfu in 2 samples. All 32 strains belonging to *S. aureus* on the basis of the ID 32 STAPH identification which agreed with their positive reaction for the clumping factor, coagulase and TDNAse. Hemolytic activity was not observed in 5 strains (16%) while one showed α-hemolysis, 7 (22%) β-, 17 (53%) αβ- and for 2 strains hemolysis was incomplete. RPLA detected SEC production in the surnatant of 10 strains (31%) cultures while none of the

strains produced SEA, SEB or SED. *In vitro* SEC concentrations detected on surnatants were: ≥ 2 ng/ml and < 4 ng/ml in one strain; ≥ 8 ng/ml and < 16 ng/ml, in one strain; ≥ 16 ng/ml and < 32 ng/ml, in one strain; ≥ 32 ng/ml in 6 strains. Molecular analysis confirmed that the strains belonged to *S. aureus* through identifying the *gyrA* gene. The isolates belonged to 3 of the 4 allelic *agr* groups. In detail, *agr* I was found in 3 strains, *agr* II in 3 strains and *agr* III in 26 strains. All the strains had *hlgv* and *hlgb*. *sea*, *seb*, *sed*, *see*, *sek*, *sem*, *seo* were not detected in the 32 isolates by PCR. In 8 strains (25%) *tst* was found associated to *sec* and *sel*. In 3 strains (9.4%) only the *seh* gene was present. The results of the RPLA and PCR tests were always negative for SEA, SEB and SED production and for their corresponding genes. The results of the two tests for SEC and the *sec* gene did not agree for 4 strains. Indeed in 3 strains the RPLA test found that SEC was produced while the *sec* gene was not present. For one isolate the RPLA test found no SEC production, despite the corresponding gene being present. As a result the 4 strains were tested by ELISA. The results agreed with those of PCR. The genes *mecA*, *LukS-PV LukF-LukPV*, *hlg*, *eta*, *etb*, *etd* were not found in any of the strains. In addition 23 strains contained the EDIN factor genes, 19 the *Luk-ED* genes, and 8 the *LukM* genes. Using the different associations of the genes it was possible to identify (table 1) 6 virulence factors profiles (P). The distribution of the profiles in the 4 cultures is shown in Table 2.

Table 1. Pathogenic patterns of S. aureus isolated from sheep subclinical mastitis.

pattern	n.	agr group	enterotoxins	TSST-1	exfoliatines	leukocidins	hemolysins	edin factor	mecA
P1	2	agr III	-	-	-	Luk D-E	hlgv, hlb	edin	-
P2	8	agr III	sec, sel	tst	-	luk D-E, lukM	hlgv, hlb	edin	-
P3	3	agr III	seh	-	-	luk D-E	hlgv, hlb	-	-
P4	13	agr III	-	-	-	-	hlgv, hlb	edin	-
P5	3	agr I	-	-	-	luk D-E	hlgv, hlb	-	-
P6	3	agr II	-	-	-	luk D-E	hlgv, hlb	-	-

Table 2. Distribution of S. aureus pathogenic patterns in four farms.

pattern	Farm			
	1	2	3	4
P1	2[a]			
P2	1		1	6
P3		3		
P4	1		12	
P5			2	1
P6			3	

[a], number of strains

Discussion

All 32 strains were identified as *S. aureus* according to the pheno- and genotypic properties. All strains showed *hlb* and *hlgv* genes. Finding *hlb* gives evidence that the strains

are of animal origin. However β-hemolisys was only detectable in 24 (75%) of these 32 strains which supports the findings of Salasia *et al*. (2004). *agr* locus is characterized by a polymorphism which results in 4 major *agr* groups (Jarraud *et al*., 2002). Group III *agr* was the most prevalent (81.2%). There was no association between *agr* alleles and virulence factors, which differs from the findings of von Eiff (2004). Nineteen (59.4%) of the strains carried *lukD/E*, as has previously been described in isolates from ruminant mastitis (Rainard *et al*., 2003). Eight out of thirty-two strains harboured *lukM*. LukM genes were found by Rainard *et al*. (2003) in almost all isolates of ovine origin while they were detected in only two-thirds of caprine and 10% of bovine strains. Exfoliatin genes were not found which agrees with the results of other authors, who were unable to find them at all in cow mastitis strains or, if they did, at levels not higher than 1% (Endo Y. *et al*., 2003; Salasia *et al*., 2004). None of the strains harboured PVL genes which agrees with Rainard (2003). PCR detected SE genes in 11 (34.4%) out of the 32 strains. In 8 (25%) strains was found *sec* in association with *tst* and *sel*. In 3 non hemolytic strains was found *seh*. Finding *seh* is noticeable, as this is not a common finding in strains isolated in sheep (Rosec and Gigaud, 2002; Scherrer D. *et al*., 2004). Finding *sec* and the SEC production is characteristic of *S. aureus* isolates from sheep. In ovine strains only rarely other SEs production or the corresponding *se* are found. *sec, tst* and *sel* finding in 8 strains confirms the results of similar research on cattle and human strains. Indeed *tst*, *sec* and *sel* were localised in the same pathogenic island (Kuroda *et al*., 2001). The strains were placed in six profiles depending on the arrangement of the virulence factors. Three profiles showed a specific distribution in the farms. The most numerous were P2 (found on 2 farms) and P4 (found on 3 farms). The former had the most virulence factors and the latter the least virulence factors among the profiles. Virulence factors can kill phagocytes (hemolysins and leukotoxins) or reduce immune response (SAgs). Relevant data on *S. aureus in vitro* leukotoxic activities and on virulence profiles are available for cows strains. Conflicting results and conclusions have been published on the relationships between leukotoxins and SAgs production and mastitis induction, clinical characteristics and outcomes. More recent data show an higher cow recovery rate from mastitis when strains lacking in SAgs determinants are involved (Haveri *et al*. 2005). In this respect, one noteworthy result of our research is that *S. aureus* isolates from sheep with SM are less enterotoxigenic (34,4%) than has been reported by other authors (Pisanu S. *et al*., 1987; Gilmour and Harvey, 1990) for acute clinical mastitis (70-80%).

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Subclinical mastitis affects the plasmin system, milk composition and curd yield in sheep and goats: comparative aspects

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Abstract

Subclinical mastitis in dairy sheep and goats has major deleterious effects on both the yield and the quality of milk. Improved insight into the interaction between bacteria and the affected gland was achieved by comparing infected and uninfected glands in the same animal. Lower concentration of lactose in the infected glands was associated with higher whey and albumin concentrations. In both sheep and goats curd yields from the infected halves were lower than those from the uninfected ones, although casein concentrations were almost equal in the two glands. It is likely that modifications in the casein micelles, caused by bacterial activity, are more detrimental to curd formation than to casein formation; this is also indicated by the longer clotting time of milk in the infected glands. Plasmin, the major proteolytic enzyme, in milk exists mostly as inactive plasminogen, and is activated by plasminogen-activator. Beta-casein is a natural substrate of plasmin, and a beta-casein-derived peptide down-regulates milk secretion. In goats, plasminogen activity was close to zero, possibly because of unusually high plasminogen-activator activity. The higher casein content, together with the higher plasmin activity, in sheep than in goats resulted in higher contents of casein degradation products, which include factors that down-regulate lactose and milk secretion. The higher degradation of casein explains the higher loss of curd and the lower milk yield in response to bacterial infection, in sheep than in goats.

Keywords: sheep, goat, intramammary infection, milk and curd yield

Introduction, material, methods and results

Subclinical mastitis (SM) in dairy sheep and goats has major deleterious effects on both the yield and the quality of milk (Leitner *et al.*, 2004a). However, most studies that evaluated economic loss and the mechanisms involved with SM addressed the whole udder rather than the single gland, so that these studies were limited by their consideration of the animal as a whole and not animals with only one infected gland. Moreover, those studies required the use of a large number of animals, because of genetic variability, and variations in management, lactation number and days in milk. Therefore, in order to overcome those

limitations, a study based on a glandular-level model was designed, in which each animal had one udder-half infected with a known coagulase-negative staphylococci (CNS) species and the contra-lateral gland free of bacteria (Leitner *et al.*, 2003, 2004b,c). Focusing on CNS was justified because in dairy sheep and goats in Israel and in other countries, this is the pathogen group that is the commonest cause of SM infection (Kalogridou-Vassiliadou, 1991; Lerondelle *et al.*, 1992; Contreras *et al.*, 1997, 1999; Haenlein, 2002; Leitner *et al.*, 2003). The present half-udder model studies included 36 Israeli-Assaf dairy sheep and 25 Israeli goats of various cross breeds; in all cases, one udder-half was infected with identified CNS and the contra-lateral gland was free of bacteria. Milk yield was measured and sampling was carried out during the morning milking. Milking was by hand and the yield was determined by measuring the amount of milk obtained from each udder half for each individual animal. The milk was tested for: the presence of bacteria according to Hogan *et al.* (1999); SCC by means of the Fossomatic 360; and gross composition (protein, fat and lactose) with the Milkoscan 6000 (Foss Electric, Hillerød, Denmark). The curd percentage was measured and the curd yield (Yc) was calculated as the curd percentage multiplied by the milk yield. The clotting time (Tc) was determined according to Berridge (1952). Skim milk was analyzed for the concentrations of casein, total whey protein, albumin and proteose peptones (p-p) according to Shamay *et al.* (2000, 2003), and measurement of the activities of PA, PLG and PL according to Silanikove *et al.* (2000). In the same samples, within 5 h after sampling, the concentration of free (ionized) calcium ($[Ca^{2+}]$) was determined by the repeated addition procedure, and the calcium activity (aCa^{2+}) by the uncorrected procedure, with a calcium-specific electrode (Silanikove *et al.*, 2003).

Somatic cell counts were significantly higher ($P < 0.0001$) in the infected halves than in the uninfected ones for both sheep and goats, but cell numbers were higher in the infected sheep glands (Table 1). The concentrations of fat, protein and casein in the infected glands were significantly lower than those in the uninfected ones in sheep but not in goats. Milk yield from the infected glands was lower than that from the uninfected ones in both sheep and goats, with a greater decrease in sheep than in goats (52.6 and 29.6%, respectively) (Table 1). Moreover, in sheep, the Yc in the infected glands (34.8%) only tended ($P < 0.1$) to be lower than that in the uninfected glands (36.4%), whereas in goats that difference was significantly ($P < 0.0001$) lower (23.2 vs. 20.8%). However, the overall yield (milk yield/day times the percentage of Yc) was significantly lower ($P < 0.0001$) in the infected glands than that in the uninfected ones for both sheep, (278 vs. 602 g/day) and goats (287 vs. 453 g/day). The Tc of the milk from the infected halves was significantly longer than that of the milk from the uninfected ones for both sheep (909 vs. 413 s) and goats (295 vs. 167 s). The differences between sheep and goats in the effects of CNS infection on milk yield and on SCC ($\sim 5x10^6$ and $\sim 1x10^6$, respectively), together with the significant reductions in fat, protein and casein contents in sheep but not in goats, suggest a stronger response to the bacteria in sheep than in goats.

In sheep, PA and PL activities were significantly higher in the infected than in the uninfected glands, whereas PL + PLG was similar in both, and the PLG activity and the PLG:PL ratio were significantly lower in the infected glands (Table 2). The data clearly indicate that the increase in PL activity in the infected glands was due to conversion of PLG to PL by PA without an apparent increase in total PL + PLG activities. In goats, PA and PL activities were significantly higher in the infected than in the uninfected glands (Table 2). PLG activity ranged from very low to undetectable; therefore these data are not presented.

Table 1. Mean values ± SE of bacteriological status vs. the various independent variables and its effects.

Animal	Parameter	Bacteriological status		Effect		
		Uninfected	Infected	Infection		Animal
				P	Difference*	
Sheep	SCC (×1000)	311±37	4999±1219	0.0004	4688	NS
	Milk (kg/day)	1.52±0.04	0.72±0.03	0.0001	-0.72	0.0009
	Fat (g/L)	64.9±0.26	61.7±0.21	0.05	-3.2	0.0004
	Protein (g/L)	58.5±0.07	53.5±0.10	0.0009	-5.1	0.0001
	Lactose (g/L)	44.7±0.08	33.5±0.16	0.0001	-11.2	0.02
	Whey (g/L)	11.9±0.38	12.8±0.16	0.0731	0.85	0.03
	Casein (g/L)	45.9±1.36	40.5±1.59	0.0002	-5.5	0.0001
	Albumin (µg/mL)	517±31	759±59	0.0047	268.7	0.0568
Goat	SCC (×1000)	417±72	1750±197	0.0001	1333	0.07
	Milk (kg/day)	1.96±0.04	1.38±0.04	0.0001	-0.58	0.0001
	Fat (g/L)	38.9±1.1	38.8±1.2	NS	0	0.0002
	Protein (g/L)	34.2±0.5	35.0±0.5	0.07	0.7	0.0001
	Lactose (g/L)	47.0±1.0	41.7±1.3	0.004	-5.2	0.004
	Whey (g/L)	6.1±0.3	6.8±0.4	0.0001	0.69	0.0001
	Casein (g/L)	28.1±0.7	28.2±0.8	NS	0	0.0001
	Albumin (µg/mL)	280±22	473±50	0.003	192	0.04

*Difference = uninfected minus infected

Table 2. Means ± SE of plasmin (PL), plasminogen (PLG), PLG/PL, plasminogen activator (PA), free (ionized) calcium ([Ca^{2+}]), calcium activity (aCa^{2+}) and proteose peptone (p-p) and their effects.

Animal	Parameter	Bacteriological status		Effect		
		Uninfected	Infected	Infection	Animal	
				P	Difference*	
Sheep	PL (U**/mL)	33.9±5.1	58.9±4.8	0.0007	25.1	NS
	PLG (U**/mL)	92.2±8.1	62.5±5.3	0.001	-29.7	0.0004
	PA (U**/mL)	148±29	354±56	0.0002	206	0.0091
	PL+PLG (U**/mL)	126.05	121.44	NS	-4.59	NS
	PLG/PL	3.54	1.04	0.002	-2.5	NS
	[Ca^{2+}](mmol)	3.52±0.58	4.14±0.41	NS	0.62	NS
	aCa^{2+} (mmol)	1.01±0.06	0.70±0.05	0.002	-0.32	NS
	p-p (mg/L)	0.98±0.01	2.42±0.12	0.0001	1.4	NS
Goat	PL (U**/mL)	20.3±2.4	39.8±6.1	0.0003	19.4	0.005
	PA (U**/mL)	3376±404.1	4334±565.5	0.05	958	0.002
	[Ca^{2+}](mmol)	4.80±0.4	5.05±0.3	NS	0.24	NS
	aCa^{2+} (mmol)	1.89±0.1	1.62±0.1	0.002	-0.27	0.0001
	p-p (mg/mL)	0.35±0.05	0.53±0.05	0.0005	0.18	0.0002

*Difference = uninfected minus infected

**1 Unit = activity unit; 1 unit is the amount of PL that produces a change in absorbance of 0.1 in 60 min, at 405 nm; [Ca^{2+}] - Ca concentration; aCa^{2+} - Ca activity

The concentrations of [Ca^{2+}] did not differ between the infected and uninfected glands, whereas those of aCa^{2+} were significantly lower ($P < 0.002$), and the p-p concentrations were significantly ($P < 0.0001$) 2.4 and 1.5 times as high in the infected than in the uninfected glands of sheep and goats, respectively (Table 2).

Discussion

Comparison between sheep and goats: milk yield and composition

One of the basic features of mammary secretion is that the total osmotic pressure of the secretion remains approximately constant and equal to that of the blood. As lactose is the main single osmotic component in milk apart from mineral salts, the secreted milk volume follows the changes in the secretion of lactose very closely (Shamay *et al.*, 2000). It may be concluded that the greater reduction in lactose secretion in the infected glands of sheep (65%) than in those of goats (37.5%) is the main reason for the greater reduction in milk volume in the infected glands of sheep than in those of goats.

In goats, PLG activity was close to zero, which may be attributed to the unusually high PA activity, and which is consistent with the findings of Fantuz *et al*. (2001) and Baldi *et al*. (2002). Silanikove *et al*. (2000) suggested that regulation of PA activity serves as a bridge between systemic hormonal influences and the local regulatory system, and that CN degradation products down-regulate milk secretion. Thus, the high basal level of PA in goats may make their system irresponsive to systemic effects. The higher CN concentration, together with the higher PL activity in sheep than in goats resulted in higher concentrations of CN degradation products that include factors that down-regulate milk secretion; this accounts for the greater effect of infection on milk yield (MY) in sheep than in goats. Therefore, it appears that, with respect to MY, sheep are more vulnerable than goats to sub-clinical infections.

Comparison between sheep and goats: curd yield

The curd yield from the infected halves was lower than that from the uninfected ones for both sheep and goats, although the CN concentrations in the two glands were almost equal. The present data suggest that knowledge of the gross CN content in the milk is insufficient to enable prediction of the curd yield, probably because of modifications to the CN micelles or to the various casein micelle components that are more detrimental to curd formation than they are to the CN concentration itself.

It seems strange at first sight that the reduction in curd yield is more severe in goats than in sheep, despite the fact that the reduction of milk yield is more dramatic in the latter. There are two explanations for this finding. First, fat is a component of the curd and its content in sheep milk is significantly higher than in goat milk. Second, as was found in the present study, in sheep, the fat content in the infected glands increased in comparison with that in the uninfected ones, whereas in goats the opposite occurred. Thus, the more efficient conversion of milk into curd in sheep than in goats accounts for the inversion of differences between the species.

Calcium Activity (aCa^{2+}) as a measure of casein degradation

Calcium is present in milk at a supersaturating concentration, because it is bound to the CN micelle through to its chelation by phosphoserine residues. Silanikove *et al*. (2003)

demonstrated a negative linear relationship between the CN concentrations in the milk of humans, goats, cows, sheep and mice, on the one hand, and calcium activity (aCa^{2+}), on the other hand. In goats, aCa^{2+} was negatively related to measures of proteolysis (p-p) (Leitner *et al.*, 2004c), which is consistent with similar findings in sheep (Leitner *et al.*, 2004a). Casein degradation occurs in the gland during the intervals between milkings (Le Roux *et al.*, 1995; Urech *et al.*, 1999; Leitner *et al.*, 2004a, b, c). Thus, the association between CN degradation and the reduction in aCa^{2+} may be related to the exposure of phosphoserine groups that are hidden within the casein micelles, and the differences in aCa^{2+} between the infected and uninfected glands may represent the additional CN degradation in the infected glands. Thus, measurement of aCa^{2+}, a rapid and inexpensive procedure, may serve as a valuable tool for monitoring the extent of CN degradation under various conditions.

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Host response reactions of the lactating ovine udder during experimental challenge with *Staphylococcus epidermidis*

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Abstract

The responses of five lactating Merino wool sheep (M) and 5 lactating East Friesian milk ewes (EF) to experimental mammary infection with *Staphylococcus epidermidis* were examined. Infection caused an influx of neutrophils into milk, the numbers of which started to rise 4 h after infection (p. i.) and peaked 8 to 24 h after infection. The initial response was accompanied by mild fever and mild leucopaenia in blood. No other signs of systemic infection were observed. At all times milk appeared normal. Numbers of staphylococci decreased logarithmically until 24 h, were absent from three (M) and four ewes (EF) two and three days p. i., but re-emerged intermittently in four of five ewes at subsequent samplings in both studies. Cytokines in milk were measured by ELISA. IL-8 was elevated in infected glands at 2 h and peaked at 8 h to 24 h. In the ewes intermittently shedding bacteria, IL-8 remained elevated until the final sampling at 10 weeks. IL-1β was transiently elevated at 1 and 2 d. One EF sheep showed an intense peak of IL-1β. Milk samples from this EF ewe were bacteriologically negative, and the concentrations of IL-1β, as well as IL-8, were low from one week p. i. until the final sampling. Histological examination revealed leucocytic infiltrates in the four EF glands remaining infected, and a high level of CD5+ lymphocytes in three ewes. The results of both studies showed that the intense early neutrophil infiltrate eliminated most but not all bacteria and a state of subclinical infection ensued. The course of infection is determined by the relationship between the initial neutrophil influx and the proinflammatory cytokines.

Keywords: cytokines, coagulase negative staphylococci, subclinical

Introduction

In recent years sheep milk and milk products have gained increasing popularity. As a result of this demand, udder health in dairy sheep flocks has become more important. Although subclinical intramammary infections due to coagulase-negative staphylococci (CNS) in sheep dairy flocks are increasing (Winter *et al.*, 2002; Wittek *et al.* 1998; Leitner *et al.* 2001; Moroni and Cuccuru, 2001), the pathogenic mechanisms that lead to subclinical mastitis after infection with CNS are largely unknown. In other types of bacterial mastitis, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and

interleukin-8 (IL-8) have been found to be released (Persson Waller *et al.* 1996; Persson Waller *et al.* 1997; Boudjellab *et al.* 1998; Boudjellab *et al.* 2000; Riollet *et al.* 2000; Hagiwara *et al.* 2001; Kehrli and Harp, 2001; Winter and Colditz, 2001; Winter *et al.*, 2003). The presence of these cytokines is also associated with changes in mononuclear cell populations, which are important for both inflammatory and immunological reponses in the udder (Persson Waller and Colditz, 1998; Persson Waller and Colditz, 1999; Rivas *et al.* 2000). However, cytokine and cellular responses in the mammary gland following acute CNS infection, and their role in inducing resolution or progression to subclinical infection has not been determined. In the present study, the effect of intramammary challenge of ewes with *Staphylococcus epidermidis* on bacterial shedding and on several parameters of host defence were examined over a period of 10 weeks.

Materials and methods

The study was conducted in two experiments approved by the animal ethics committee, CSIRO Armidale (trial A) and the Austrian Federal Ministry for Education, Science and Culture (trial B). Ten clinically healthy multiparous Merino ewes (A) and ten East Friesian ewes (B) were used. An inoculum of 2.8×10^6 (A) or 3.3×10^7 (B) bacteria in 1 ml volume per sheep was introduced into the left mammary gland of five ewes of each group. In each trial, five control sheep received an intramammary infusion of 1 ml pyrogen free saline. Milk and jugular blood samples were collected before infection and at 2, 4, 8, 24, 48, 72, 144 h and in trial B additionally at 2, 3, 4, 6, 8 and 10 weeks after infection. Milk samples were collected in sterile containers aseptically by hand stripping. At each sampling the ewes were clinically examined, and feed intake and rectal temperature were recorded.

Leucocyte counts were measured by means of a haematology analyser (CA 580 A, Medonic, Stockholm, Sweden) calibrated for sheep blood. The somatic cells (SCC) in each milk sample were counted by means of a fluorescencoptical method (Fossomatic). A loopful (0.01 ml) of each milk sample was inoculated onto Columbia blood agar and incubated aerobically at 37° C for 24 - 48 h. For cytokine analysis milk samples were centrifuged (2500 *g*, 20 min, 4° C) and the fat- and cell-free milk fractions were collected. These samples were then frozen at -30° C until assayed. The concentrations of cytokines IL-8 and IL-1β in these milk samples were determined by ELISA as described by Persson Waller *et al.* (1996). Monoclonal antibodies to ovine IL-8 and IL-1β were obtained from Serotec (Biomedica, Vienna, Austria), and recombinant ovine cytokine standards were a kind gift of Peter McWaters, CSIRO Livestock Industries, Geelong, Australia.

In trial B the five infected animals were killed 10 weeks after infection and promptly necropsied. Milk samples from the left (unchallenged) glands of these five ewes were examined 2 d before euthanasia and were found to be bacteriologically negative and to have SCC comparable to the control group. These unchallenged glands were subsequently used as control tissues for histology. After gross examination, three tissue samples from both mammary glands were removed for histological investigation. The samples were selected from the deep parenchyma near the dorsal surface of the gland, in the centre of the gland and at the point of transition from teat cistern to gland cistern. Tissue samples were fixed in 70 g/l neutral buffered formaldehyde. After embedding in paraffin, 4-µm sections were cut and routinely stained with hematoxylin and eosin (HE). For cell types of interest, sections were examined at x 40 magnification. For immunohistochemical

investigations, tissue samples from the same locations were frozen in isopentane/liquid nitrogen and stored at -20° C. Sections were prepared and stained as described by Jörundsson *et al.* (1999). The presence and distribution of leucocytes expressing CD4, CD5, CD8, γδ T cell receptor and MHCII surface antigens was determined. Tissue sections from right supramammary lymph nodes of challenged ewes served as positive controls and sections from organs usually not containing lymphocytes were used as negative controls. Positive cells staining brown following the peroxidase reaction were scored as above.

Results

During both experiments the controls remained clinically healthy, and results of bacteriological examination of their milk samples were negative. The mean SCC ranged from 37.6 to 330 (x 10^3) cells/ml. The cytokines IL-1β and IL-8 were within a range from 0.00 to 0.38 ng/ml, and from 0.00 to 11.57 ng/ml, respectively.

Feed intake and behaviour were not affected in challenged ewes. Eight hours after infection a slight and shortlived increase of the rectal temperature and swelling of the infected glands were observed. Simultaneously leucocyte counts in blood dropped to their lowest level. Milk appeared normal, was not discoloured and did not contain any flakes or clots. Immediately after infection (2, 4, 8 h) bacteria were isolated from milk samples of all infected animals. At subsequent samplings, milk from some infected ewes became bacteriologically negative. However, bacteria re-emerged in both trials in milk from four challenged glands until the end of the experiment (table 1), while milk from one sheep of each experiment remained free of bacteria after 8 h. SCC in milk increased 4 hours after infection and peaked 8 hours (trial A) and 1 day after infection (trial B), respectively. Leucocyte counts returned to a low level from 48 to 144 h in trial A, whereas in trial B SCC

Table 1. SCC, IL-1β, IL-8 and bacteriological positive results (BE +) in milk of experimentally infected glands in trial A and B.

	Trial A				Trial B			
Time	SCC	IL-1β	IL-8	BE+	SCC	IL-1β	IL-8	BE+
pi	x10^6/ml	ng/ml	ng/ml	n	x10^6/ml	ng/ml	ng/ml	n
0	0.09	0.15	13.35	0	0.05	0.00	0.38	0
2h	0.08	0.25	38.56	5	0.08	0.00	10.38	5
4h	22.50	0.52	40.94	5	4.83	0.02	38.41	5
8h	202.76	1.24	45.56	5	7.70	0.17	46.19	5
1d	19.21	2.41	43.82	4	7.99	2.62	40.03	3
2g	2.27	1.51	31.16	3	6.49	0.85	42.46	1
3d	1.35	1.54	18.31	4	5.05	0.21	39.02	1
6d	0.35	0.59	6.48	3	1.47	0.21	12.49	4
2w					4.06	0.20	30.75	3
3w					4.37	0..21	53.45	0
4w					1.74	0.04	31.60	3
6w					4.43	0.13	43.15	2
8w					3.05	0.00	25.01	2
10w					2.22	0.00	22.09	3

remained elevated until the end of the experiment (table 1). The concentrations of the cytokines IL-1β and IL-8 were significantly elevated in infected glands and peaked at 8 hours and 1 day after infection (table 1).

In concordance with the bacteriological clearance of the milk from one sheep in trial B, SCC in its milk dropped to a physiological level at the end of the experiment together with the concentration of IL-8. Milk from this sheep showed an intense peak of IL-1β (11.52 ng/ml) 1 day after infection. The mammary gland appeared normal by histopathological examination. No CD5 cells were observed, whereas these cells were particularly prevalent in 3 ewes. No apparent differences in the presence of CD4+, CD8+, MHCII and γδ+ cells in infected and control glands could be detected.

Discussion

The interaction between invading bacteria and the defence systems of the host determines the outcome of infection. To limit infection of the mammary gland, bacteria must be recognised early and the local inflammatory response, especially neutrophils, mobilised promptly. Experimental challenge with *S. epidermidis* caused an intense but transient elevation of leukocyte counts in milk, which peaked 8 h and 24 h pi and coincided with mild fever and a moderate nadir in circulating leucocyte counts in blood in both trials. Some differences in the host reactions of both trials might be due to the fact that the ewes in trial A were kept together with their lambs, which suckled from the infected udders, whereas in trial B the ewes were milked twice a day. The leucocyte influx, comprised overwhelmingly of neutrophils, depressed bacterial counts in milk and led to a transient clearance of bacteria from milk. Leucocyte counts in milk rapidly declined over the next day and were accompanied by a re-emergence of bacteria in milk in most ewes. Elevated SCC was accompanied by elevated IL-8 concentrations. Early appearance of IL-8 is in accord with studies on *S. aureus* mastitis and might be stimulated by bacterial products (Persson Waller *et al.*, 1997; Persson Waller and Colditz, 1999). Monocytes and alveolar macrophages appear to be the predominant cellular sources of IL-8 in the presence of LPS, but this chemotactic cytokine can also be produced by several non-immune cells such as epithelial cells (Eckmann *et al.*, 1993). Production of IL-8 by mammary epithelial cells cultured in vitro with endotoxin has recently been reported (Boudjellab *et al.*, 1998; Barber *et al.*, 1999). Together with the early appearance of IL-8 in milk following infection in the current study, these reports suggests a prominent role for IL-8 in recruitment of neutrophils in the mammary gland. After neutrophils clear most of the bacteria, the ongoing production of IL-8 might be linked to IL-1β. Although based on observations in only one animal, the pronounced peak in IL-1β seen in this sheep suggests the possibility that IL-1β played an important role in terminating S. epidermidis infection. Once the bacteria were completely removed from the gland the stimulus for production of IL-8 decreased. The peak might have activated neutrophils for enhanced phagocytosis and intracellular killing of bacteria. Failure to eliminate bacteria rapidly may lead to hyporesponsiveness of defence mechanisms in the mammary gland (Maas and Colditz, 1987; Young *et al.*, 2001) and survival of bacteria within leucocytes and epithelial cells (Bayles *et al.*, 1998). This pattern is consistent with a low toxigenicity of CNS, which when present in large numbers following artificial infection are able to provoke production of pro-inflammatory cytokines that recruit large numbers of neutrophils into the gland. These phagocytes cleared the majority of bacteria, however,

survival of some bacteria within phagocytes may have occurred leading to re-emergence of bacteria in milk when numbers of phagocytes dropped to a low level. This phenomenon may accentuate the need for rapid elimination of bacteria by the initial leucocyte influx in order to prevent the development of chronic infection.

In addition to neutrophils, mononuclear cells are thought to play an important role in mammary defence. Changes in prevalence of mononuclear subsets occur during mastitis, but the associations with resistance of infection are difficult to demonstrate (Rivas *et al.*, 2001, Riollet *et al.*, 2001). In the current experiment B CD 5+ cells were present in higher numbers in the mammary glands still suffering from subclinical mastitis. CD5+ lymphocytes have previously been noted to cluster around vascular tissue in the ovine mammary gland (Lee *et al.*, 1989) and to increase in number in milk during mastitis (Ayoub *et al.*, 1996).

Lactating udders are capable of a prominent local inflammatory response. The association between the concentrations of IL-1β and IL-8, and the intensity of neutrophil infiltration of the infected gland are thought to influence the clinical appearance of infection (Kehrli and Harp, 2001). Further work is required to identify strategies to enhance mammary immune defence against coagulase negative staphylococci. The study highlights the importance of the initial host response in eliminating intramammary infections with *S. epidermidis*.

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Efficacy of antibiotic treatment at drying-off in curing existing infections and preventing new infections in dairy goats

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Abstract

Milk samples were collected from each gland of 476 dairy goats (n = 4 herds), 7 days prior to the end of lactation and again at the final milking of the lactation for bacteriology and somatic cell count (SCC). Animals were blocked by intramammary infection (IMI) status and age (1, 2, >2 years) and ranked on the average SCC of both glands within a doe. Half the goats were assigned to be treated by intramammary infusion with 300 mg of procaine penicillin, 100 mg of dihydrostreptomycin and 100 mg of nafcillin (Nafpenzal DC, Intervet, Auckland, New Zealand) and the others were left as untreated controls. Milk samples were collected between 0 and 4 days post-kidding and again 3 to 4 days later for bacteriology.

The gland level prevalence of IMI before drying-off was 27.2% (258/948). Pathogens isolated included coagulase negative staphylococci (59.3% of all IMI), *Corynebacterium* spp. (29.1%), *Staphylococcus aureus* (6.2%), *Streptococcus agalactiae* (4.3%) and other species (1.2%). Cure proportion increased following treatment (RR=2.98; P<0.01), was lower in >1-year-old compared to 1-year-olds (RR=0.87; P=0.07) and was lower for *Staphylococcus aureus* than other species (RR=0.54; P<0.01). Treatment reduced the likelihood of new IMI (RR=0.14, P<0.01). Older animals were more likely to acquire a new IMI than younger animals (RR=1.88 and RR=3.22 for 2 and >2-year-olds relative to 1-year-olds, respectively; P<0.01). Glands with an IMI at dry-off were less likely to acquire a new IMI than those uninfected (RR=0.16; P<0.01). Treatment reduced the $\log_{10}$ SCC at the first herd production recording test in the subsequent lactation (2.21 vs. 2.49 (SED = 0.06) $\log_{10} \times 10^3$ SCC/ml for treated vs. control respectively, P<0.001).

It is concluded that infusion of antibiotics increased the bacteriological cure proportion, reduced the incidence of new infection over the non-lactating period and reduced SCC in dairy goats.

Introduction

Infusion of antibiotics into the udder of cows at the end of the lactation ("dry cow therapy"; DCT) increases the cure rate of existing intramammary infections (IMI) and reduces the incidence of new infections over the dry period with a resultant reduced prevalence of IMI at the commencement of the subsequent lactation (Smith *et al.*, 1967; Harmon *et al.*, 1986). The cure rate of existing infections after DCT is 60-90% (Pankey *et al.*, 1982; Sol *et al.*, 1994; Browning *et al.*, 1990; Williamson *et al.*, 1995) and DCT reduces the new IMI rate by approximately 50% (Browning *et al.*, 1994; Williamson *et al.*, 1995).

Infusion of antibiotics at the end of lactation in dairy goats ("dry-goat therapy"; DGT) results in cure rate of existing IMI of between 66%-100% (Fox *et al.*, 1992; Poutrel and de Cremoux, 1995; Poutrel *et al.*, 1997). Untreated, but infected animals had cure rates of between 20% and 25% (Poutrel and de Cremoux, 1995).

The aim of this study was to assess the efficacy of intramammary antibiotic treatment of dairy goats at the end of lactation in curing existing mammary gland infections and reducing the incidence of new infections over the dry-period and early in the subsequent lactation.

Materials and methods

Duplicate milk samples (~5 ml) were collected from both glands of 476 dairy goats (n = 4 spring-kidding herds), for bacteriology and somatic cell count (SCC), 7 days prior to the end of lactation. Animals were blocked by bacteriological status (0 = uninfected, 1 = one gland infected, 2 = two glands infected), ranked on age (1, 2, >2 years) and ranked on the average SCC of both glands 7 days before the end of lactation, then randomly assigned to one of two treatments within sequential pairs of goats within herds. Following the final milking, each gland of each goat within the treatment group was infused with 300 mg of procaine penicillin, 100 mg of dihydrostreptomycin and 100 mg of nafcillin (Nafpenzal DC, Intervet, Auckland, New Zealand) while the other does were left as untreated controls. Milk samples were collected before the final milking for bacteriology. Duplicate milk samples were again collected between 0 and 4 days post-kidding and again 3 to 4 days latter, for bacteriology. The SCC was determined for each goat ~ 40 days postpartum in preserved milk samples using a Fossomatic fluoroptic counter (Testlink, Hamilton, New Zealand).

Milk (10µl) was streaked onto Columbia sheep blood (5%) agar (Fort Richard Laboratories, Auckland) and incubated for 48 h at 37°C. Bacteria were speciated on the basis of colony morphology, Gram stain reaction and catalase, coagulase and CAMP tests using NMC recommendations.

A gland was defined as infected if culture of two of the three pre dry-off samples resulted in growth of 3 or more colonies of the same bacterial species. A gland was defined as cured if the bacterial species present at drying-off was not isolated from any of the samples taken post-kidding. A gland was defined as being newly infected if no bacteria were isolated at drying-off and then 3 or more colonies of the same bacterial species were present in either of the posting kidding samples, or if the bacterial species isolated post-kidding was different from the species isolated pre dry-off. A sample was defined as contaminated if > 2 distinct colony types were observed.

Bacteriological data were analysed at gland level and it was assumed that glands within a goat were independent. The outcomes of interest were the cure proportion and the new infection incidence over the non-lactating period. Backward stepwise was used to analyse cure and new infection rates. Univariate analysis (either χ^2 or logistic regression) of potential explanatory variables (age, herd, treatment, bacterial species at drying off and length of the non-lactation period) was undertaken and variables found to be associated ($P<0.2$) were then offered to a backward stepwise logistic regression models using likelihood ratio to remove variables. A number of glands were missed or contaminated at subsequent samplings and were removed from the analyses. The Log_{10} SCC were analysed at goat level by a general linear model with herd, number of glands infected at end of lactation and treatment as main effects.

Results

The gland prevalence of IMI at the end of lactation was 27.2% (258/948). Pathogens isolated included coagulase negative staphylococci (CNS; 59.3% of all IMI), Corynebacterium spp. (29.1%), *Staphylococcus aureus* (6.2%), *Streptococcus agalactiae* (4.3%) and other species (1.2%).

Cure proportion increased following treatment (RR = 2.98 for treated compared to control; P<0.01; Table 1), decreased with age (38/56 (67.9%) vs. 97/167 (58.1%), RR = 0.87; P=0.07) for 1 year olds compared to > 1 year olds respectively) and was lower for *Staphylococcus aureus* infections than other bacterial species (RR = 0.54; P<0.01).

Treatment reduced the likelihood of new IMI compared to untreated controls (RR = 0.14, P<0.01; Table 1). Older animals were more likely to acquire a new IMI than younger animals (8/287 (2.8%), 12/245 (4.9%) and 29/314 (9.2%) for 1, 2 and >2-year-olds respectively; RR = 1.88 and RR = 3.22 for 2 and >2-year-olds relative to 1-year-olds, respectively; P<0.01). Glands with an IMI at dry-off were less likely to acquire a new IMI than glands uninfected at dry-off (6/238 (2.5%) vs. 43/609 (7.1%), RR = 0.16; P<0.01). There was a treatment by infection status interaction (P<0.05; Table 1) as control glands within the uninfected group had a higher new IMI than treated but uninfected glands but among the infected glands, treatment had no effect on new IMI rate.

Treatment decreased SCC (2.21 vs. 2.49 (SED = 0.06) $\log_{10} \times 10^3$ SCC/ml for treated vs. control respectively, P<0.001). Goats infected at drying off had higher SCC in the subsequent lactation than uninfected does (2.21 (SE = 0.03), 2.35 (SE = 0.05) and 2.50 (SE = 0.06) $\log_{10} \times 10^3$ SCC/ml for does with 0, 1 or 2 glands infected at the end of the previous lactation, P<0.001). However, there was a treatment by infection status interaction (P<0.001) such that in uninfected does treatment did not affect SCC, while in infected does treatment significantly reduced SCC compared to untreated does (Figure 1). Herds tended to differ in SCC (P=0.07).

Table 1. Cure proportion and new infection proportion for glands infected or uninfected at drying-off and following kidding in goats treated by intramammary infusion of antibiotics (penicillin, dihydrostreptomycin and nafcillin) at drying-off (treated) or left as untreated controls (Control).

Status at end lactation	Group	n enrolled	Cured			New infections		
			n	total n	%	n	total n	%
Uninfected	Treated	311				6	311	1.9
	Control	298				37	298	12.4
	Total	609				43	609	7.1
Infected	Treated	114	99	107	92.5	3	113	2.7
	Control	129	36	116	31.0	3	125	2.4
	Total	243	135	223	60.5	6	238	2.5

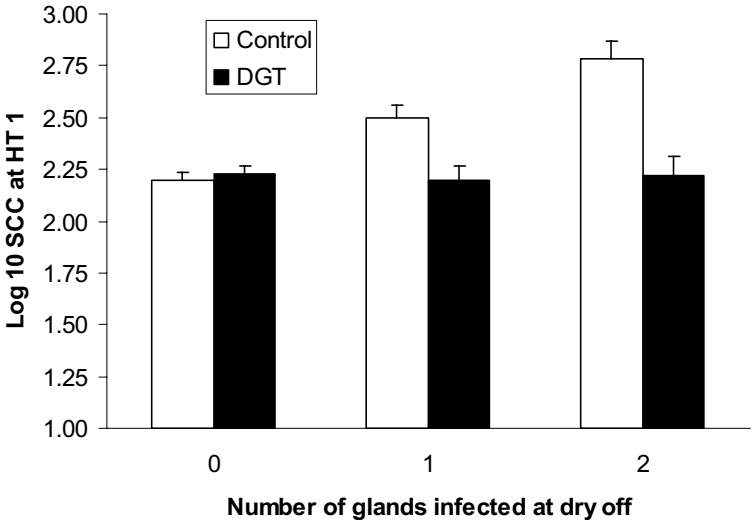

Figure 1. The estimated marginal mean log_{10} SCC (and standard error of the mean) at the first herd test (HT1) in the subsequent lactation for does that had 0, 1 or 2 glands with intramammary infection at the end of the previous lactation and that were either treated by intramammary infusion with antibiotics (DGT) or left as untreated controls (Control).

Discussion

This study demonstrated that infusion of antibiotics at drying-off into the goat mammary gland reduced the number of new infections over the dry period and increased the cure proportion of the existing infections.

The cure proportion was 92.5% in treated, and 31% in untreated glands which is similar to previous reports from goats (Fox *et al.*, 1992; Poutrel and de Cremoux, 1995) and cattle (Smith *et al.*, 1967; Sol *et al.*, 1994; Williamson *et al.*, 1995). In common with reports from cattle, the cure proportion of *Staphylococcus aureus* was lower than for other IMI (Sol *et al.*, 1994; Sol *et al.*, 1997; Owens *et al.*, 1997). Low *Staphylococcus aureus* cure proportions have been associated with poor penetration and distribution of antibiotics throughout the gland (Owens and Nickerson, 1990) and the ability of *Staphylococcus aureus* to survive intracellularly (Craven and Anderson, 1984; Owens and Nickerson, 1990). Cure proportion was higher in younger animals in agreement with studies in dairy cattle (Sol *et al.*, 1994; Sol *et al.*, 1997) and may be due to a shorter duration of infection before treatment in younger does or reduced immunocompetence with age. The reduced cure proportion of chronic IMI may be due to a greater degree of parenchymal damage and/or an increased probability of microabscess formation with a resultant lower cure rate due to poor antibiotic penetration and distribution into parenchymal tissue (Owens and Nickerson, 1990). Phagocytosis within milk also declines with increasing age (Paape *et al.*, 1978).

The number of new IMI across the non-lactating period was reduced from 9% to 2% following DGT which is consistent with cattle studies (Smith *et al.*, 1966; Browning *et al.*, 1990; Williamson *et al.*, 1995). New IMI rate was reduced by the presence of an IMI at

drying-off as has been reported in dairy cattle (Pankey *et al.*, 1982; Pankey *et al.*, 1985; Rainard and Poutrel, 1988).

The SCC was lower in treated than control goats was likely due to the combined effect of a higher cure rate of existing infection and lower new infection rate with a net lower prevalence of infection in the subsequent lactation.

It is concluded that infusion of antibiotics into the mammary gland of dairy goats at the end of lactation results in an increased cure proportion of existing infections and a reduction in the number of new infections. Hence dry-goat therapy is a useful tool for managing milk quality in dairy goats.

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Animal welfare issues related to mastitis

Associations between hygiene scores and udder health parameters in organic dairy herds

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Abstract

One important goal of organic farming is the promotion of animal health and the reduction of antibiotic treatments. 22 organic dairy herds participated in a project with the aim to phase out antibiotic treatments. Under these conditions somatic cell count (SCC) and records of treated cases of mastitis may be insufficient to document udder health and animal welfare. The aim of our study was to assess and characterise udder health by using systematic clinical examinations. The udders of 50 cows per farm were clinically examined during milking at the beginning of the project period. The hygiene of body and udder was scored. The data was analysed using Principal component analysis and a general linear model. The prevalence of cows with knotty tissue ranged from 0-0.46, the prevalence of cows with nodes in the udder from 0-0.17 and the prevalence of cows with a dry quarter from 0-0.18. Danish Holsteins had a higher number of udder treatments per 100 cow years than Jerseys. Principal component analysis extracted two components: component 1 was characterized by udder and body hygiene scores, number of antibiotic treatments and Danish Holsteins, whereas component 2 was characterized by nodes in the udder, knotty tissue and blind quarters. Farms loading high on component 2 had a higher calculated bulk milk SCC. Nodes, knotty tissue and blind quarters were not associated with the number of udder treatments. This indicates that a lower treatment frequency did not lead to a higher proportion of cows with chronic mastitis. The different management practices in the farms will be further discussed in the presentation. Systematic clinical udder examinations were useful tools to monitor udder health.

Keywords: organic farms, treatment, udder characteristics, hygiene

Introduction

One important goal of organic farming is the promotion of animal health and a reduced usage of antimicrobial agents. In organic dairy farming, mastitis is the most important disease regarding the amount of antibiotics used for treatment (Bennedsgaard 2003). The risk of antibiotic resistance, poor mastitis treatment results and the concerns of the consumers regarding residuals in milk products have started a discussion among Danish organic farmers to promote explicit non-antibiotic treatment policies in their herds. As a result of these discussions, a project was initiated in 2004 with the aim to phase out antibiotic treatments in 22 organic farms. Under an explicit non-antibiotic treatment strategy, the number of treated cases of mastitis does not reflect the true number of mastitis cases. One problem of a non-antibiotic treatment strategy can be reduced animal welfare, because farmers just may stop calling the veterinarian leaving the cows untreated. The

farmer's decision to treat a cow with mastitis with antibiotics depends on the seriousness of the symptoms, the characteristics of the cow, the infection level in the herd and his attitudes towards alternative treatment (Vaarst *et al*., 2002). All participating farmers expressed that they wanted to keep the opportunity for antibiotic treatments in cases of emergency, but their thresholds when regarding a case as emergency may differ. Systematic clinical examinations of the udders may be an important tool to assess udder health in organic farms with non-antibiotic treatment strategy.

The aim of this paper is to present the results from the clinical udder examinations and information already available in data bases and to demonstrate how this information could be combined/analysed to assess the udder health under non-antibiotic premises. Furthermore we wanted to evaluate, if an explicit non-antibiotic policy has a negative effect on udder health.

Material and methods

The participating farms were located in central and northern Jutland. Mastitis treatments with antibiotics were registered in the Danish Cattle Base and were summarized as the mastitis treatments per cow year within 6 months prior to the clinical udder examination. In Danish organic farms all antibiotic treatments are applied by the veterinarian. Herd characteristics are presented in table 1.

At the start of the project, the first author examined the udders of all cows immediately after milking. In farms with more than 50 cow years, a random sample of 50 cows was examined. Table 2 shows the herd prevalence and the definitions of several clinical features.

Statistical analysis

Principal component analysis (PCA) was performed using the factor procedure of SAS (Proc factor, SAS, 2000). The number of components to retain was determined by evaluating the eigenvalue-one criterion, the scree test, the proportion of variance accounted for and the interpretability criteria (Hatcher, 1994). The scree test displayed two components with the largest eigenvalues before a visible 'break'. Further, a component was regarded as important if at least three variables had high loadings on that component and shared the same conceptual meaning. A factor loading greater than +/- 0.35 was considered to be important. Loadings range from

-1 to +1 and represent the correlation of a given variable with the underlying factor. PCA attempts to identify a minimum number of components explaining the majority of the variation in the original data.

The relation between the extracted components with the CBSCC was analyzed with a general linear regression model.

Results and discussion

The organic farmers had on average 0.12 mastitis treatments per cow year (Table 1), which is very low in comparison to Bennedsgaard *et al*. (2003) who reported 0.33 mastitis treatments in 24 organic herds converted before 1990 and 0.50 mastitis treatments in organic herds that converted in 1995. Conventional herds had 0.59 mastitis treatments per cow year. 6 farms did not have any antibiotic mastitis treatments. Most of the participating

Table 1. Herd characteristics of 22 organic dairy herds that want to reduce antibiotic udder treatments.

Herd ID	Cow Housing[1] years	Breed[2]	CBSCC[3]	MAST[4]	SCC rank culled cows [5]	ECM[6]
1	35 DL	J	235	0	0.89	6235
2	44 DL	J	157	0	0.74	5817
3	109 DL	DH	237	0.02	0.54	7667
4	68 SF	DH	160	0	0.67	7912
5	96 DL	DH	247	0.23	0.54	7504
6	39 DL	DH	291	0.15	0.67	6127
7	158 DL	J	242	0.02	0.78	7500
8	86 SF	DH	143	0.07	0.68	6277
9	34 SF	J	254	0	0.71	5560
10	43 DL	J	222	0.09	0.61	6352
11	81 DL	DH	464	0.37	0.60	6564
12	62 DL	DH	261	0.22	0.70	7370
13	84 SF	DH	454	0.21	0.73	7577
14	71 DL	J	266	0	0.64	7449
15	58 SF	DH	132	0.03	0.75	8559
16	134 SF	DH	347	0.17	0.73	7960
17	48 DL	DH	342	0.47	0.69	7247
18	115 SF	J	266	0.11	0.75	8373
19	62 SF	DH	162	0.31	0.69	9000
20	45 SF	DH	84	0.20	0.59	7677
21	77 SF	J	317	0	0.61	6272
22	52 DL	J	228	0.11	0.56	6773
Avg.	73		250	0.12	0.67	7178

[1]DL = Deep litter system, SF = loose housed with slatted or concrete floor

[2]J = Jersey, DH = Danish Holsteins

[3]calculated bulk somatic cell count (x1000/ml)

[4]mastitis treatments per cow year

[5]SCC rank of last test day before culling

[6]energy corrected milk yield in kg, 305 d production

farmers already have reached far in implementing a non-antibiotic treatment policy in their daily management. In three farms no cow with a dry quarter was found whereas in other farms up to 18% of cows had a dry quarter. On average 7 % of cows had a dry quarter, which is comparable to the results of Houe *et al*. (2002) in 4 conventional herds and Klaas *et al*. (2004) in 8 farms with automatic milking system.

The PCA extracted 2 components that explained 70% of the variation in the data (Table 3). Component 1 was labelled 'Soiling, DH & treatments' because it consisted of the variables dirty leg index, dirty udder index, DH and mastitis treatments. Component 2 consisted of variables that described an udder with chronic clinical changes and therefore was called 'chronic mastitis udder'.

Poor hygiene, DH and high incidence of udder treatments were related. There are several explanations to be discussed. Dirtier legs and udders reflect a higher risk of getting mastitis.

Table 2. Results from clinical examinations in 22 organic herds.

Herd ID	Dirty legs index[1]	Dirty udder index[2]	Prev. nodes[3]	Prev. knotty tissue[4]	Prev. of dry quarters[5]
1	0.95	0	0.04	0	0.03
2	0.44	0.03	0.02	0.12	0.12
3	1.08	0.15	0.05	0.15	0.12
4	1.11	0.37	0.12	0.37	0.14
5	1.09	0.25	0	0.47	0.03
6	1.76	0.70	0.09	0.07	0
7	0.34	0.04	0.02	0.03	0
8	1.09	0.23	0.03	0.06	0.02
9	0.56	0.07	0.07	0.23	0.03
10	0.54	0.16	0.11	0.16	0.11
11	1.86	0.46	0.04	0.12	0.06
12	1.11	0.18	0.14	0.43	0.18
13	1.27	0.45	0.18	0.29	0.14
14	1.41	0.39	0.04	0.08	0.08
15	1.55	0.84	0	0.07	0.02
16	1.17	0.52	0.03	0.12	0.12
17	1.26	0.46	0.09	0.09	0.17
18	0.92	0.16	0	0.13	0.03
19	1.11	0.43	0.02	0.05	0.03
20	0.78	0.25	0	0.05	0
21	0.79	0.05	0.15	0.23	0.08
22	0.79	0.07	0.07	0.24	0.08
avg	1.04	0.28	0.06	0.16	0.07

[1]Herd mean of degree of soiling with manure on legs scored from 0-4. 0=no soiling, 4=whole body with thick layer of manure

[2]Herd mean of degree of soiling with manure on udder scored from 0-2. 0=no soiling, 2 = manure on bigger parts of the udder

[3]prevalence of cows with distinct nodes in at least one quarter

[4]prevalence of cows with knotty tissue in at least one quarter

[5]prevalence of cows with dry quarters, cows that did not produce milk in 1 quarter

Second, farmers that do not focus on keeping their cows clean may be more likely to treat mastitis cows with antibiotics. In another study (Klaas *et al*. 2004), cows in early lactation were associated with higher degree of soiling of the udder. Stage of lactation was not included in our analysis. DH had a higher milk yield than Jerseys. Milk yield was loading significantly on component 1 in the initial PCA, but when milk yield was adjusted for breed, the adjusted milk yield did not show significant loadings on any of the two components and therefore was excluded from further analysis, while breed effect still remained important. The role of breed has to be further analyzed

Calculated bulk tank SCC (CBSCC) was excluded from the PCA because it loaded non-significantly on both components. The relationship was examined in a general linear model and revealed that a unit increase of component 2 increased CBSCC by 45. Component 1

Table 3. Principal components analysis: Correlations (standardized regression coefficient) between udder health parameters and 2 components after orthogonal rotation (varimax rotation).

	Component 1 'Soiling, DH & treatments'	Component 2 'Chronic mastitis udder'
Dirty leg index	0.88 *	-0.04
Dirty udder index	0.88 *	-0.15
Danish Holstein (DH)	0.86 *	0.13
Mastitis treatments per cow year	0.69 *	0.15
Prevalence of dry quarters	0.08	0.86 *
Prevalence of cows with nodes	0.02	0.84 *
Prevalence of cows with knotty tissue	-0.01	0.78 *
Eigenvalue	3.2	2.1
Variance explained (%)	40	30

*correlation coefficients > 0.35 were regarded as significant

showed a quadratic relation to CBSCC. The relation is demonstrated in figure1, where CBSCC is cut in three categories. The lowest CBSCC was observed in farms loading high on component 1 and low on component 2. Farms with negative loadings on both components had medium CBSCC between 200 and 300; only one had a CBSCC less than 200. This indicates that a non-antibiotic treatment policy does not affect the number of cows with chronic changes in the udder.

Conclusions

PCA extracted to components that may represent two different herd characteristics: the 'Dirty, DH & treatments' type and the chronic udder type. High loading on component 1 'Soiling, DH & treatments' was associated with both high and low CBSCC, whereas high

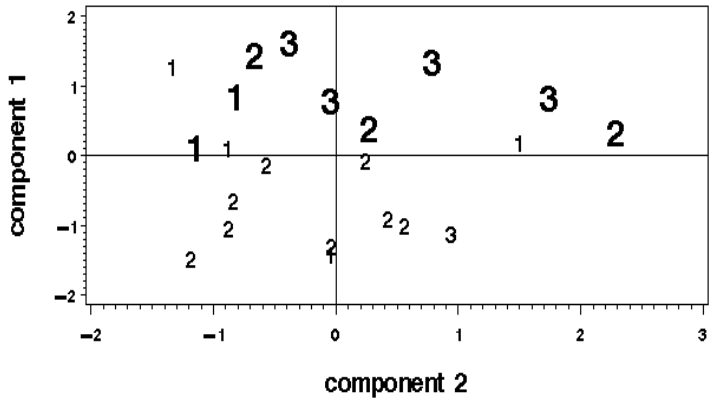

Figure 1. Relation between the 2 principal components and the calculated bulk tank SCC. Component 1: Soiling, DH & treatments, Component 2: Chronic mastitis udder. CBSCC (in 100/ml) 1: < 200, 2: 200-300, 3: ≥ 300. Numbers in regular font: < 0.12 mastitis treatments per cow year. Boldface numbers: ≥ 0.12 mastitis treatments per cow year.

loading on the component 2 'chronic udder' was associated with high CBSCC. Clinical features can be a useful tool in udder health assessment in farms with non-antibiotic treatment policy together with evaluation of SCC. Herds with Jersey had the lowest usage of antibiotics for mastitis combined without large problems with elevated CBSCC. The Jersey herds also had the cleanest cows, but it was not possible to decide whether these results were due to management or breed effects.

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Teat lesions and teat necrosis in heifers in The Netherlands

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Summary

In recent years an increasing number of Dutch farms had to deal with teat lesions, which in extreme cases resulted in sloughing of the teat (2004: on average 1 animal on 5% of the farms). This phenomenon was almost exclusively seen in heifers and it affected usually one heifer on a farm in the year. In a few cases the heifers licked or bit off the teat.

There are 2 manifestations of the phenomenon.

The first is periparturient and usually affects the whole teat. The skin of the teat is hardened and has a purple colour. It appears as if there is a fluid filled blister, parts of the affected skin slough and the teat gets hard and stiff thereafter.

The other manifestation starts with a vesicle on the teat, which ruptures becoming a small fissure, which then deepens. This fissure is usually at the base of the teat, close to the udder although it has also been seen elsewhere on the teat but not on the udder itself. The fissure deepens slowly and in some cases perforates into the teat canal and results in sloughing of the teat. In a few cases it extends over the udder skin from one teat to another. This manifestation also usually starts periparturient but can be seen later in lactation. There is minimal response to treatment and, where there is a response, the healing process is slow taking weeks to months. There is no adequate therapy. This phenomenon was not related to bovine herpes mamillitis virus (BHV2), pseudocopox or papova viruses. In a few cases BHV4 was suspected. Fungi could not be detected, although in a few cases there was a response to antifungal treatment. *Staphylococcus aureus* and Arcanobacterium pyogenes were both isolated twice in 2 of the 25 examined cases.

There seems to be a relationship to a disturbed blood supply, selenium supply, sudden change in ration, the periparturient period, uddercleft dermatitis and, according to farmers, interdigital dermatitis.

Introduction

Infectious and noninfectious teat lesions and lesions of the bovine udder skin, including subcutis, has been described and presented by Blowey and Weaver (1996). Particularly viral infections can play an important role in the aetiology of teat lesions (reviewed by Gibbs, 1984). For example, pseudocowpox, belonging to the genus of *Parapox* is a common virus isolated throughout many countries of the world, has been detected in bovine teat lesions. Another virus, bovine herpesvirus 2 (BHV2), which can cause bovine herpes mammillitis or bovine ulcerative mamillitis, was initially isolated in Scotland in 1963

and thereafter in many other countries like the USA, Bulgaria, Australia, Brazil, UK and Switzerland (Janett *et al.*, 2000; Gibbs, 1984).

In The Netherlands the number of observed teat lesions increased in the past 10 years, and in extreme cases it resulted in necrosis of the teat. These newly observed types of teat lesions were almost exclusively recorded in heifers and it affected usually one heifer on a farm in a year. It was not observed each year on the same farm. Similar type of teat lesions have been described in Wisconsin (Moriello *et al.*,1993), Germany (Bruns *et al.*, 2002) and the UK (Holliman, 2003).

In this paper the clinical symptoms of 2 types of bovine teat lesions by Dutch heifers are described. Possible causes are discussed, and based on a questionnaire an estimation of the incidence in teat lesions is made.

Material and methods

In the period 1997-2004 25 farms, with teat lesions in 1 or more heifers, were visited by the Animal Health Service Ltd on request of the local veterinarian. Clinical symptoms were recorded and diagnostic (virology, bacteriology, histology, fungi, minerals) investigations were carried out. Sampling of affected animals were not done if the the condition had commenced over 1 week earlier. Only cases in the past half-year were recorded. Usually farms were only visited if there were more than 1 animal affected.

In 2004 a questionnaire was sent to 1500 randomly selected farmers of the 25.000 farmers with dairy cattle in The Netherlands. The questions concentrated on possible teat lesions. A photograph of the various teat lesions was shown at the relevant questions.

Results

On the 25 farms 89 cases had been seen in the previous 6 months. In 87 cases heifers were affected. In most cases (66) the problem had started around calving.

There are 2 manifestations of the phenomenon.

The first is periparturient and usually affects the whole teat. The skin of the teat is hardened and has a purple colour. It appears as if there is a fluid filled blister (Figure 1). Parts of the affected skin slough and the teat gets hard and stiff thereafter. The teat falls off in about 50% of the affected animals and in many cases the heifer will develop mastitis. In a few cases heifers become recumbent and must be euthanised. On occasion the wound

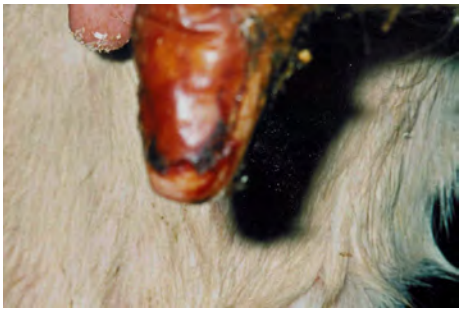

Figure 1. Fluid filled blister.

will heal following the loss of the teat and the quarter will stop milksecretion (Figure 2 and 3, right hind teat dropped).

The other manifestation (manifestation 2) starts with a vesicle on the teat, which after rupturing becomes a small fissure, which then deepens (Figure 4 and 5). This fissure is usually at the base of the teat, close to the udder although it has also been seen elsewhere on the teat (Figure 6) but not on the udder itself. The fissure deepens slowly and in some cases perforates into the teat canal and results in sloughing of the teat. In a few cases it extends over the udder skin from one teat to another (Figure 3). This manifestation also usually starts periparturient but can be seen later (weeks to months) in lactation.

These 2 manifestations can be seen on the same farm at the same time. In a few cases the heifers lick or bite off the teat. It is seen in all teats (left, right, front,hind).

Amazingly the large fissures on the teat of manifestation 2 are apparently not painful at milking. The wounds show no inflammation and the wound is clean.

From 12 farms 25 tissue and or vesicular fluid samples were investigated. BHV2 was never isolated, and in addition, no antibodies directed against BHV2 were detected in the affected heifers. In 2 samples from 2 farms BHV4 was isolated but 16 respectively 25 blood samples from a longitudinal section of these 2 herds were all BHV 4 seronegative. Other

Figure 2. Right hind teat dropped off.

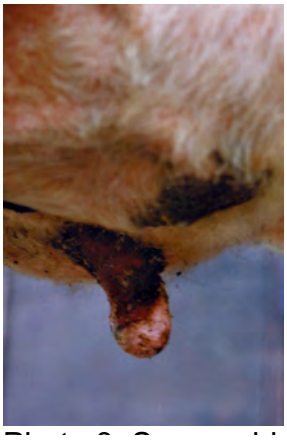

Figure 3. Same udder as Figure 2: right hind teat left and left hind teat also affected.

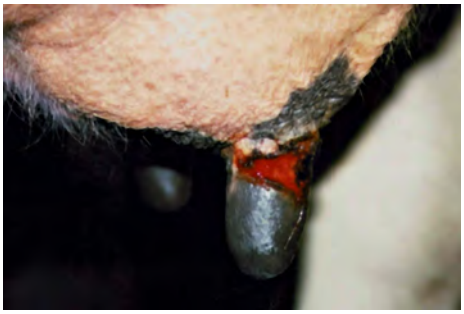

Figure 4. Small fissure.

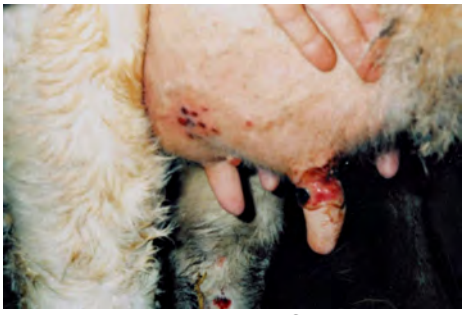

Figure 5. Deepened fissure.

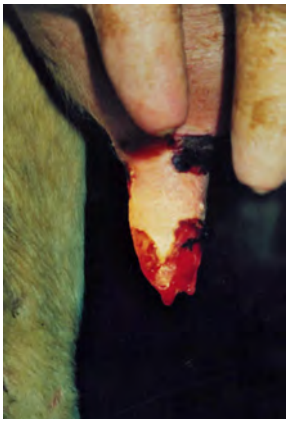

Figure 6. Fissure with peeling off of the skin at the end of the teat.

viruses, such as pseudocowpox or papova were not isolated by e.g. electron microscopy and/or virus isolation.

In 104 blood samples from 13 farms BHV2 antibodies were not detected and on 6 farms BHV4 antibodies were detected. But there was no relationship between the heifers with teat-necrosis and BHV4 antibodies in affected heifers.

In 2 cases Staphylococcus aureus was isolated and also in another 2 cases Arcanobacterium pyogenes. Fungi were never isolated.

Histologically examination of 3 teats showed in 2 cases thrombosis of vessels and in 1 case there were signs of a virus infection based on inclusion bodies in epithelial cells.

On 11 farms the selenium blood level (GSH-Px in U/g Hb) was determined in 4-6 heifers. On 5 farms the average level was below the recommended 120 and in 2 cases above the upper limit of 350.

On the 25 farms visited the feeding pattern and condition of the heifers was not different from the average feeding patterns and conditions for heifers on other Dutch dairy farms.

From the 1500 farmers who received a questionnaire 601 responded. Sloughing of the teat was seen in 2004 on 5% of the farms with on average 1 animal.

There is minimal response to treatment and, where there is a response, the healing process is slow taking weeks to months. There is no adequate therapy. Although fungi could not be detected, in a few cases there was a response to antifungal treatment.

On 4 farms with more than 3 cases in a few months (1 farm with 10 cases in a row) no more cases were seen after diminishing the energy supply to heifers and a mineral supply adjustment to restore normal blood selenium levels.

Discussion

In The Netherlands heifers with teat problems around calving were not unusual in the past. In most cases it was associated with extreme udder oedema and eczema of the adjacent leg region. However, in the past 10 years, the sloughing of the teat without a clear association with udder oedema is unusual. Also unusual is the second described phenomenon (manifestation 2) starting with a small vesicle, both around calving but also months later. This manifestation was not noticed in the past. Also Bruns *et al*. (2002) saw ulcerative necrotizing teat dermatitis develop in a heifer which started 3 weeks after calving.

Based on the vesiculae a viral agent aetiology seems likely. An association with herpes mamillitis (BHV2) could not be proven. Virological cultures were all negative for BHV2 and antibodies against BHV2 were not detected in blood samples. Holliman (2003) described a similar phenomenom and according to him BHV2 is typically a sporadic condition of the bovine udder/teats of newly-calved heifers. In a study by Scott *et al*. (1978) antibodies against BHV2 were present in 37.7% of 576 sera collected during 1972 and 1973 from cattle in The Netherlands. They concluded that antibodies to BHV2 were clearly present, although they stated in the article that there was no published report of the presence of herpes mamillitis or the isolation of virus in The Netherlands. In a study by ID-Lelystad (unpublished report, 2001) over 4000 blood samples randomly chosen in The Netherlands only 3 samples had antibodies against BHV2. Thus BHV2 seems to be present in The Netherlands but at very low prevalence.

In Wisconsin (Moriello *et al*., 1993) BHV2 seroconversion was reported in 19% of the 69 animals with teat lesion syndrome, but all tissue and vesicular fluid samples submitted were negative on bacterial, fungal and virus isolation. The syndrome described in Wisconsin very much resembles the phenomenon in The Netherlands. Moriello *et al*. (1993) report a start with a small patch of bluish discoloration on the skin of the teat in almost exclusively first calf heifers. This rapidly progresses to a large, flaccid vesicle. The vesicle ruptures and the resultant ulcer fails to heal and an annular ring of inflammation and erosion develops, encircling the entire teat ending in sloughing of the teat. They saw no clustering on particular farms.

BHV4 infections are frequently recorded in The Netherlands (Wellenberg *et al.,* 2000). The relationship of BHV4 with the descibed teat necrosis is not clear.

Moriello *et al*. (1993) suggested that a toxin of S. aureus could be the etiological agent. Both Staphylococcus aureus and Arcanobacterium were isolated twice but a toxin of *S. aureus* seems to be unlikely.

The large fissures on the teats of manifestation 2 are apparently not painful. This is maybe another indication that the bloodsupply is disturbed, causing necrosis. Maybe also in other sites in the udder or body the boodsupply is disturbed causing swollen joints and abcesses. These animals become recumbent and must be euthanised like also descibed by Bruns *et al*. (2002).

On 3 farms the heifers licked or bit off the teats; this is also described in Israel by Yeruham and Markusfeld (1996). They saw this phenomenon in 42 heifers on 30 farms with a total of 9756 cows. Of these 42 animals 41 were culled due to ulceration and teat necrosis. These heifers each had a severely oedematous udder. They suggested a hereditary factor because most of the heifers were the progeny of 2 bulls. Maybe the heifers licked or bit their teat off because of irritation or pain.

The lesions observed in this study seemed to commence without any particular organism being involved. It was suggested that some mechanical cause was involved e.g. where the teat is bent under as the heifer lies down (maybe on a hard surface) and so the blood supply is interrupted.

There seems to be a relationship to a disturbed blood supply, selenium supply, sudden change in ration, the periparturient period, uddercleft dermatitis and, according to farmers, interdigital dermatitis. But none of these factors could be proven. The fact that on 4 farms with more than 3 cases in a few months (1 farm with 10 cases in a row) no more cases were seen after diminishing the energy supply to heifers and a mineral supply adjustment to restore normal blood selenium levels is an indication that energy supply and selenium are possible riskfactors. But it is no proof given the fact that is also stops on farms without any treatment.

Moriello *et al*. (1993) concluded that this teat lesion syndrome in dairy cows in Wisconsin had a major economic impact and warranted further investigation. According to the questionnaire in The Netherlands in 2004 teat necrosis is seen on a yearly basis in about 1250 heifers in The Netherlands, which represents a considerable loss.

Despite, the many observed cases of teat lesions in The Netherlands in 2004, usually this phenomenon affects only one case per farm, and it does not happen each year. Therefore, reliable investigations on farm level are not easy, and it is even difficult to measure the effects of any treatments on farm level. A good research program should give more insights in this phenomenon in The Netherlands.

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Effect of stockperson-dairy cow interaction to mammary gland health

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Abstract

Mastitis harms animal welfare. This disease is usually caused by faults in milking hygiene. The purpose of this paper was to evaluate the effect of stockpersom-dairy cow interaction of different milking parlours, in the same farm, on the occurence of mastitis and mammary gland infection. Five milking parlours from the same farm in Poços de Caldas-MG/Brazil were analysed, in a total of 295 dairy cows. evaluation of stockperson-dairy cow interaction's quality, as well as its effects upon cow's behaviour were carried out through direct and continuous observations during milking sessions using focal sampling. Strip cup test, California Mastitis Tests (CMT) and microbiological exams were made for the mastitis diagnosis. The occurrences of seven stockpersons behavioural categories ("talk" (T), "touch" (TO), "call" (C), "hit" (H), "shout" (S) , "push" (P) and "twist tail" (TT)) and four of cows (defecation (D), urination (U), rumination ® and movements of legs (MOV) were recorded. The results were analysed from the statistical test Qui-Square and ANOVA (one way). There were statistical differences (P<0,05) in the stockperson actions and occurrence of mammary infection by *Staphylococcus aureus*. However, the stockperson-dairy cow interaction didn´t influence in the occurrence of mastitis. The presence of the calf during milking and the space for the cow in the milking parlour can provide good animal welfare and soften negative actions from milking man.

Keywords: behaviour, stress, welfare, milking parlour, dairy cattle

Introduction

Dairy farms are complex and they need many different professionals, for example veterinarians, agronomists, farm managers, cattle feeders and stockperson (Rosa, 2002). For Albright (1997), the most important factor that can determine herd stress is the behaviour, attitude and consistency.

In the system for milk production, stockperson and dairy cow interact for a long period, during routine activities (Hemsworth and Coleman, 1998). The importance of positive interaction is reflected in daily production increase and animal welfare (Rosa and Paranhos da Costa, 2001).

Behavioural analysis are much used and they are associated with stockperson actions during milking. Many stockperson and dairy cows behaviours observed in the milking parlours indicated, in general, that positive men actions promoted adequate dairy cow behaviour during milking. These behaviours' cows are: rumination occurrence, urination and defecation absence, reaction decrease and production and milk quality increase (Rushen *et al.*, 1999; Breuer *et al.*, 2000; Hemsworth *et al.*, 2002).

Negative actions, for example, shout and hit, can result in a bigger stress level on the animals. This situation endangers the production and milk quality due to increase residual milk. Aversive stockpersons can present an indifferent behaviour in relation to milking hygiene. This situation promotes a high level of mastitis.

The purpose of this paper was evaluated the effects stockperson-dairy cow interaction on mammary gland health and animal welfare.

Material and methods

This study was carried out on a farm, located in Poços de Caldas district, Minas Gerais State- Brazil. Stockperson-dairy cow interaction was analyzed in five different milking parlours on the same farm.

The animals are Caracu race. After parturition they were distributed at random in the different milking parlours up to their limit capacity. In these places the animals stayed until fifth lactation month.

The feeder system and milking routine (calf with the cow during milking, teats hygiene, manual milking of the cow and filtrate the milk) are the same in all milking parlous. The management differential point is the man responsible for the parlours.

Strip cup and California Mastitis Test (CMT) were carried out on the individual quarters of all cows and the mammary gland or milk showing testing positive had milk samples aseptically collected for laboratory examination. In the laboratory, milk samples were streaked and agar plates were incubated at 37ºC for 72 hours according to Murray *et al*. (1999) and the final identification according to Krieg and Holt (1994).

In the beginning of the behaviour study, there were two days for preliminary observations. This fact allowed habituated between animals and observer during milking and the integration of the observer with milking routine. After this period the milking routine was followed for five days. To reduce the influence's observer stockperson's behaviour, the details of the purpose of this study it were not told to them.

Stockperson's behaviour was analysed during cow stay on the milking parlours and it was registrated the occurrence of these behaviours:

- Set of Positive actions:
  - "Call" (C): say the name or number of the cow.
  - "Talk" (T): talk with the animal with soft voice.
  - "Touch" (TO): soft touch with the hands.
- Set of Negative actions:
  - "Hit" (H): beating that the animal can suffer from the milking man.
  - "Shout" (S): talk with the animal with harsh voice.
  - "Push" (P): agressive and intentional pushing.
  - "Twist tail" (TT).

Behaviours' cow was observed during milk and it were registrated the occurrence or not of these aspects: rumination (Ru); Defecation (D); Urination (U). Also registered the movements of cow's legs (MOV) during calf retreat, teats hygiene and milking, when the cow satyed with their rear members still or moving them (Rosa, 2004).

After the observation period, the relative frequency of all stockperson actions and cows behaviour was calculated. After this, it was calculated the average of relative frequencies for both stockperson actions (negative and positive actions). And then it was calculated the reason of average relative frequency of positive actions on the average relative frequency of negative actions and then classified the parlours according to:

- minor than 0,25: insignificant interaction;
- between 0,26 and 1,55: unadvisable interaction;
- between 1,56 and 6,45: unstable interaction;
- higher than 6,45: friendly interaction.

In the insignificant interaction, the stockperson performs the milking without interaction with the cow. In this situation the man didn't use, or use in decrease relative frequency, the behaviours that it were considered how strong behaviours cows influential. The milking was consider monotonous.

In the unadvisable interaction, the negative behaviours exercised more influence on the cows behaviour, although milking man positive actions occurred.

In the unstable interaction, the negative behaviours exercised more influence on the cows behaviours, although relative frequency of milking man positive actions was higher than in the unadvisable interaction.

In the friendly interaction, the positive behaviours exercised more influence on the cows behaviours, although milking man negative actions can occurred (Rosa, 2004).

The results were analysed from the statistical test Qui-Square and ANOVA (one way).

Results

The extremes values for the relative frequencies of the positive actions set (T, TO e NM) changed from 0.00% to 10.97% and for negative actions set (B, S, P and TT) changed from 0.68% to 7.08%. These relative frequencies were used for classified the quality of the interaction milking men and cows in: insignificant in parlours B; unadvisable in A and C parlours and unstable in D and E milking parlours.

In relation to mastitis, it wasn't observed statistical differences between the milking parlours, in exception for *Staphylococcus aureus* mammary infection level, it was smaller in B milking parlours than A, D and E milking parlours (Table 1).

Discussion and conclusions

In the studies accomplished with bovine, it were observed welfare progress when positive actions were accomplished during the routine activities. Positive actions during milking caused minor escape distance, minor time for approximation, rumination increase, defecation and urination decrease, minor reaction during milking and milk production and milk quality increase (Hemsworth *et al.*, 1996; Breuer *et al.*, 2002; Waiblinger *et al.*, 2002; Hemsworth *et al.*, 2003; Raussi, 2003; Waiblinger *et al.*, 2004).

Table 1: Results about the clinical and subclinical mastitis levels and about mammary infections on the different milking parlours. Minas Gerais Brazil 2005.

	A	B	C	D	E
% mammary glands with clinical mastitis	1,69	1,5	0,69	0	0
% mammary glands with subclinical mastitis	35,59	32,5	29,55	35,77	30,56
% lost mammary glands	5,85	1,96	1,69	3,91	3,52
% mammary glands infection	80,95	66,13	66,67	69,77	65,48
% *Staphylococcus* spp infections	40,91[a]	21,54[b]	27,91[a,b]	40,91[a]	36,9[a]
% *Streptococcus* spp infections	10,61	10,77	20,93	4,55	8,33
% *Corynebacterium* spp infections	39,39	38,46	23,26	29,55	23,81

different letter (a,b) indicate statistical difference (p<0,05) in relation to the different milking parlous.

However Rosa (2004) affirm that isolated positive actions it weren't sufficient for welfare animal progress. In this study, stockperson from 4 of the 5 milking parlours presented interactions classified how unstable and unadvisable. Although it were observed positive actions by stockperson. It weren't observed correlation in welfare evaluations and mastitis levels. Also Rosa *et al*. (2003) didn´t observed correlation in behaviours' cow and subclinical mastitis.

Stress can increase residual milk level in the mammary gland after milking. It is a predeterminative factor for intramammarian infections, because milk is an excellent substratum for the microorganisms. Rushen *et al*. (1999) concluded that higher residual milk levels were caused by oxitocina secretion when the aversive milking man was present.

The stockperson-dairy cow interactions during milking can compromise mammary gland health. It occurs because the mains mastitis control measures are reported with the quickness execution during milking, for example teat hygiene. It is expected that stockperson with negative actions also presents minor exposure with milking actions and the consequence is the higher mastitis levels. In this study it wasn't observed friendly stockperson-dairy cow interaction for prove this hypothesis, it is necessary more studies.

In the milking parlours where it was observed insignificant interaction also it was observed the minor level of *Staphylococcus aureus* infection (p<0,05). When the milking is monotonous, the milking man can execute his activities how the recommendations. In this case intramammarian infection occurrence is reduced.

It was considered the farm management, it was observed that calf presence during milking and the space available per cow in the milking parlours can provide welfare suitable. This fact can have minimized the negative milking man actions and it wasn't observed statistical differences in the mastitis occurrence and milking men and dairy cows.

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Control programmes

The systems approach to udder health control

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Abstract

Mastitis research has been conducted for many decades and different udder health control concepts have been issued. However, mastitis is still a major problem in dairy herds and both farmers and veterinarians have not been successful in combating this disease.

This paper addresses udder health control from a systems analysis perspective. The great advantage of such is the structured and formalized approach of the herd problem, and the integration of the 3 main areas of concern, being management factors, cows and their environment especially at milking, and udder health performance features.

It is shown how well the HACCP-like approach fits well into this udder health control concept, where evidence-based veterinary medicine issues are paramount.

Introduction

During several decades, many different udder health control programs have been developed and implemented in the field. Formal programs have been described as the "five point scheme for udder health control" by Blood *et al.* (1978) and Leslie (1994). Such programs were predominantly focused on contagious streptococci like Strep. agalactiae. The program goals were [1] prevention of new infections through drying off therapy, good milking hygiene and milking machine function, and [2] reduction of existing infections through both adequate clinical mastitis therapy advice and reduction of subclinical infections. This program has proven to be successful in creating awareness among farmers about udder health care and in eliminating large proportions of Strep. agalactiae infections in herds, but were less successful in eliminating other streptococci and staphylococci infections. Nevertheless, this program is still widely implemented throughout the world, but with variable results. It can equally be stated that in many situations the approach of udder health problems appears to be fragmented in nature. Sometimes, focus in such approaches is on risk factor identification and risk elimination or reduction of their effects. In other situations, udder health measurements are being done on cow-associated parameters like somatic cell count or electric conductivity without studying associations with managerial practices. In other situations again, there is not sufficient knowledge to provide scientifically sound arguments for certain decisions taken. Sometimes people just copy statements and advice from others.

Furthermore, it was shown that by focusing solely on streptococci infections through control programs and hygiene, there occurred a shift in the bacteriological profile on dairy farms regarding intramammary infections. More and more other pathogens could be cultured from mastitic milk samples. Examples are *E. coli* (and coliform bacteria) and *Staph. aureus* (Schukken *et al.*, 1991).

Intramammary infections (IMI) with E. coli have been widely studied the past years. Major characteristics were that such infections largely occurred on dairy farms where cows showed a decreased resistance to infections, possibly -partly- induced by an increased milk production level which was based on a higher level of metabolism, which in turn caused the energy balance postpartum to become more or less severe. The latter has been indicated as a cause of loss in general disease resistance (Schukken *et al.*, 1991; Zadoks *et al.*, 2001). Wide variation exists in appearance and causes of IMI between cows.

The call for more successful udder health control programs becomes even more paramount in the context of quality risk management programs, comprising animal health and public health, which are currently under development (Fourichon *et al.*, 1996; Noordhuizen and Welpelo, 1996; Noordhuizen and Frankena, 1999; Noordhuizen, 2003; Seegers *et al.*, 2003).

Quality control concepts may refer to Good Farming Practice guidelines. These guidelines are very general in nature and address more generally present risk conditions for more than one hazard at the same time, such as mastitis and lameness or bovine virus diarrhea. They can be considered as elements of a certain attitude, philosophy and mentality, valuable to improve production conditions and awareness; they are however not suitable for proper certification because screening and control are not executed, and validity not comprised. Other quality control concept regards ISO 9000, in which predominantly is elaborated what is done in a production unit. However, it usually does not comprise how something must be done and how it is evaluated. Hazard analysis critical control points, HACCP, addresses both the product and the production process, and therefore may be much more interesting within the framework of a dairy farm and disorders occurring there. It addresses the what and the how of disease occurrence and the risk conditions associated with it; therefore it looks far more promising for application on dairy farms than the others, both from an operational (how to adjust daily management practices) and a strategic (quality assurance) point of view (Noordhuizen and Welpelo, 1996; Fourichon *et al.*, 1996).

The question rises whether we have so far applied the appropriate approaches for the combat, control and or elimination of IMI in an acceptable way showing effectiveness and success.

Therefore, the objectives of this paper are to approach the herd IMI control programs from the perspective of a systems analysis, and to show the relevant steps in such an approach..

Systems analysis: general features

In order to obtain an appropriate insight into the system or production process of a dairy farm, one should first be aware of the different process steps and functions that are being dealt with on that farm. A system is a complex series of organizational structures and processes, which in turn may comprise several components or units. Moreover, it can be further characterised by certain inputs and certain outputs (Sorensen and Enevoldsen, 1992; Hurd and Kaneene, 1993; Enevoldsen and Grohn, 1994). In Figure 1 an example of a dairy farm is presented, where different inputs and outputs can be distinguished, next to the different functions in the production process itself.

Systems in general suffer from complexity. For appropriate understanding it is indicated to use a systems modelling approach, where different issues are being simplified.

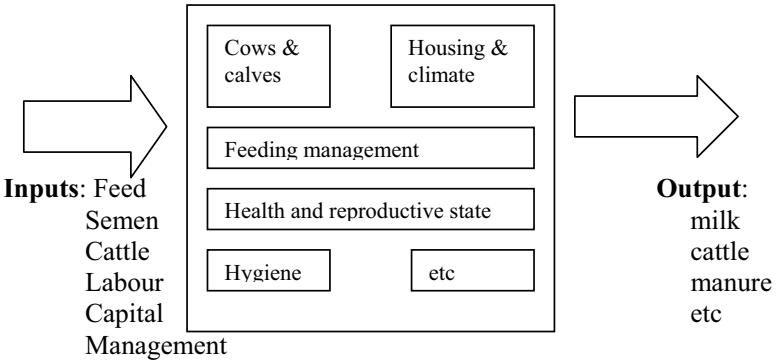

Figure 1. Schematic outline of an organizational system, its components, and its inputs and outputs, as applied to a dairy farm.

Design of production systems like of a dairy farm needs regulations for reassuring that the systems behaviour remains within certain standards, in spite of the complexity. Only then the system will behave as would be expected from the field observations.

A proper analysis of such a system should be focussed on the different components as well as its inputs and outputs (Hurd and Kaneene, 1993). The outputs basically define the objectives of a systems design, while the systems design represents the materials and methods for achieving those objectives (i.e. outputs). For each component particular goals must be set, materials and methods defined, results monitored and validation put into place, highly comparable to a formal veterinary herd health and production management program (Brand *et al.*, 1996). Subsequently, it is determined where the inputs should come from and which are already available. Finally, each system (-component) must be validated at farm level internally and externally.

Systems analysis: the focus on udder health

The systems approach in Figure 2 has 3 major components [a] the management factors which can be involved in the occurrence of mastitis problems in a cow or the herd, [b] the interaction between cows in the herd, the housing and climatic conditions in the system as a whole, [c] the output measurements which are commonly used to assess the udder health situation on a dairy farm. It must be clear that items under [a] can to a large extent be influenced because they are at a tactical level, while the items under [b] can hardly or not because they are at a strategic level. Moreover, it must be considered that cows in a herd are not independent from each other; there is an interaction, which in case of contagious mastitis can affect the herd status based on contact rate, transmission efficiency and pathogenicity of pathogens, and proportion of susceptible cows or heifers. Finally, many of the output measurements refer to proxi-parameters which are not pathognomonic but at least show a correlation with mastitis.

Often the advisors of the dairy farmer only address the centre piece of the picture in Figure2. Veterinarians examine and treat cows with clinical mastitis, sometimes look into the barn climate as a contributory factor to IMI's as well as into housing conditions as a potential source for e.g. environmental pathogens. Other advisors will address farm management considering that pivotal in the optimisation of udder health. Again others look

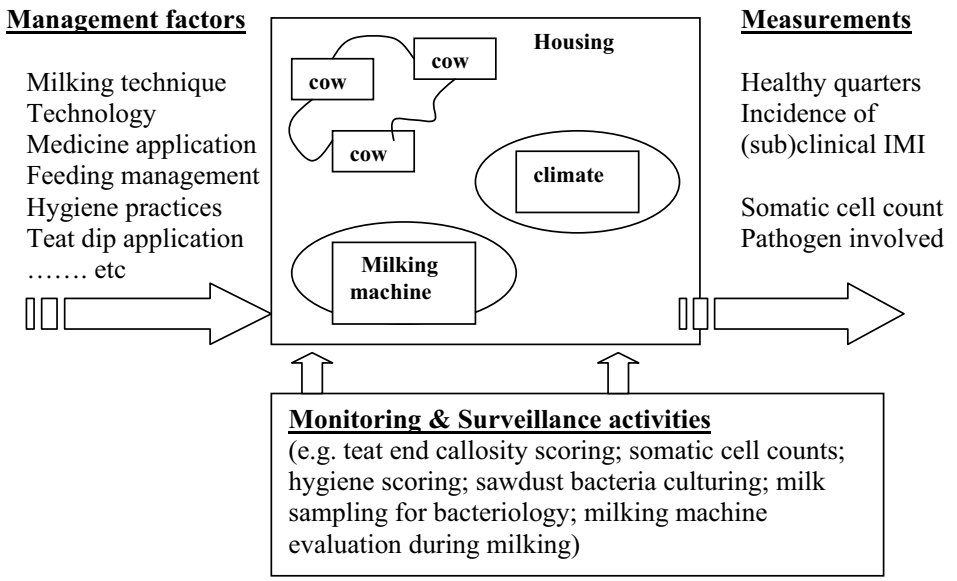

Figure 2. presents the application of the forenamed approach with respect to udder health, as one production process component of the dairy farm system.

to udder health performance indicators like pattern of somatic cell counts, cases of clinical and subclinical mastitis, and pathogens involved in such cases. It is not rare that sometimes the approach of an udder health problem on a dairy farm is carried completely at random and by different persons operating separately. In other words, coordination and structure in the approach is missing, as well as a formal step-wise analysis of the problem, the evaluation of the balanced interactive approach between the three main components in Figure 2, the awareness that for some issues scientific knowledge is still lacking, and the evidence based arguments underlying the advice given. These are the major issues in a systems approach to udder health control.

A formal and structured udder health control system

The principle elements of the systems approach should be followed: structured and formalized, planning, step-wise analysis, balanced and integrated between the 3 components, evidence based arguments for advice.

The ultimate objectives of an udder health control system should be that new intramammary infections should be prevented as much as possible and existing infections eliminated or at least substantially reduced in impact under constraints of economic efficiency, and/or that the (udder tissue) damage is kept at acceptable levels (Fourichon *et al.*, 1996; Fetrow, 2000; Seegers *et al.*, 2003). Basically we have to set the standards or acceptable levels for such infections. As a consequence, the raw milk in the bulk tank should hardly or rather not contain these udder pathogens (public health issue).

It therefore can already be concluded at this stage that udder health control is an economic farm activity.

Next step is the decomposition of the respective components in the system. The examples are given in Figure 2.

Let us use these 3 components for elaborating the practical approach of both the problem solving in the field. It is shown that monitoring is a component of problem solving as well as a routine activity.

Suppose that the dairy farmer reports an udder health problem.

First step is to *define what exactly the problem* is made of: Too many clinical mastitis cases in a certain period of time? Too high somatic cell count in the bulk tank? Too many cows with a too high individual cell count? Too many cows with teats tread in a short period? Which cows are affected: low parities, older parities or mixed parities? Which calving season and which milk yield level? Where has the problem occurred (are cows kept in production groups or in different barns)? Which pathogens are involved in the problem and how does the herd mastitis pathogen profile looks like? This requires the (continuous) monitoring of cows and their history. Monitoring as a routine activity is meant to collect information of pending problems or to detect trends, but also to collect signals of problem cows in the herd; it can be part of an early warning system and a starting point for problem analysis and solving!

Next, the *risk conditions* prevailing on the dairy farm are monitored; a risk profile is designed for the farm, where risk factors are listed in order of their relevance. Risk factors from management as well as risk factors from the environment and the cows themselves are identified. Monitoring is broad because mastitis is multifactorial in nature, and also conditions outside udder health issues can be involved (see Figure 2). An example is the feed quality and feeding management because these affect e.g. the general disease resistance of cows. *Interpretation of this risk factor profile* is the subsequent step, where knowledge of pathophysiology and epidemiology are paramount. The results of this interpretation are then used for surveillance, i.e. a Plan of Action has to be defined. That plan of action will comprise both general managerial issues as comprised in good farming practice guidelines, as well as specific issues derived from the risk assessment procedure. The latter may be elaborated into working instructions or protocols. At all times the relationships between the 3 components are addressed jointly (see Figure 2). A plan of action will comprise advice for the shorter term as well as for the longer term, based on priorities, economic cost-benefit assessments, and managerial feasibilities. Advice should be evidence-based; empirical issues deleted as much as possible. Empirical issues may lead to a waste of labor and costs when not validated in the field. It should be borne in mind that on average a dairy farmer can handle only a limited set of advises. Therefore, the plan of action should be broken down into different advises in ranking order of relevance set against a time table (time management) and planning of actions, and implemented in concordance with the farm management.

Finally, the *time table of actions* should also comprise a follow-up where the effects of advice given are being checked according to a preset protocol; when needed, adjustments can be made in the plan of action. In this way the farmer knows that he is being screened for his compliance to the rules set out for him. A herd treatment plan is issued by the veterinarian for newly occurring mastitis problems (what to do if.....). All results from the routine monitoring process, and the investigations and laboratories (pathogens cultured; cell counts; antibiograms) must be recorded, with date, cow ID or event ID. That way, it

can be demonstrated to third parties not only what the current udder health status is, but also what actions with what results have been carried out.

Discussion and concluding remarks

The forenamed systems approach looks easier to perform than will appear in reality, but it has a much broader perspective than all different part-plans for udder health control before. In The Netherlands, attempts have been made to guide the farmer and his veterinarian through an integrated approach to udder health control by means of a so-called "*mastitis planner*". Even this attempt has not proven to be highly successful, possibly because it comprised too much paperwork at one time or because adoption by farmers was too low (Beaudeau, 1995). Other critical success factors regard the attitude and mentality of the farmer toward udder health, and the interaction between farmer and veterinarian with regard to udder health control activities. When we would take all under-ligned items together, it will appear that these may be part of a HACCP-compatible program for dairy farms. The advantage would be that both operational (daily management) and strategic (quality) issues are integrated which is beneficial to the dairy farmer. Moreover, it has been stated before that animal health, animal welfare and public health are features from the production process on the dairy farm. Production process control means quality risk control and risk management. Disease combat becomes preventive health care through the application of a quality control program. This fits into the concept of consumer protection and food safety certainties, as lied down in the context of EU regulation 178/2002 and the new EU Hygiene directive. The application of HACCP-compatible programs on dairy farms may provide the consumer with more certainties and the farmer with a rather simple, farm-specific but holistic, not too costly program, to achieve his operational goals and his strategic responsibilities. It refers to his "license to produce and to market".

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The importance of a low SCC premium policy in the reduction of population wide SCC levels

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Abstract

A study was conducted to evaluate the importance of a low somatic cell count (SCC) premium policy in the reduction of SCC levels in New York State. One large cooperative in New York State has implemented an aggressive premium policy. The amount of premium offered has changed considerably over the years, and thus allowed us to evaluate the impacts of a changing premium structure on SCC in milk produced by that cooperative. The actual value of the premium varied between $0.50 and $1.00 per 100 pounds of milk that met the cooperative's milk quality criteria. Multivariable mixed model regression analysis showed that only the highest level of the premium was linked to reductions in SCC of milk produced in the herds shipping to the cooperative studied here. Specifically, the highest premium level ($1.00) was shown to result in a reduction of the population mean SCC by 11,000 cells/ml. Our analyses support the conclusion that a milk quality premium policy has a significant impact on SCC.

Introduction

The number of somatic cells in milk is determined by the status of health and function of the mammary gland (Heeschen, 2005). Somatic cells, or polymorphnuclear leukocytes, are a key measure of milk quality and offer challenging and complex questions of economic and managerial objectives to the dairy industry. While premium offerings from a milk processing plant present producers significant monetary incentives to maintain bulk tank somatic cell count (BTSCC) at 300,000 or fewer cells per milliliter (cells/ml), the degree that this inducement affects management is still unknown. Nearly all developed countries producing milk have adopted SCC limits to define milk as either suitable or not suitable for human consumption (NMC, 2001). SCC standards in the United States (US) are the most lenient for any developed county in the world (NMC, 2001). Currently, US policy allows dairy producers to sell milk to the domestic market without penalty at or below 750,000 cells/ml. The European Union (EU), Australia, and New Zealand require that milk used for dairy products sold in their respective territories have somatic cell levels below 400,000 cells/ml, while all Canadian provinces have a legal limit of 500,000 cells/ml.

Mastitis, defined as an inflammation of the udder, is usually caused by a bacterial infection. This inflammation results in elevated SCC, which interferes with milk production in the udder (Schukken *et al.*, 1990). Mastitis is considered to be the most frequent and most costly production disease in dairy herds of developed countries (Seegers *et al.*, 2003). Economic consequences are rarely transferred past the producer because raw milk quality is the determining factor for processing value. Therefore, decreasing SCC is of keen interest

to the producer and the processor. Additionally, somatic cells in milk have not been demonstrated to be a direct threat to public health (Smith and Hogan, 1998), but previous studies (Van Schaik *et al.*, 2002) indicate that high SCC milk has increased bacterial counts, as determined by plate loop count. In a 2001 study of herds in South Dakota and Minnesota, Jayarao and Henning found that 26.7% of all bulk tanks were contaminated with one of five major foodborne pathogens (*E. coli, C. jejuni, L. monocytogenes, Salmonella spp, Y. enterocolitica*), with lower quality (Grade B) milk having a roughly 300% higher prevalence of pathogens than milk with higher quality (Grade A). The distinction between Grade A and Grade B is a result of SCC level. Although not all bacteria in milk are pathogenic and most pathogenic bacterial populations are controlled by pasteurization, there have been multiple documented outbreaks of foodborne illness due to contaminated pasteurized milk (Jayarao and Henning, 2001). By decreasing SCC in milk, we may be able to reduce the level of pathogens initially present in raw milk and thus the level of the pathogens in pasteurized milk products.

Several countries throughout the world have successfully implemented SCC reduction programs. The European threshold was 500,000 cells/ml prior to 1992 when the European Economic Community (EEC, the precursor to the European Union) directive 92/46 reduced the limit to the current 400,000 cells/ml level for fluid milk. In 1998, the EU revised the standard to mandate that milk over 400,000 cells/ml could not be used for human consumption (Schukken *et al.*, 2003). In a province-wide program beginning in 1989, Ontario Canada successfully implemented a gradual step-wise process to reduce the BTSCC regulatory threshold from 800,000 cells/ml to 500,000 cells/ml over a six year period (Sargeant *et al.*, 1998). Changes in the regulation of BTSCC in the US have been proposed for many years, with a major hurdle being a need to estimate an appropriate plan to effectively reduce BTSCC without sustaining damage to the dairy industry (Adkinson *et al.*, 2001). The most recent policy to lower the US regulatory threshold for BTSCC was put in place effective July 1, 1993 (NMC, 2001). Voting delegates of the National Conference on Interstate Milk Shipments (NCIMS) approved an amended National Mastitis Council (NMC) proposal that reduced the BTSCC limit from 1,000,000 cells/ml to the current 750,000 cells/ml limit (NMC, 2001).

Managing production disease is involved and often very costly. However, opportunities exist for producers to capture quality premiums if somatic cell and bacteria counts fall in line with a milk plant's offering structure. This becomes increasingly attractive in times of low milk price. A simulation study by Schukken and colleagues (Schukken *et al.*, 1990) suggests that herds with a low annual SCC level are more likely to stay within that level, while herds with a high annual SCC are more variable, everything else constant. This indicates that additional economic incentives may play a major role in reducing the overall population BTSCC. However, to date no work has been done to demonstrate how producers are influenced by various premium levels. By evaluating premium offerings in combination with milk quality and milk quantity data from a New York State milk cooperative, we can explore and make assumptions about the existing relationship. This study was conducted to (i) outline previous somatic cell count reduction programs and (ii) model the inducement that premium programs have on population bulk tank somatic cell counts.

Materials and methods

Study population

Data were collected monthly from one large milk cooperative in New York State from April 1998 through December 2003. Milk processed by this cooperative represents an average of 10.34% of the milk produced in New York. Included in the data set were: a unique anonymous producer ID, the zip code of the producer farm, volume of milk shipped (lbs/month), component analysis including butterfat, protein, lactose, other solids, BTSCC, bacteria count by plate loop count (PLC), and the presence of inhibitor residue (IR, also known as antibiotic residue). All milk quality and component analyses were performed by the milk cooperative's in-house laboratory. Milk quality premium offerings were also collected and recorded for the same test period. This cooperative has had a very aggressive and variable low SCC premium policy, which allowed us to evaluate the impact of this premium on the population wide SCC in milk produced over time. The actual amount of the low SCC premium varied from $0.50 per hundredweight (cwt.) to $1.00 per cwt. of milk during the data collection period.

Data and statistical analyses

All data were analyzed used the Statistical Analysis System software (SAS, SAS Institute, Cary NC). Descriptive analysis of milk variables by month was performed using PROC MEANS. All variables with the exception of pounds (n=29,166), plate loop count (n=29,093), and inhibitor residue (n=29,076) had 29,175 reported individual observations. When variables were analyzed by producer-month, there was a maximum of 69 (April 1998 - December 2003) monthly samples per individual producer. The weighted SCC (WSCC) is calculated on samples from individual milk shipments and is weighted or adjusted based on each producer's level of milk production. The WSCC for all milking cows in a herd should approximate the SCC and is calculated as WSCC = (SCC x $lbs_{producer\text{-}month}$) / $\sum lbs_{month.}$

To estimate the impact of premium programs on the subsequent milk quality a PROC MIXED (multivariate regression) model was developed to test the fixed effects of seasonality, pounds of milk produced, and the premium structure in a given time period. The model also incorporates random effects for producer to account for herd variability: SCC = Intercept + Seasonality + Milk (Kg) + Premium code + RE.

Where: Seasonality is a sine function modeling annual variation in SCC. Milk is milk production on a monthly farm basis (Kg). Premium code is a categorical variable coding for baseline (before February 1999), $0.50/cwt. for February 1999 to July 2001, $1.00/cwt. for August 2001 to July 2002, and $0.60/cwt. thereafter. RE is a complex error term incorporating a repeated measures term for repeated observations within a farm. The residual error is assumed to be iid normal.

Results

The mean, number (sample size), and standard deviation for each variable in the dataset, calculated by month to account for seasonal variation is shown in Table 1.

Figure 1 is a time series plot of mean SCC and mean WSCC. Figure 1 illustrates that mean SCC and mean WSCC follow the same trend. Monthly mean WSCC allowed visualization of

trends in the overall milk supply, while mean SCC allowed visualization of trends at the producer level.

The final regression model results are shown in Table 2. Significant variables include seasonality (2 variables), milk produced, and SCC premium at all levels. Compared to baseline, a premium of $1.00/cwt. reduced the population mean SCC by 11,000 cells/ml. Mid range premium offerings resulted in a 9,000 cell/ml increase for the $0.50/cwt. level and an 8,000 cells/ml increase at the $0.60/cwt. level. It is clear that only high premium levels offer enough incentive to lower mean population cell counts.

The actual SCC and the model SCC predictions are shown in Figure 2. The model follows the actual data quite well. A noticeable exception is the lower than expected SCC at the beginning of the premium program and during the $1.00 premium offering period.

Table 1. Descriptive analysis of plant raw milk testing variables by month. Mean, number of observations, and standard deviation are reported.

Month	Kg of milk			Somatic cell count			Plate loop count			Inhibitor residue		
	Mean	No	St. Dev	Mean	No.	St. Dev	Mean	No.	St. Dev	Mean	No.	St. Dev
January	117681	2121	188098	322630	2122	173200	26520	2122	149920	0.0024	2122	0.05
February	110833	2115	177716	325510	2115	185320	25190	2115	105670	0.0048	2115	0.07
March	121111	2113	195963	325340	2113	179920	20580	2113	50540	0.0014	2113	0.04
April	115464	2593	184165	342420	2593	185690	23850	2593	70410	0.0043	2593	0.07
May	119300	2576	185566	347630	2576	177090	26480	2576	84180	0.0020	2576	0.04
June	116853	2559	186096	344190	2559	170710	32190	2559	151630	0.0039	2559	0.06
July	118074	2547	187956	372130	2549	185100	29270	2549	98520	0.0035	2549	0.06
August	115879	2533	181620	377280	2533	189140	33160	2533	132000	0.0055	2533	0.07
September	112549	2529	181096	359490	2529	177850	26410	2529	72230	0.0036	2529	0.06
October	114874	2505	186428	339280	2507	170550	23650	2507	84640	0.0056	2507	0.07
November	112771	2491	186829	319260	2491	166010	18463	2491	36119	0.0032	2491	0.06
December	114807	2484	194943	324610	2488	171290	22380	2488	64600	0.0028	2488	0.05

Table 1. Continued.

Month	Other solids component			Somatic cell count			Plate loop count			Inhibitor residue		
	Mean	No.	St. Dev	Mean	No.	St. Dev	Mean	No.	St. Dev	Mean	No.	St. Dev
January	6.15	2122	1.19	322630	2122	173200	26520	2122	149920	0.0024	2122	0.05
February	6.16	2115	1.19	325510	2115	185320	25190	2115	105670	0.0048	2115	0.07
March	6.15	2113	1.17	325340	2113	179920	20580	2113	50540	0.0014	2113	0.04
April	6.05	2593	1.06	342420	2593	185690	23850	2593	70410	0.0043	2593	0.07
May	6.06	2576	1.07	347630	2576	177090	26480	2576	84180	0.0020	2576	0.04
June	6.05	2559	1.06	344190	2559	170710	32190	2559	151630	0.0039	2559	0.06
July	6.03	2549	1.03	372130	2549	185100	29270	2549	98520	0.0035	2549	0.06
August	6.02	2533	1.04	377280	2533	189140	33160	2533	132000	0.0055	2533	0.07
September	6.00	2529	1.08	359490	2529	177850	26410	2529	72230	0.0036	2529	0.06
October	6.07	2507	1.08	339280	2507	170550	23650	2507	84640	0.0056	2507	0.07
November	6.06	2491	1.10	319260	2491	166010	18463	2491	36119	0.0032	2491	0.06
December	6.06	2488	1.08	324610	2488	171290	22380	2488	64600	0.0028	2488	0.05

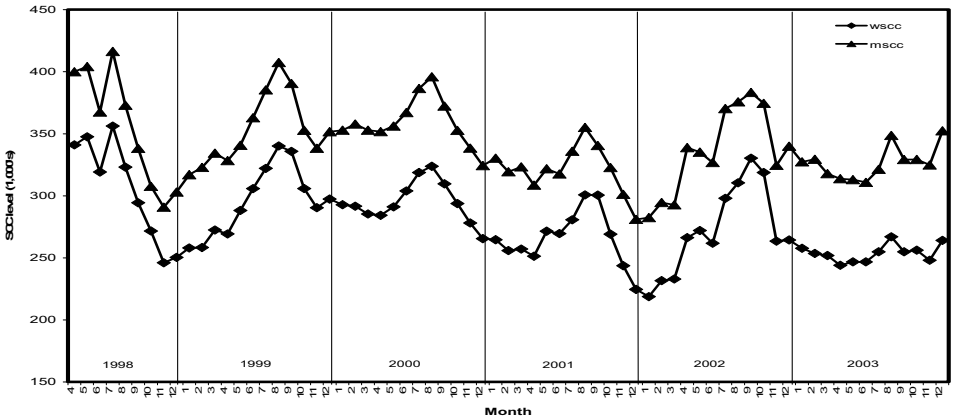

Figure 1. Time series plot of mean SCC and weighted SCC by month.

Table 2. Final model results.

Effect	Point estimate	St. Dev	t-value	P-value
Intercept	358.9500	5.7629	62.2900	<0.0001
Season 1	-15.0805	0.9715	-15.5200	<0.0001
Season 2	-18.1614	0.9492	-19.1300	<0.0001
Pounds	-0.0001	0.0000	-11.3700	<0.0001
Premium $0.50/cwt.	9.0879	2.0130	4.5100	<0.0001
Premium $0.60/cwt.	8.2483	2.4046	3.4300	0.0006
Premium $1.00/cwt.	-11.0633	2.4909	-4.4400	<0.0001
Baseline Premium	0			

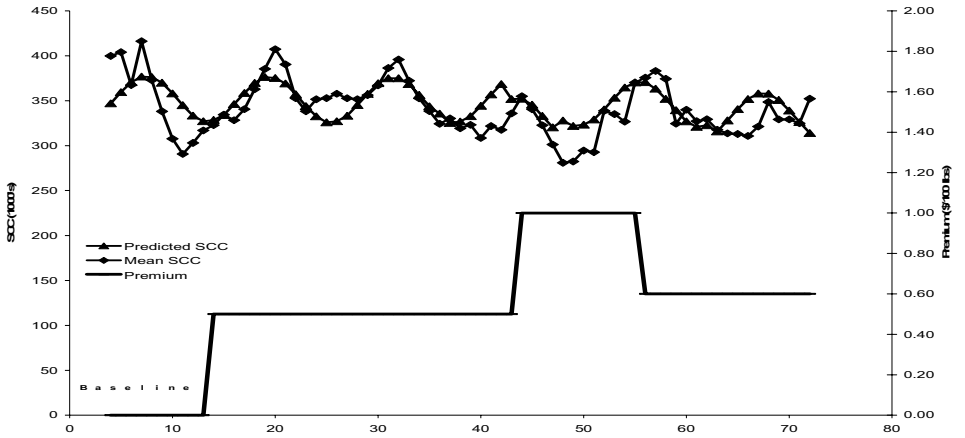

Figure 2. Time series plot of predicted mean SCC, raw SCC, and premium level by month number.

Discussion

Our analysis shows that an aggressive premium policy is effective in reducing mean SCC of all milk produced for this individual cooperative. At the maximum premium level of $1.00/cwt., we saw a reduction of 11,000 cells/ml compared to the pre-program period. A premium of approximately 7% of the daily milk price (assuming $15.00/cwt. milk) resulted in a drop of average SCC by approximately 3%. Clearly, milk buyers can steer milk quality in the supplying dairy farms.

The data set we used is unique in that it contains a variable premium structure over time. Obviously, it is only one premium program in one cooperative. Results would be more reliable if data on multiple cooperatives with varying premium programs were available.

If the principle finding of this paper holds true, policy on SCC premiums can be an important tool in the hands of milk buyers. Optimal premium policy may be designed based on the milk quality needs of the processing facility for which the milk is purchased.

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Use of SCC in genetic selection for reduced incidence of mastitis: A mixture model approach

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Abstract

Mixture distributions consist of two or more sub-distributions (groups), where group memberships of the observations are unknown. The udder health status of each cow at the test-days on which SCC are recorded are usually unknown. Further, as SCC increases as a result of udder infection, the observed SCC/SCS can be assumed being a two (or more)-component mixture depending on mastitis status (e.g, healthy/mastitic). Using a mixture model for statistical analysis of observed test-day SCS implies that the observations can be categorized into putative disease categories according to the probability of disease, given the observed data and all other parameters in the model. A Bayesian hierarchical two-component normal mixture model for SCS was developed, where baseline SCS is associated with some fixed and random effects, and test-day health status is assumed fully determined by an underlying liability to mastitis. The prior probability of mastitis may vary between different sub-groups, such as herd, stage of lactation, lactation number, etc. Hence, the liability may be associated by some fixed and random effects distinct from those affecting baseline SCS. Based on analysis of simulated data, the model seemingly gives unbiased estimates of all parameters, and the predicted breeding values for liability to mastitis seem better suited for genetic selection than crudely selecting for lower SCS. The model also provides estimates of test-day probability of mastitis, based on the recorded SCS, which may be used for disease detection. The proposed model could easily be extended to handle a wider range of problems related to statistical analyses of mixture traits.

Keywords: mixture model, somatic cell count, detection of disease, genetic selection

Introduction

For certain traits an unknown underlying group structure may affect distribution of observations. An observation may therefore be drawn from K mutually exclusive and exhaustive distributions (or "groups"). In animal breeding, mixture models have so far primarily been used in QTL-analyses. Disease is another factor that may cause mixture distributions in quantitative traits, through mechanisms controlling the relationship between unobserved disease traits (categorical) and continuous traits. For example, SCS may be regarded as a trait sampled from either an "uninfected" or "mastitic" cow, where SCS in

the "uninfected" and "mastitic" groups may be normally distributed with different means (e.g., Detilleux and Leroy, 2000), and possibly different variances. Mixture models can be used to categorize the observations into putative disease categories. Acquiring information about unknown disease categories by making better use of information from related continuous (mixture) traits (e.g., SCS and electrical conductivity in milk) could be of great value both with respect to treatment strategy, herd management decisions and may also improve genetic evaluation of traits such as mastitis.

So far, mixture models for detection of mastitis based on SCS has been developed and analyzed on simulated data (e.g., Ødegård *et al.*, 2003). In these models probability of mastitis has been estimated based on the observed SCS, and observations categorized into "healthy" and "mastitic" classes according to this probability. When calculating probability for mastitis, the model seeks to adjust for "base level" SCS of the cow. Furthermore, the *a priori* probability for mastitis has often been assumed equal for all observations, which is not realistic for real data. Thus, such models do not provide any good criteria for selection for lower incidence of mastitis. A hierarchical mixture model may be needed to implement a more flexible, practical and realistic model. In this model, probability for mastitis may depend on effects such as herd-test-day, stage of lactation, and additive genetic effects, and implies a direct approach for predicting breeding values for liability to mastitis using data coming from mastitis-related mixture traits. In the following we will shortly describe a simple version of this model. A more detailed description can be found in Ødegård *et al.* (2005).

Method

The setting and notation are as in Ødegård *et al.* (2003). Briefly, the data consists of n measurements for a quantitative trait, such as SCS of a cow. A 2-component Gaussian mixture model poses that the ith measurement (i = animal or record within animal), given location and dispersion parameters (α), and probabilities, $P = [P_1, P_2, \ldots, P_n]^t$ has the distribution:

$$SCS_i \sim (1-P_i)\, N[f_i(\alpha), g_i(\alpha)] + P_i N^*[f^*_i(\alpha), g^*_i(\alpha)] \quad (1)$$

where P_i is the *a priori* probability that SCS_i is drawn from the distribution $N^*(\cdot)$, and $(1-P_i)$ is the *a priori* probability that it is drawn from $N(\cdot)$ Ødegård *et al.* (2003) assumed that $P_i=P$ for all i, while in this study, P_i is allowed to differ between observations. Further, $f_i(\alpha)$, $g_i(\alpha)$, $f^*_i(\alpha)$ and $g^*_i(\alpha)$ are functions of the parameter vector α. Typically, $f_i(\alpha)$ and $f^*_i(\alpha)$ are linear combinations of fixed and random effects, $g_i(\alpha) = \sigma^2_{e0scs}$ and $g^*_i(\alpha) = \sigma^2_{e1scs}$ for all $i = 1,2, \ldots, n$. Conditionally on the parameters, α and P, the joint density of the data vector SCS is:

$$p(SCS|P,\alpha) = \prod_{i=1}^{n} [(1-P_i) \cdot N[f_i(\alpha), g_i(\alpha)] + P_i \cdot N^*[f^*_i(\alpha), g^*_i(\alpha)] \quad (2)$$

Estimation by maximum likelihood or by Bayesian approaches are facilitated by augmenting the density above with auxiliary indicator (0,1) variables Z_i (i = 1, 2, ..., n). It is assumed that

$$\Pr(\mathbf{Z}=\mathbf{z}|\mathbf{P},\boldsymbol{\alpha})=\Pr(\mathbf{Z}=\mathbf{z}|\mathbf{P})=\prod_{i=1}^{n}\Pr(Z_i=z_i|P_i)=\prod_{i=1}^{n}P_i^{x_i}(1-P_i)^{z_i} \quad (3)$$

In this model we postulate the existence of an underlying continuous random variable, called liability (λ), which determines the actual mastitis status for each observation (probit link function). This is a threshold-liability model (Wright, 1934; Dempster and Lerner, 1950; Gianola, 1982; Gianola and Folley, 1983), which has been used for genetic analysis of clinical mastitis as a binary response (e.g., Heringstad *et al.*, 2003). Here, the liability is incorporated into what we term a liability-normal mixture (LNM) model. In both models true mastitis status goes from 0 to 1 if liability exceeds a given threshold *T*(= 0). In the standard threshold liability model, data consists of observed binary responses (say, "presence" or "absence" of clinical mastitis), whereas in the LNM model, data consist of observed SCS. However, distribution of SCS changes from N(() to N*(() according to mastitis status, and putative mastitis status may therefore be inferred based on the observed SCS. In this model the *a priori* probability of mastitis, for a specific observation *i*, is:

$$P_i=\Pr(\lambda_i>T|\boldsymbol{\alpha})=\Pr(\lambda_i>T|\boldsymbol{\beta}_\lambda,\mathbf{s}_\lambda,\mathbf{p}_\lambda)=\Phi\left(\mathbf{x}_{i\lambda}'\boldsymbol{\beta}_\lambda+\mathbf{z}_{is_\lambda}'\mathbf{s}_\lambda+\mathbf{z}_{ip_\lambda}'\mathbf{p}_\lambda\right)=\Phi(\tilde{\lambda}_i), \quad (4)$$

Where $\Phi(\cdot)$ is the standard normal cumulative distribution function. Thus, P_i is not a parameter in this model, but a function of the expected liability.

Further,

$$\Pr(\mathbf{Z}=\mathbf{z}|\mathbf{SCS},\mathbf{P},\boldsymbol{\alpha})=\frac{\mathrm{p}(\mathbf{SCS}|\mathbf{z},\mathbf{P},\boldsymbol{\alpha})\Pr(\mathbf{Z}=\mathbf{z}|\mathbf{P})}{\mathrm{p}(\mathbf{SCS}|\mathbf{P},\boldsymbol{\alpha})} \quad | \quad (5)$$

is the conditional probability distribution of Z, given SCS, α and P. Assuming that (SCZ_i, Z_i) *i=1,...,n*, are mutually independent given α and P, then

$$\Pr(Z_i=1|SCS_i,P_i,\boldsymbol{\alpha})=\frac{\mathrm{p}(SCS_i|z_i=1,P_i,\boldsymbol{\alpha})P_i}{\mathrm{p}(SCS_i|z_i=0,P_i,\boldsymbol{\alpha})(1-P_i)+\mathrm{p}(SCS_i|z_i=1,P_i,\boldsymbol{\alpha})P_i} \quad (6)$$

is the posterior probability (given SCS_i, α and P) that the draw is made from (mastitis), whereas the complement is the posterior probability that the draw is from . Here, SCS_i is the somatic cell score for record *i*.

Modeling SCS and the liabilities

Conditionally on Z=z and parameter vector α, we assume that the SCS and λ variables can be modeled as a bivariate model with a Gaussian (SCS) and a binary trait (Z (=mastitis)), with the latter being fully determined by the underlying liability.

$$\mathbf{y}=\begin{pmatrix}\mathbf{SCS}\\ \boldsymbol{\lambda}\end{pmatrix}=\begin{pmatrix}\mathbf{X}_{0_{SCS}}\boldsymbol{\beta}_{0_{SCS}}+\mathbf{M}_z\mathbf{X}_{1_{SCS}}\boldsymbol{\beta}_{1_{SCS}}+\mathbf{Z}_{a_{SCS}}\mathbf{a}_{SCS}+\mathbf{Z}_{p_{SCS}}\mathbf{p}_{SCS}+\mathbf{e}_{zSCS}\\ \mathbf{X}_\lambda\boldsymbol{\beta}_\lambda+\mathbf{Z}_{a_\lambda}\mathbf{a}_\lambda+\mathbf{Z}_{p_\lambda}\mathbf{p}_\lambda+\mathbf{e}_\lambda\end{pmatrix} \quad (7)$$

$$=\mathbf{X}_z\boldsymbol{\beta}+\mathbf{Z}_a\mathbf{a}+\mathbf{Z}_p\mathbf{p}+\mathbf{e}_z$$

where; **y** = column vector of SCS and liability variates, $\mathbf{M}_z$ = (*n*×*n*) diagonal matrix of indicator variables, with typical element Z_i (*i* = 1, 2, ..., n), β_{0SCS} = vector of "fixed" effects affecting SCS common to all cows, β_{1SCS} = vector of "fixed" effects affecting SCS peculiar to cows with mastitis, $\beta = [\beta_{0_{SCS}}', \beta_{1_{SCS}}', \beta_\lambda']$, $a = [a_{SCS}', a_\lambda']'$ = vector of random additive genetic effects, $p = [p_{SCS}', p_\lambda']'$ = vector of random permanent environmental effects,

$e_z = [p_{zSCS}', e_\lambda']'$ = vector of random residuals, and X_{0scs}, X_{1scs}, $X\lambda$, Z_{ascs}, Z_{pscs} and $Z_{p\lambda}$ are incidence matrices of appropriate order. Further, $e_{zSCS} = (z_i e_{i0_{SCS}} + (1\text{-}z_i) e_{i0_{SCS}})_{i = 1, \ldots, n}$, where $e_{i0_{SCS}} \sim N(0, \sigma^2_{e0_{SCS}})$, $e_{i1_{SCS}} \sim N(0, \sigma^2_{e1_{SCS}})$; $e_{i0_{SCS}}$ and, $i = 1, \ldots, n$, are assumed to be mutually independent.

Standard prior distributions were assumed for all location and dispersion parameters. Residual correlation between SCS and λ was not estimable with this model, and was therefore set to zero. Estimation was carried out with Gibbs sampling.

Simulation study

The model was tested using simulated data. Four different scenarios were chosen, and each scenario was replicated 20 times (Table 1). For all scenarios, four generations were simulated, each consisting of 800 cows from 10 sires. Mastitis frequency was set to 25%. Residual variance for SCS was assumed homogeneous, independent of disease category. For comparison purposes three models were fitted; a standard repeatability model for SCS ignoring the mixture (IM), a mixture model for SCS ignoring the structure of the liability (NM) (equivalent to Ødegård *et al.*, 2003), and finally a LNM model.

The posterior mean estimates for all parameters were close to their true values. However, if the structure of the underlying liability was ignored, parameters for SCS were confounded with those of the liability. Further, specificity and (even more) sensitivity were slightly reduced (Table 2).

An advantage of the LNM model, compared with other mixture models is that it provides EBVs for liability to putative mastitis, which may be directly used in selection for improved disease resistance. In Figure 1, correlations between true breeding values for liability to mastitis and predicted breeding values based on the IM and LNM models are shown for the different scenarios. Compared to the standard test-day model for SCS (IM), EBVs for liability to mastitis from the LNM model had consistently higher correlations (9-530%) with the true breeding values, particularly when assuming a negative genetic correlation between

Table 1. Input parameters (IP) for 4 different scenarios.

Scenario		σ^2_{aSCS}	$\sigma^2_{a\lambda}$	$r_{aSCS\lambda}$	σ^2_{PSCS}	$\sigma^2_{P\lambda}$	$r_{PSCS\lambda}$	σ^2_{eSCS}
1	IP	0.100	0.125	0.000	0.100	0.125	0.000	0.800
2	IP	0.100	0.125	0.500	0.100	0.125	0.000	0.800
3	IP	0.100	0.125	-0.500	0.100	0.125	0.000	0.800
4	IP	0.100	0.059	0.000	0.100	0.125	0.000	0.800

Table 2. Sensitivity and specificity estimated with a non-hierarchical normal mixture model (NM) and a hierarchical liability - normal mixture model (LNM). Values are reported as means over 20 replicates from each scenario.

Scenario	LNM		NM	
	Sensitivity	Specificity	Sensitivity	Specificity
1	0.660	0.885	0.630	0.879
2	0.664	0.893	0.622	0.887
3	0.646	0.882	0.618	0.877
4	0.641	0.893	0.616	0.890

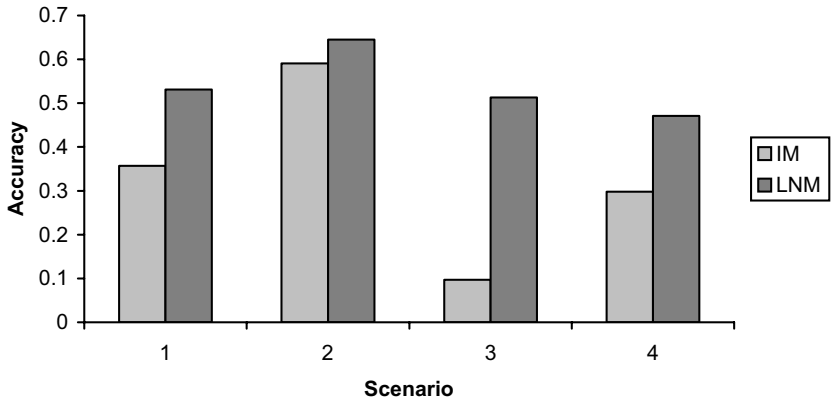

Figure 1. Correlations between true breeding values for liability to mastitis and predicted breeding values (accuracy) estimated with a standard model for SCS ignoring the mixture (IM) and a liability - normal mixture model (LNM). Average correlations for 20 replicates from each scenario are presented.

"baseline SCS" and liability to putative mastitis (indicating that high SCS in healthy cows reduces risk of infection).

Future aspects

In addition to selecting for cows able to avoid infection, we may also be interested in the cows' ability to recover when infection occurs. This may be achieved by developing a mixture model where size of SCS response to infection has a genetic component, which in turn may be related to probability of recovery from disease. The model could also easily be extended to multivariate mixtures, consisting of multiple mixture traits depending on same mixture variable (e.g., SCS and electrical conductivity in milk), different mixture variables (e.g., SCS and a trait affected by QTL), or both mixtures and non-mixture traits. More advanced mixtures, consisting of more than two components (e.g., healthy, subclinical mastitis, clinical mastitis) may also be developed.

Conclusion

Inferring an underlying liability to mastitis in mixture models for mastitis-related mixture traits gives a more realistic and accurate model, both in terms of genetic evaluations and identification of diseased animals. Based on simulation studies the model seemingly gives unbiased estimates of the parameters. The proposed model could easily be extended to handle a wider range of problems related to genetic analyses of mixture traits.

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Genetic improvement of mastitis resistance in Norwegian Red (NRF)

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Abstract

A method for reducing incidence of mastitis in dairy cows is genetic selection. The Norwegian Red (NRF) breed has been selected for improved mastitis resistance over the longest time period, worldwide. NRF represents one of the few cattle populations where assessment of effects of long-term selection against mastitis is possible in a large scale. Clinical mastitis (CM) has been included in the breeding program for NRF since 1978. In Norway, selection against CM relies on a nationwide health recording system, where each case of veterinary treated disease has been recorded on an individual cow basis since 1975. A threshold model analysis of CM, including 1.6 million first-lactation NRF cows, indicates genetic improvement for mastitis resistance after 1990 equivalent to a reduction of 3 %-points CM each 10 years. Analysis of multiparous cows shows that effective selection against CM in first lactation elicited favorable correlated response for mastitis resistance in second and third lactations. Results from a Norwegian selection experiment demonstrate that considerable genetic response can be achieved for mastitis resistance if sufficient selection pressure is placed on the trait. On the other hand, if mastitis is ignored in a breeding program, selection for increased milk yield results in an unfavorable correlated selection response in CM The genetic difference between a line selected for increased milk yield and a control line (low milk yield) was 3.1 %-points CM after 4 cow-generations. A broad breeding objective enables to obtain simultaneous genetic progress for milk yield and mastitis resistance, despite the unfavorable genetic correlation between traits.

Keywords: genetic improvement, selection, heritability, genetic correlations

Introduction

A method for preventing disease in dairy cows is genetic selection, provided that genetic variability exists. Strategies include selection on genetic markers, indirect selection using correlated traits (e.g., somatic cell counts to improve mastitis resistance), or direct selection on clinically observed traits. The last option has been followed in Norway, where clinical mastitis has been included in the breeding program for Norwegian Red (NRF) since 1978 (Heringstad *et al.*, 2000). NRF, representing 95% of the dairy cows in Norway, has been selected for increased milk production, reduced frequency of clinical mastitis, and for several other functional traits, including female fertility. The relative weight given to clinical mastitis in the total merit index, used for selection of sires, has increased gradually from

less than 3% in 1978 to 22% in 2005 (http://www.geno.no). Each year, around 125 NRF sires are progeny tested, based on information from 200-300 daughters per sire, and the 7-10 highest ranking bulls, based on total merit index, are selected and used as elite sires.

In Norway, selection against mastitis relies on a health recording system where each case of veterinary treatment has been registered on an individual cow basis, since 1975. Figures for 2004 show that 95% of the cows participate in the Norwegian Dairy Herd Recording System, where individual health recording is integrated (http://org.tine.no). Since antibiotics can be prescribed only by veterinarians in Norway, health recordings are viewed as reliable. In the current genetic evaluation procedure mastitis is defined as a binary trait (0 = healthy, 1 = diseased), based on whether or not a cow had at least one veterinary treatment of clinical mastitis in a period going from 15 days before to 120 days after first calving.

The objective of this paper is to report on effects of 25 years of selection against clinical mastitis in NRF. Worldwide, NRF is the population that has been selected for improved mastitis resistance over the longest time period, and it represents one of few dairy populations where documentation of the effects of long-term selection against clinical mastitis is possible.

Heritability and genetic correlations

The most common approach of dealing with mastitis data in genetic evaluation has been to consider the trait as binary, and use linear models for statistical analysis. The heritability of mastitis estimated with linear models is low, with most estimates between 0.02 and 0.04 (Heringstad *et al.*, 2000; Hansen *et al.*, 2002; Carlén *et al.*, 2004). A linear model does not take the binary nature of the data into account. An alternative is to use threshold models, which postulate an underlying continuous variable, liability. Here, an observed binary response takes the value 1 if liability is larger than a fixed threshold, and 0 otherwise. When analyzing mastitis with threshold-liability models, heritability estimates on the underlying scale have ranged between 0.06 and 0.14 (Lund *et al.*, 1999; Heringstad *et al.*, 2003a; 2004; Chang *et al.*, 2004; Zwald *et al.*, 2004). Low heritability implies that many daughters are needed in progeny testing to obtain reliable breeding values for the trait.

It is well known that the genetic relationship between milk production and mastitis in dairy cows is antagonistic. In a review, Heringstad *et al.* (2000) reported estimates of genetic correlation between mastitis susceptibility and milk yield ranging between 0.24 and 0.55, with an average of 0.43. Recent estimates of the genetic correlation between mastitis and protein yield range from 0.19 to 0.43 (Hansen *et al.*, 2002; Carlén *et al.*, 2004; Heringstad *et al.*, 2005). The unfavorable genetic correlations imply that if mastitis is ignored in a breeding program, selection for increased milk production is expected to result in a genetic deterioration of resistance to the disease.

Selection experiments

Two Norwegian dairy cattle selection experiments (Heringstad *et al.*, 2003b) evaluated the correlated response in clinical mastitis resulting from selection for increased milk production, as well as the response to direct selection against clinical mastitis. The first experiment was carried out from 1978 through 1989 and included groups of cows selected

for high and low milk yield (HMY and LMY, respectively). Each year, the highest ranking proven sires for milk production, within the most recent group of NRF test-bulls, were selected and mated to cows in the HMY group. A group of sires with low milk production indices from progeny testing in 1978 and 1979 were used as sires in the LMY group (control group) during the entire experiment. After four cow-generations, the genetic difference in mastitis between HMY and LMY group cows was 3.1%-points clinical mastitis (Figure 1); this is a correlated response to selection for increased milk production. The second selection experiment started in 1989 and includes one high protein yield (HPY) group and one low clinical mastitis (LCM) group. The highest ranking proven sires for protein yield and mastitis resistance were selected each year from the most recent group of progeny tested NRF bulls and used as sires in the HPY and LCM groups, respectively. The genetic difference between HPY and LCM was 8.6%-points clinical mastitis after three cow-generations (Figure 1); this is mainly a result of direct selection against clinical mastitis in the LCM group. The difference in genetic trend of clinical mastitis between HMP and HPY groups, both selected for increased milk production, reflects the gradual change in the NRF breeding objective towards more weight on health relative to milk over the last 20 years. The results illustrate that it is possible to obtain considerable selection response for clinical mastitis, and that selection for increased milk production will result in an unfavorable correlated increase in mastitis incidence, if resistance to the disease is ignored in the breeding program.

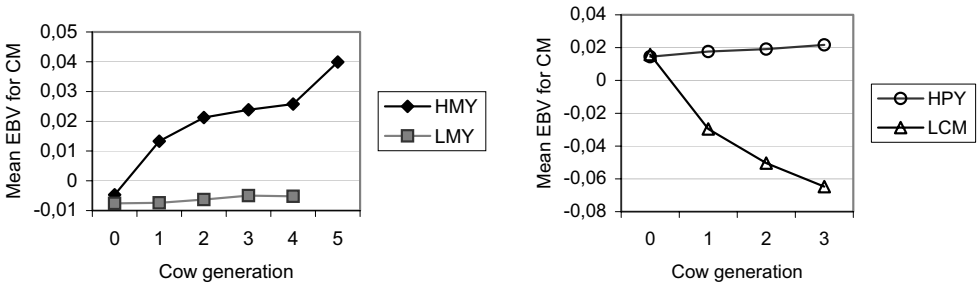

Figure 1. Mean estimated breeding value (EBV) for clinical mastitis (CM) per cow generation for high milk yield (HMY) and low milk yield (LMY) cows from experiment I, and high protein yield (HPY) and low clinical mastitis (LCM) cows from experiment II (from Heringstad et al., 2003b).

Genetic change in mastitis resistance in the NRF population

Heringstad *et al*. (2003a) analyzed records of clinical mastitis on 1.6 million first-lactation daughters of 2411 NRF sires that were progeny tested from 1978 through 1998 with a threshold-liability model. A Bayesian approach via Gibbs sampling was used. Mastitis was defined as a binary trait based on whether or not the cow had at least one veterinary treatment of clinical mastitis in a period going from 15 days before to 120 days after first calving. The model for the underlying liability had age at first calving, month×year of calving, herd×3-year-period, and sire of the cow as explanatory variables.

Genetic evaluations (posterior means) of sires both in the liability and observable scales were computed. The evolution of the average sire posterior means by birth-year of daughters was used to describe genetic change in the NRF population. Figure 2 shows an approximately constant genetic level from 1976 to 1990 (-0.02% per year), and a genetic improvement

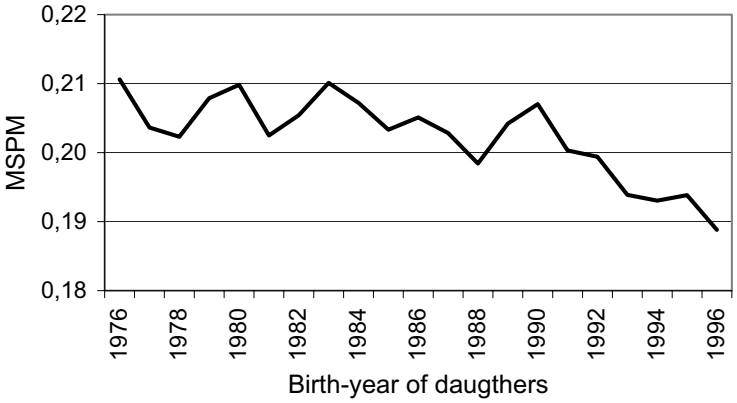

Figure 2. Genetic change of clinical mastitis in the NRF population, given as mean sire posterior mean (MSPM) by birth-year of daughters (from Heringstad et al., 2003a).

thereafter (-0.27% per year). Little or no genetic change for cows born before 1990 (Figure 2) suggests that the relative weight placed on clinical mastitis in that period may have been large enough to counteract the expected unfavorable correlated response due to selection for increased milk production. The favorable genetic change for cows born after 1990 (Figure 2) is a result of the increased weight placed on clinical mastitis, relative to milk production, in the total merit index in recent years.

Is mastitis the same trait within and between lactations?

It is well known that the frequency of mastitis is higher in later lactations. An important question is, therefore, whether mastitis can be assumed to be the same trait in first and later lactations. A related question is whether mastitis can be assumed to be the same trait at different stages of lactation, since the probability of clinical mastitis is much larger in early than in late lactation.

Clinical mastitis records from 372,227 daughters of 2411 NRF sires were analyzed with a Bayesian multivariate threshold model (Heringstad *et al.*, 2004). All cases of veterinary treated clinical mastitis occurring from 30 days before first calving to culling or 300 days after third calving were included. Lactations were divided into four intervals: (-30 - 0), (1 - 30), (31 - 120), and (121 - 300) days after calving. Within each interval, absence or presence of mastitis was scored as "0" or "1' based on the clinical mastitis episodes. A 12-variate (3 lactations x 4 intervals) threshold model was used, assuming that mastitis was a different trait in each lactation-interval. Residuals were assumed correlated within lactation, but independent between lactations. The model for liability to clinical mastitis had specific lactation-interval effects of month-year of calving, age at calving (first lactation) or calving interval (second and third lactation), herd-5-year-period and of sire of the cow, plus a residual.

Point estimates of genetic correlations of liability to clinical mastitis between lactation-intervals ranged from 0.24 (between intervals 1 and 12) to 0.73 (between intervals 1 and 2). Within lactation, genetic correlations tended to be higher for adjacent than for non-adjacent intervals, and genetic correlations between intervals in different lactations tended

to be higher for intervals at the same stage of lactation. Genetic correlations much lower than 1 indicate that clinical mastitis cannot be regarded as the same trait in different parts of lactation or in different lactations.

Trends of mean sire posterior means by birth year of daughters were used to assess genetic change. The 12 traits showed similar trends, with little or no genetic change from 1976 to 1986, and genetic improvement in resistance to clinical mastitis thereafter. Annual genetic change was larger for intervals in 1st than in 2nd or 3rd lactations, as illustrated in Figure 3 showing genetic change for the 30 d interval before 1st, 2nd, and 3rd calving (intervals 1, 5, and 9). Within lactation, genetic change was larger for intervals early in lactation, and more so in 1st lactation. This reflects that selection against mastitis in NRF has emphasized mainly clinical mastitis in early 1st lactation, with favorable correlated selection responses in 2nd and 3rd lactations suggested.

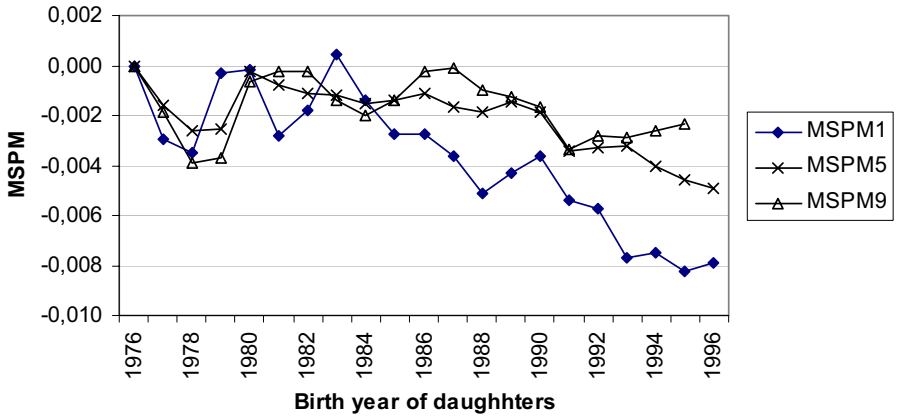

Figure 3. Genetic change of clinical mastitis, given as mean sire posterior mean (MSPM) by birth-year of daughters for intervals 1, 5, and 12, which were the 30 d intervals before 1st, 2nd, and 3rd calving, respectively (from Heringstad et al., 2004).

Conclusions

Genetic improvement of mastitis resistance is being achieved in NRF. Effective selection against clinical mastitis in early first lactation has elicited favorable correlated selection responses in other intervals in 1st, 2nd, and 3rd lactation.

The results presented here, together with those of Andersen-Ranberg *et al*. (2005) showing genetic improvement for female fertility and protein yield, demonstrate that with a broad breeding objective it is possible to obtain simultaneous genetic improvement for milk production, health, and fertility, despite unfavorable genetic correlations between traits and the low heritability of some of the traits. This is possible if: a) estimated breeding values are precise (traits recorded for large enough daughter groups), and b) selection is sufficiently intense (traits receive an appropriate weight in total merit indexes).

Acknowledgments

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Social factors related to mastitis control practices: The role of dairy farmers' knowledge, attitude, values, behaviour and networks

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Abstract

It is often assumed that knowledge is an important factor influencing the preventative and curative behaviour of farmers with regard to mastitis. In this study we explore systematically the relative importance of farmers' knowledge vis-à-vis other social and contextual factors. A longitudinal study was set up in which the effectiveness of an ambitious mastitis reduction programme will be monitored by an integral analysis of technical and social variables.

This paper presents the outcomes of a quantitative baseline survey of 378 Dutch dairy farmers. This survey was preceded by a qualitative exploratory study amongst farmers and veterinarians, which served to develop relevant survey questions and working hypotheses. A first analysis of the survey data suggests that -contrary to the dominant view - a lack of general knowledge and problem awareness are not the key factors explaining the non-utilization of specific preventative and curative practices. Instead, external triggers (such as sanctions, problems and incentives), internal normative beliefs and farmers' perceived self-efficacy seem to be the key factors affecting mastitis control practices. The survey also shows that, on the topic of mastitis, veterinarians appear to be the most influential actor in the network surrounding farmers. Finally, the paper discusses several implications for an intervention programme and for further research.

Background

The dairy sector is an important part of Dutch agriculture. It is also a sector under pressure, with many dairy farmers hoping to secure the farm's future by reducing production costs and increasing milk quota. Milk quality is checked regularly and dairy farmers receive a financial penalty when their bulk tank Somatic Cell Count (SCC) is, during three consecutive months, over 400,000/ml. Around 80% of dairy farmers subscribe to a service which provides them with the SCC results for individual cows. Adult cows with a SCC above 250,000/ml are marked on the information sheets that farmers receive, and are called "attention cows".

Recently, the average Dutch SCC is on the rise. During the summers of 1997-2000 it used to peak at around 220,000/ml, but for the last four years it has reached values of around 260,000/ml in summer. It is unclear why this has happened. The long hot summers we have enjoyed lately might be a factor; there are new pathogens that might contribute

to the rise; or the recent changes which made the penalty system a bit more lenient might have more impact than expected.

The Gezondheidsdienst voor Dieren (Animal Health Service Ltd, an independent organisation with the aim to increase animal health, a veterinary laboratory, and a staff of around 50 veterinarians) was asked to initiate a project to lower the overall Dutch SCC. The chosen target for this project is a reduction of mastitis by 10% in five years. Mastitis is a costly disease, it is estimated that such a reduction will save the dairy industry over €30 million per annum.

For the project to be successful, it essential to determine what the factors are that influence mastitis management on the farm. The project plan, therefore, includes a baseline survey which tries to clarify how farmers perceive and manage mastitis. This survey will provide insight that can guide the remainder of the project. But the survey can also be used as a baseline for an assessment of the changes in farmer perception, knowledge and behaviour after five years.

Conceptual framework

The Department of Communication Science, Wageningen University was asked to design this baseline survey. To help with this design process, a conceptual framework was developed. This framework distinguishes between four aspects: knowledge, perceptions, behaviour and communication.

The aspect "knowledge" does not refer to objective, proven knowledge, but to subjective knowledge: what an individual farmer holds true in relation to mastitis and mastitis management. Instead of knowledge, one could therefore use the term personal belief system. It contains ideas and beliefs like: "in order to prevent resistance it is best to change antibiotics often". These beliefs often feed the development of perceptions and ultimately guide behaviour.

"Perceptions" is a broad term, related but different from knowledge. It stands for the instances where an idea or belief is evaluated, and coupled with a positive or negative emotion. Another word that is often used in this context is attitude. A perception once developed can have quite an influence on behaviour, even when there is hardly any knowledge to support it. For example, the idea that one has little control over the development of mastitis on the farm can lead to feelings of frustration and even fear. An additional issue here is that people tend to look for information that supports their existing perceptions.

With "behaviour" we refer to actual actions taken, such as cleaning the teats with a paper towel before every milking, or using antibiotics the moment it becomes clear a cow has clinical mastitis. Together, all these actions can be called mastitis management.

The last aspect we looked at is "communication". Communication with others often has a major impact on how knowledge and perceptions are developed and altered. For example, discussions with the veterinarian can put existing ideas and attitudes to the test, and may lead to changes in mastitis management.

The baseline survey

The conceptual framework was further developed during the discussions we had with mastitis experts (mainly veterinarians) and farmers. From these discussions, hypotheses for each of the four aspects (knowledge, perceptions, behaviour, communication) were developed. The hypotheses were translated into survey questions, which were formulated so they would test the hypotheses. The survey questions were pre-tested, and the baseline survey form finalized. During May 2004, 543 ad random selected farmers were phoned and asked to participate in the research. From this group, 378 completed survey forms were received back (response rate of 70%).

Results

The purpose of this section is to highlight some of the results of the survey. We will first give a brief profile of the participating farmers, and then continue with a discussion on the results for the aspects of the conceptual framework: behaviour, perceptions, knowledge, and communication.

Profile

On average, the dairy farmers who participated in the survey were 42 years old and milking 77 cows. The average bulk tank SCC for this group was 178,000/ml, keeping in mind that the survey was conducted in May when SCC levels are normally already elevated. Most farmers tried to keep the bulk tank SCC for their farm far below the penalty level (400,000/ml). They indicated that, on average, they are satisfied with a bulk tank SCC of around 155,000/ml, and feel they have a serious problem when it rises above 282,000/ml.

Behaviour

The survey included many questions that try to determine what preventive or curative actions farmers take in regards to mastitis. It is beyond the scope of this article to go into much detail, but one thing that became quite clear is that farmers differ significantly in their approach. The only thing they seem to agree on is to clean the teats before milking (99,5% does this). But for other practices the views vary widely. E.g. 16% always wear gloves in the milking shed (79% never does), 33% use fore-stripping for all the cows (16% never use it, the others only occasionally), 70% milk all cows together (24% separate cows with clinical mastitis from the herd and milk them separately).

About half of the farmers never send a milk sample away for bacterial testing. Those who do are very selective. For example, the farmers who use bacteriological tests when cows have clinical mastitis, estimate that they have a test done for, on average, one out of every five cows with clinical mastitis. Most farmers start antibiotic treatment the moment it becomes clear a cow has clinical mastitis. But with sub clinical mastitis they are much more relaxed; 55% of the farmers always wait till the next individual SCC results come in.

Perceptions

On average, the farmers feel they have a serious mastitis problem when one or more of the following happens: the bulk tank SCC reaches 282,000/ml, during the course of a year 19 or more cows contract clinical mastitis, there are 13 cows with sub clinical mastitis at

any stage, or the latest individual SCC results show there are eight or more new attention cows. Nearly three out of four farmers (72%) have had, at one stage or another, such a serious mastitis problem on their farm.

Mastitis management is a topic that has the interest of nearly all farmers. Many (90%) perceive mastitis as a disease that can be hard to deal with, and many (91%) indicate they would love to decrease the number of cows with mastitis. But, at the moment, the majority is satisfied with the way they handle mastitis (only 5% disagrees with a statement like "I handle mastitis in the right way") and the level of control they have (only 18% disagrees with a statement like "generally speaking, I have mastitis under control).

The aspects of mastitis they find most disturbing are the possibility that an affected cow might not recover, the extra labour that is required, and the financial consequences. The fact that mastitis might lead to stress (e.g. that they might start to worry about the future) does not seem to be an important factor (see Table 1).

Nearly all farmers (94%) monitor the bulk tank SCC results, the individual SCC results and the development of (new) cases of clinical mastitis closely. But it appears that actual mastitis management is rather ad hoc. Only one out of four farmers has a clear, detailed mastitis management plan which specifies "what to do, when". Only 6% actually have it written down. Most farmers "play it by ear": they assess the situation and then decide what to do. For example, they decide on a case by case basis whether it is necessary to send a sample away for bacteriological testing, or if it is time to start with antibiotics.

Farmers were asked what would happen if the penalty system would change and they would receive a penalty when their bulk tank SCC is above 350,000/ml (instead of 400,000/ml). The majority of farmers (65%) said they would look where they could make improvements, but only a minority (10%) thought they might receive more penalties with such a stringent system. Apparently they are quite confident that they have sufficient control to keep mastitis levels on their farm low.

Table 1. What is the most disturbing aspect of mastitis?

Aspect	Score
Not knowing if a cow will recover	31%
The extra labour required	24%
The financial consequences	20%
The cow suffers	8%
I start to worry	7%
Other	10%

Knowledge

When the farmers were asked to assess whether they know enough about mastitis to prevent problems, the opinions varied considerably. About one-third (32%) is confident enough and believe they have sufficient knowledge. But there are also many (30%) who believe that they lack sufficient knowledge. When we look at this second group in more detail, two things become clear. First, these farmers have had more mastitis problems in the last two years or have a relatively high bulk tank SCC at the moment. Which might explain why they do not feel so confident about their own knowledge (anymore). Second,

they are more concerned about the possibility of a future outbreak of mastitis on their farm. Not surprising as the idea that one might lack the knowledge to deal with a disease like mastitis successfully, would give cause for some concern.

After this impression of how farmers rate their overall knowledge, they were asked to assess their knowledge in specific areas of mastitis management. The topics that many farmers feel they lack knowledge on are the more technical, science-based aspects. Like the role of pathogens by the development of mastitis, the use of bacteriological tests, or the influence of rations on cow health. They often feel much more confident when it comes to knowledge of the day to day management of the farm, like the milking process or maintaining a sufficiently high hygiene level in the cow shed (see Table 2).

These results are, of course, based on self assessment. But we also included a few questions that try to gauche actual knowledge levels. For example, we asked the farmers if they know how Staphylococcus aureus or Coli bacteria are transmitted, and gave them two options: mainly through (a) infected milk or (b) through infected manure, sawdust, etc. Knowing the correct answers is quite important as this knowledge determines where one should look for solutions when confronted with mastitis caused by these pathogens (e.g. better hygiene in the milking shed or in the cow shed). It turned out that around 75% of farmers gave the right answers to these two questions. Which also means that 25% lack what seems to be quite essential knowledge in this area.

In addition to this, half of the farmers state that they often do not know what the cause is behind an increase in the number of cows with mastitis. Despite this uncertainty, many feel a reluctance to conduct bacteriological tests to find out which pathogens are involved: around 50% of the farmers never send a milk sample off for bacteriological testing.

Communication

Farmers receive SCC results on a regular basis. Nearly all of them see these results as essential information on which to base their mastitis management decisions. Most farmers study the SCC results the same day they become available.

Communication with others can also be a key to learning, to expanding one's knowledge. Interest is a precursor to gaining new knowledge through communication. The survey data clearly show that mastitis management is a topic that has the interest of nearly all farmers.

At the moment the veterinarian is the most important source for information on mastitis, followed by farming journals.

Table 2. How would you rate your knowledge level? (from 1 = insufficient to 5 = excellent).

Topic	Average
Milking process and mastitis	3.9
Cow shed hygiene and mastitis	3.9
Using SCC results	3.6
Treatment of clinical mastitis	3.6
Using medication	3.5
Buying and selling cows	3.5
Treatment of sub clinical mastitis	2.9
The connection between feeding and mastitis	2.8
Pathogens and bacteriological tests	2.7

Table 3. How important are the following information sources for increasing your knowledge of mastitis? (from 1 = not important to 5 = important).

Source	Average
Veterinarian	4.5
Farming journals	3.9
Animal Health Service Ltd	3.6
Colleagues	3.2
Study club	3.1
Feed supplier	2.9
Course	2.7
Advisor	2.1
Internet	1.8

The farmers highly regard the knowledge and advice of the veterinarian, and nearly all (93%) g*et al*ong well with their veterinarian. Around 51% of the farmers talk often with their veterinarian about mastitis. And when he would organise a group course on mastitis management, consisting of three afternoons, two out of three farmers would like to participate. But the willingness to pay for such a course is quite low: 89% of these farmers would not pay more than €100.

Many farmers (88%) would read a mastitis article in the farming magazines they subscribe to. But the majority (56%) thinks that there are enough articles on mastitis published. Furthermore, we asked what their three preferred options would be to learn more about mastitis management and gave them a range of options to choose from. Three out of four farmers (75%) chose farming journals, 58% chose the veterinarian, and 43% chose a lecture from an expert. Rather "traditional" choices, one might say. Options like a mastitis website (chosen by 17% of the farmers), free mastitis help desk (5%), a video course (3%) or an expert system on CD (5%) were clearly less favoured.

Conclusion

Mastitis management is an important part of dairy farm management. The farmers that took part in the survey closely monitor the development of the bulk tank SCC and the cows with clinical mastitis. When the individual SCC results arrive, they look at these results seriously. And they take action when they feel they need to.

When we look at actual mastitis management on the farm, there are many difference between farmers in the way they operate, especially in the preventative measures they take. Furthermore, it is save to say that decision making is often less structured, and more ad hoc, than most mastitis experts would like it to be. Only a few farmers have clear, written protocols that outline what actions should be taken when certain problems arise. Around half of them never use bacteriological tests, whereas the others mostly use it on a case to case basis.

Despite this, most farmers are satisfied with the way they handle mastitis and the level of control they feel they have. And they do not seem to be too concerned about the dangers of a possible serious outbreak of mastitis on their farm. This, despite the fact that many

actually have had serious problems before. It could be that having solved these problems has increased the feeling of control these farmers have (or maybe memories of such problems fade quickly once they are over).

Nearly all farmers are interested in the topic "mastitis". They often read the articles on mastitis management that they find in their farming journal. But it seems that they are not very actively looking for information and knowledge. This despite the fact that around 30% of the farmers believe they lack sufficient knowledge. And although many farmers express a desire to lower the bulk tank SCC, it appears that the real motivation to pursue this aim is not present at the moment. It can be expected that when the grading system changes, and farmers would receive a penalty more quickly, this motivation might increase. Our assessment is that many farmers have the necessary knowledge and skills to make sure they would comply to such a system. Many farmers actually feel they could improve their management, for example the way they treat sub clinical mastitis, when they need to. It is also not surprising that only a small minority think they might receive more penalties when the SCC penalty level would reduced to 350,000/ml.

Finally, when asked which three policy measures out of a list of eight, they think might be the most effective in lowering the average SCC in The Netherlands, only 34% opted for more extension, while nearly 72% chose a premium for milk with a low SCC. Again, this might indicate that most farmers feel they are quite capable of lowering their SCC levels, if they are sufficiently challenged.

Mastitis and farmers perceptions and actions: An anthropological perspective to the phenomenon of mastitis

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Introduction

Farmers' decisions influence all actions and management strategies in a herd. Qualitative research interviews offer a possibility to create insight into human decision making, which cannot be described by quantitative research methods. This paper is based on research results from Danish research studies, where qualitative and quantitative research approaches were combined. The concept of qualitative research is different from the biological and biomedical research tradition. The aim of the qualitative interview investigation is to describe, interpret and understand the spectrum of experience and choices related to a given phenomenon. Two Danish studies about mastitis treatment form basis for this paper (Vaarst *et al.*, 2002; Vaarst *et al.*, 2003). Both studies combined epidemiological and qualitative part projects. Qualitative research interviews were carried through in order to describe and analyse farmers' perspectives on their own choices with regard to the decision to get cows treated for mastitis.

Presentation of the first study: Mapping farmers' treatment criteria in mastitis cases

In this study, 16 farmers participated, who had widely different herd characteristics and who were chosen because they had been involved in another study. The farmers had widely different treatment criteria and definitions of mastitis. Through the interviews, four levels of farmers' decision-making were identified, which were used in practice by the farmers when deciding whether to treat a cow with antibiotics or not. These levels were:

1. symptom level (seriousness of the mastitis case);
2. cow level (to which level the cow fulfilled the goals of the farmer and the herd);
3. herd level (the situation of the herd, e.g. in relation to milk quota), and
4. level of alternatives (whether the farmer regards e.g. blinding of teats or homoeopathy as serious alternatives to antibiotic treatment in a mastitis case).

The decision-making process is described in Figure 1. All four levels could be recognised in all herds, but with different weight on and importance. The symptom level was dominant in severe cases of acute, clinical mastitis, although 'severe' was defined differently among farmers. Cow characteristics are involved to a high degree in cases of 'mild mastitis' (defined from high somatic cell counts to clinical mastitis without fever or very hard udder). The herd situation is an aspect which makes the treatment decision very context related, including a relationship to political and cultural structures (milk quota, limits on cow

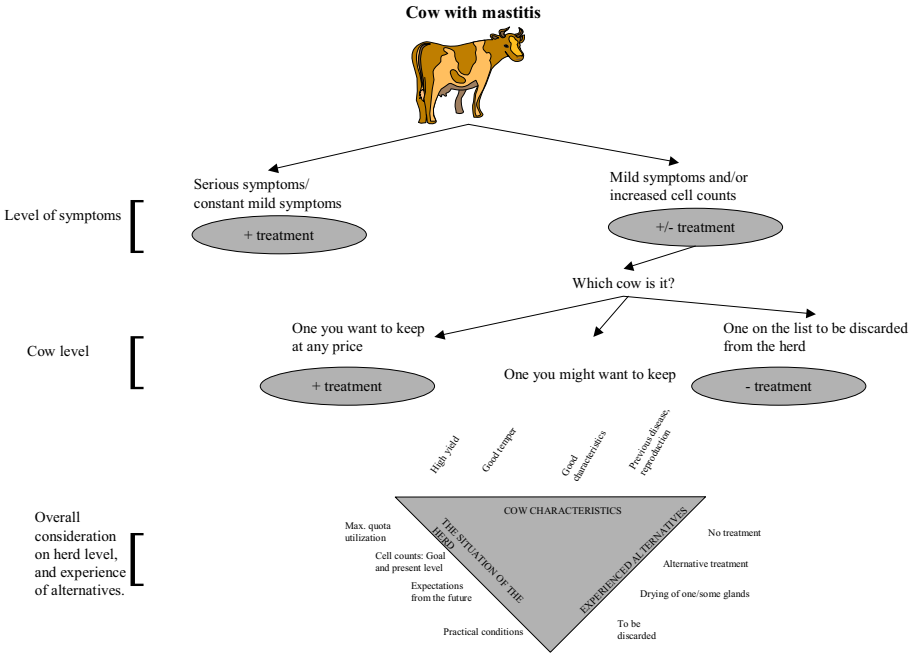

Figure 1. The farmers decision making in relation to mastitis treatments in conventional and organic herds, described through 16 qualitative interviews. Farmers' decisions on treatment is based on 4 levels: symptoms, cow, herd and perception of alternatives. From Vaarst et al., 2002.

numbers etc.; economical choices which are beyond the scope of this article). On this level, farm and society related arguments seem stronger than veterinary oriented arguments for or against medical treatment. The farmer is consequently the advocate of interests and priorities linked to this level. To be a good farmer means a lot to the person involved in the farm, but was interpreted in various ways by different stockpersons. It can be linked to either a high milk yield, a high level of hygiene, good animal well being, good farm structure, or control of the health situation.

Presentation of the second study: Farmers treatment of mastitis during conversion to organic farming

In the second study, 20 farmers were interviewed when converting to organic farming and two years after in order to explore changes in their treatment criteria and patterns. Severe, acute mastitis cases were treated by a veterinarian in all herds, mainly for animal welfare considerations. In some herds, veterinary treatment was supplemented with extra stripping out and/or peppermint ointments. Farmers seemed to perceive antibiotics as the treatment method with the best prognosis in treatment of acute mastitis and other types of mastitis. In the case of mild mastitis, animal welfare considerations are not directly involved, and the choice of treatment was based on multiple levels involving the cow and - in particular - the whole herd. In the farmers' mind, 'being organic' was a factor of minor importance for changes in herd strategies, compared the general development of the market

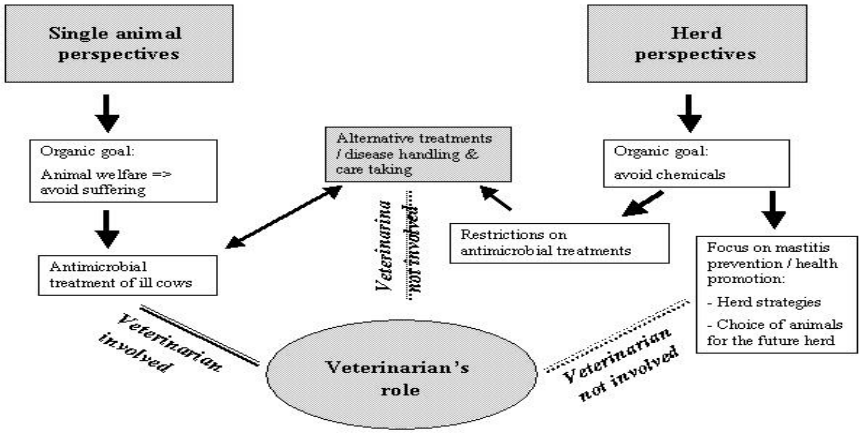

Figure 2. The veterinarian's role in organic herds in relation to mastitis treatments as described through 20 qualitative interviews with organic farmers. Veterinarians seem to be involved almost exclusively in the antimicrobial treatment regime, and little in alternative treatments or mastitis prevention. From Vaarst et al., 2003.

and the farming business today. The farmers' willingness to do a time consuming effort to prevent and cure mastitis, and think in alternative ways, which would correspond with organic ideas, seemed very small among these recently converted farmers. In general, mastitis seemed to be handled in very traditional ways and with minimum effort. In the case of acute, clinical mastitis the considerations of animal welfare (defined as described above) were given a higher priority than the considerations and preferences of the farmers as well as expectations regarding the herd structure. The farmers did not share the society's concern for development of antimicrobial resistance, and expressed that it definitely had no impact on their choices of disease treatment in the herd.

Conclusion on the use of qualitative studies to understand mastitis treatment patterns

In these studies, we wanted to understand the choices of the farmers in relation to the context and logic connected to these treatments. From the results of these interviews acute and chronic mastitis cases are obviously handled very differently by a relatively small number of farmers who participated in this project. This knowledge obtained through qualitative research interviews can be used in combination with epidemiological analyses for several purposes: 1) Creating background for dialogue with individual farmers about their choices, 2) Providing general insight into subject areas where farmers' decisions are crucial, e.g. choice of disease treatment thresholds. 3) Offering important additional knowledge, which can be directly used in development of decision support systems, where farmers' and veterinarians' need for knowledge and support decides the success of a given decision support systems development based on epidemiological methods. An increased focus on the life-world of the farmer and the study of rationality behind farmers' actions seem justified and highly relevant when working with a disease like mastitis, which is the cause of very many (often the majority) antimicrobial treatments in North-Western European dairy herds.

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Implementation of recommended mastitis prevention management practices and the herd level prevalence of contagious mastitis pathogens on Canadian dairy farms

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Abstract

Although research and extension programs have stressed implementation of certain mastitis management practices, the level of compliance is often undocumented. Additionally, herd-level prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* has been estimated in the past in Canada, but studies were limited geographically. The objectives of this study were, therefore, to estimate the level of implementation of mastitis prevention management practices on Canadian dairy farms and the herd-level prevalence of contagious mastitis pathogens. Also, the association between herd-level pathogen-specific prevalence, application of management practices and presence of risk factors was studied. A total of 291 randomly selected farms enrolled in the study submitted bulk milk samples. Of these farms, 262 completed a questionnaire on the adoption of mastitis management practices recommended by the NMC. Herd-level prevalence of *Staph. aureus* and *Strep. agalactiae* was 48.4 and 2.5%, respectively. Of the farms 95% applied post-milking teat disinfection, 73% treated all cows with antibiotics at drying off, 58% milked clinical mastitis cows last or with a separate unit, and 52% of farmers did not record new cases of mastitis on permanent records. Prevalence of *Staph. aureus* was in agreement with earlier studies, but prevalence of *Strep. agalactiae* was lower than expected. Many producers have adopted good udder health management practices, but some practices need to be promoted further.

Introduction

The NMC has a recommended mastitis plan with 10 areas of attention (NMC, 2004) which is generally considered as the standard for mastitis management. A number of studies in Canada investigated management practices on dairy farms (Spicer *et al.*, 1994; Sargeant *et al.*, 1997; VanLeeuwen and Keefe, 1998). However, these studies did not focus on mastitis management. Therefore, compliance to these 10 management areas by Canadian dairy farms is unknown.

Although several studies in the US and Europe have estimated the herd-level prevalence of *Staph. aureus* and *Strep. agalactiae*, only a few studies have been performed in Canada on the prevalence of these contagious mastitis pathogens. *Strep. agalactiae* prevalence in Canadian bulk milk ranged between 6% in Alberta (1993), 18% on PEI (1994) and 43% in Quebec (1992) (Guillemette *et al.*, 1992; Schoonderwoerd *et al.*, 1993; Keefe *et al.*, 1997). In a study on Ontario dairy farms, 58 out of 59 bulk milk samples *Staph. aureus*-positive, while 92% of the herds had at least one *Staph. aureus* culture-positive cow (Kelton *et al.* 1999).

Many studies have been conducted on dairy farms to find risk factors for mastitis. However, management of dairy farms differs among countries due to different environmental circumstances. In Canada, no studies have been conducted to investigate the risk factors of having contagious mastitis pathogens in the bulk milk.

The objectives of this study were: 1) to estimate the compliance of recommended mastitis preventive management practices on Canadian dairy farms, 2) to estimate the herd-level prevalence of contagious mastitis pathogens, and 3) to evaluate the association of certain management practices with the isolation of contagious mastitis pathogens from the bulk tank from Canadian dairy farms.

Material and methods

Herd selection

Herds were randomly selected and stratified per province. After being selected producers were invited by mail to participate in this study. Producers that did not respond were contacted by telephone four weeks later.

Questionnaire

A questionnaire was designed based on the 10-point recommended mastitis management plan as described by the NMC (NMC, 2004). After the questionnaire was sent, a second copy was sent to non-responders three months later and the farmers that still did not respond were contacted by telephone.

Sample collection

For every province, the provincial dairy laboratory was contacted to collect the bulk milk samples of the selected farms. The 10 provincial dairy laboratories stored the bulk milk samples of the selected farms frozenly at -20^{o}C and, once the batch was collected, samples were sent on ice by overnight courier to the Atlantic Veterinary College (Charlottetown, Prince Edward Island, Canada) for bacteriological culture.

Laboratory analyses

Five different media were used to determine the presence of *Staph. aureus*, *Strep. agalactiae* and *Mycoplasma spp.*: 1) Blood agar with the addition of 1 g/l esculin (Blood-esculin agar), a general medium; 2) Vogel Johnson agar, a medium selective for staphylococci; 3) Modified Edwards' medium with the addition of colistin sulphate (5 mg/L) and oxolinic acid (2.5 mg/L), a medium selective for streptococci; 4) Modified Hayflick agar, for the culturing of *Mycoplasma* spp., and 5) Modified Hayflick broth for *Mycoplasma* spp. enrichment. After mixing the milk with a vortexer for 5 seconds, 50 µl was dispensed by pipette onto blood-esculin agar, Vogel-Johnson agar and Edwards' medium, and 100 µl was dispensed onto Hayflick agar and into 2 ml Hayflick broth. The milk was spread evenly over the plates with a sterile cotton swab and allowed to air dry before incubation.

Statistical analyses

Data from completed questionnaires were entered into a database (Lauritsen and Bruus), checked and exported into Stata files for descriptive statistical analyses.

Prevalence was calculated as stratified, weighted sample. All univariate analyses were carried out by using a χ^2-test in Stata (Intercooled Stata for Windows, version 8.0. Stata Corporation, College Station, TX, USA, 2003).

Results

At this moment, 262 (90%) of the 291 questionnaires have been returned by the participating farmers. The compliance to a selected number management practices is shown in Table 1.

The distribution of barn types for the lactating cows over the 10 Canadian provinces with a significant dairy industry is summarized in Figure 1. Overall, the proportion of tie-stall and free-stall dairy farms in this study is with 42 and 45%, respectively, approximately the same (P=0.42). In the most western provinces, British Colombia and Alberta, lactating cows are predominantly housed in free-stalls. In Quebec, the province with the largest dairy industry, however, 90% of the farms have a tie-stall to house the lactating cows.

Prevalence of *Staph. aureus* and *Strep. agalactiae* in a single Canadian bulk milk sample was 48.4 and 2.5%, respectively. No *Mycoplasma* spp. were found. Figure 1 shows the prevalences per province for *Staph. aureus*. Bulk milk prevalence of *Staph. aureus* was highest in the three Maritime provinces, Prince Edward Island, Nova Scotia and New Brunswick. Three of the four western provinces, Manitoba, Alberta and British Colombia had the lowest prevalence of *Staph. aureus* isolations in bulk milk.

Prevalence of *Staph. aureus* in bulk tank milk was 63, 37 and 27% for tie-stalls, free-stall and other barns, respectively (P<0.001).

The management practices that had a significant association (P<0.10) with the isolation of *Staph. aureus* from the bulk milk are shown in Table 2.

Table 1. Adoption (%) of mastitis management practices in 262 Canadian dairy farms.

Management practice	Tie-stalls	Free-stalls	All
Pre-milking teat dip or spray	50	64	53
Dry udder before attaching	82	93	84
1 cow per cloth / towel for pre-milking treatment	88	85	86
Wear latex gloves (at least sometimes)	50*	74	62
Post-milking teat disinfection	94	97	95
Milk clinical mastitis cows last or with separate cluster	85*	32	58
Collect milk sample of clinical cases most of the time	16	17	16
Treat clinical mastitis with antibiotics most of the time	80	81	81
Treat at least 97.5% of cows with dry cow treatment	69	79	73
Use CMT more than once a month	27*	46	38
Thinks it is important to very important to cull *Staph. aureus*-positive cows	36	42	40
Milking equipment checked at least once a year	77	78	78
Uses computer for cow records	16*	48	32
Uses permanent records for clinical mastitis cases	48	49	48
Clips or flames udders	76*	46	59

*Percentages were different between tie- and free-stalls at P<0.05.

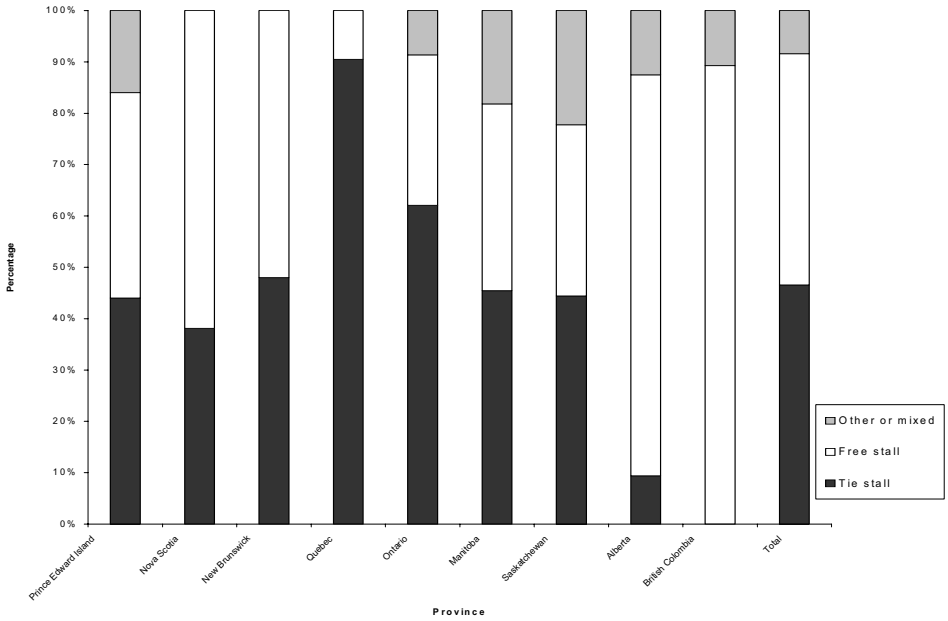

Figure 1. Distribution of barntype per province.

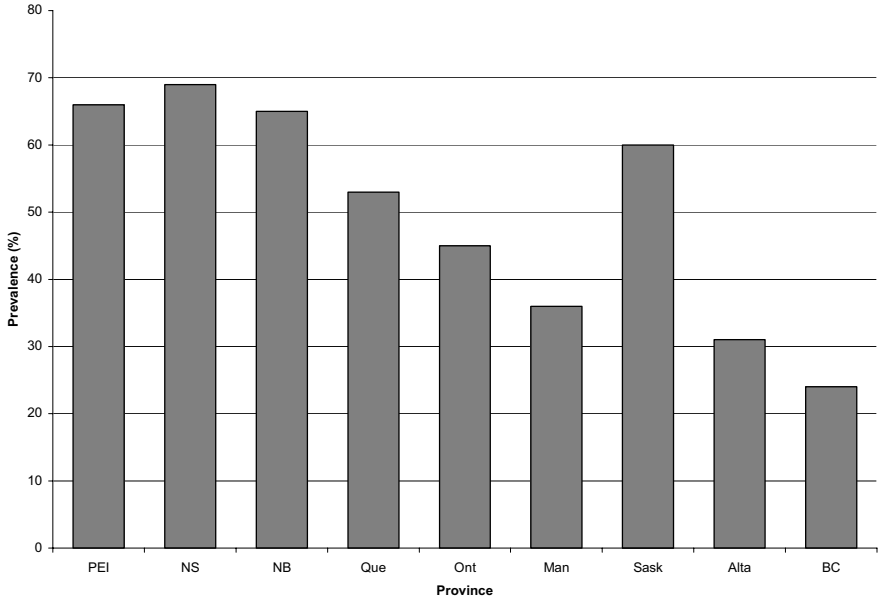

Figure 2. Prevalence of Staphylococcus aureus *per province.*

Tabel 2. Management practices that were associated with the isolation of Staphylococcus aureus (SAU) in the bulk milk.

Management practice	SAU pos farms (%)	SAU neg. farms (%)	*P*-value
Milk clinical mastitis cows last or with separate unit	36	52	0.012
Mark a clinical mastitis cow with more than one mark	54	68	0.032
Vaccinate against mastitis	19	32	0.022
Dry cow treatment > 97.5% of cows	68	78	0.06
Takes samples of high SCC cows	73	60	0.029
Uses computer for cow records	21	42	0.001
Uses straw as bedding material	63	48	0.017
Uses shavings or sawdust as bedding material	29	41	0.049
Uses sand as bedding material	1	6	0.045
Clip or flame udders	65	50	0.022
Purchases cows	56	45	0.10
Ration balanced more than 3x a year	57	70	0.042

Discussion

This study is, to our knowledge, the first nationwide Canadian study that focussed on the adoption of mastitis management practices. Most farmers implement the recommended mastitis management practices, but an important management practice like blanket dry cow treatment, which is proven to be effective against mastitis, is only implemented on 71% of the farms. Recommended mastitis management practices were most frequently implemented in herds that house their lactating cows in free-stall barns, while the prevalence of *Staph. aureus* was lower in free-stall barns than in tie-stall barns. Both the distribution of tie-stall and free-stall barns and the prevalence of *Staph. aureus* varied considerably among provinces. This implicates that extension and education should be tailored per province and barn type.

The prevalence of *Staph. aureus* of 48.4% was as high as expected. The prevalence of *Staph. aureus* found in this study agrees with earlier studies where herd prevalence ranged from 31 to almost 100% in North America (Kelton *et al.*, 1999; Khaitsa *et al.*, 2000; Jayarao *et al.*, 2004). However, the herd-level prevalence of *Staph. aureus*, and to a lesser extend *Strep. agalactiae*, will be underestimated by only using one bulk milk sample. When this study is completed, culture results will be available of four bulk milk samples, collected in the four different seasons.

No *Mycoplasma* spp was found in the bulk milk of these 291 dairy herds. However, the samples were frozenly stored which most likely will have influenced the recovery of *Mycoplasma* negatively (Biddle *et al.*, 2004). For this reason, on this batch and the other three batches of samples a *Mycoplasma* PCR will be used to provide a better estimation of the herd-level *Mycoplasma* prevalence.

Herd-level prevalence of *Strep. agalactiae* has decreased considerably the last years (Keefe, 1997; Pitkala *et al.*, 2004). The herd prevalence of 2.5% in this study was based on a weighted, stratified prevalence calculation and represented 2 farms.

The association of the presence of *Staph. aureus* in the bulk tank and certain management practices is for certain management practices negatively correlated, for example taking milk samples of high SCC cows. This could be explained by the possibility that these farmers had already a *Staph. aureus* problem on their farm. Other management practices are more associated with barn type, for example straw is used more often in tie-stall barns as bedding material than in free-stall barns.

Conclusions

Adoption of most of the recommended mastitis management practices is good. However, significant improvements can be achieved as since still significant associations with the isolation of *Staph. aureus* in the bulk tank milk were found. Prevalence of *Staph. aureus* was as high as expected, but varied among provinces. Prevalence of *Strep. agalactiae* was low, confirming a trend of declining prevalence.

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A national research network to promote mastitis control in Canada

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Abstract

Acquiring new knowledge and techniques by dairy farmers is critical to controlling mastitis. The Canadian Bovine Mastitis Research Network is a unique partnership that aims to advance mastitis control through integrated mastitis research and transfer. The Canadian dairy industry and 42 researchers in ten institutions organized the Network with a major objective of rapid and comprehensive transfer of its research results to producers. The Network funding model comprises foundational support from dairy producer organizations across Canada with substantially augmented governmental research funding. The Network programs research in consultation with industry to resolve gaps in knowledge and application and to optimize data collection, research resource sharing, and training of highly qualified personnel. The dairy industry participates in the Network 1) by providing leadership on the Board of Directors, 2) by contributing to scientific planning and evaluation, 3) by guiding the transfer of information to the industry, and 4) by collaborating in Network research projects. The industry's involvement in the Network program stimulates a sense of ownership and identity with the program, and an awareness and expectation of applicable research results. Governmental funding gives added-value to the industry's investment. The Network permits research on a national level and will produce results that reflect the mastitis situation across the full breadth of Canadian dairy production environments. This will enhance applicability and producer receptivity of the information and technology transferred by the Network.

Keywords: research networks, knowledge transfer, technology transfer, training, industry partnership

Introduction

Continued progress in udder health and milk quality control depends on continued scientific discovery and on dairy producers' implementation of both newly discovered and already existing knowledge and technology. Even though mastitis is still very important economically, it is competing with many other high-profile dairy health problems for the priority position in allocation of research resources. In this environment of competing priorities, Dairy industry leaders in Canada began developing a vision for a program that uses resources efficiently and strategically for mastitis monitoring and control research and transfer. Their desire is for a progressive research program that coordinates and collaborates nationally and internationally and whose results are visible and easily accessible to Canadian

dairy producers. Many of Canada's dairy producers' organisations have actively funded research for many years. Coordinating their research investments in a nationally-collaborative research program accompanied by pro-active knowledge and technology transfer would add value to their investments.

The industry's vision was the catalyst for creating the Canadian Bovine Mastitis Research Network. A research network provides a structure for extensive industry participation in the research program, for pooling resources (financial, infrastructural, and intellectual), and for coordinating research, training and transfer all on a national scale. Forming a research network also takes advantage of Canadian government's interest in promoting widely coordinated and innovative research collaboration partnerships between institutions and industry.

The Network's objectives are 1) to define the mastitis situation in Canada, 2) maintain excellence in its networking, research, training, and transfer, 3) conduct mastitis research with a near-term horizon, 4) transfer near-term research results to the industry, and 5) to conduct fundamental research with a longer-term horizon. Close partnership with the industry will enhance industry adoption of the research results as a consequence of a sense of ownership and expectation fostered by being partners in the effort. A unified multilingual knowledge transfer plan and durable transfer in the form of multi-disciplinarily trained students will further enhance the Network's transfer of mastitis monitoring and control knowledge and technology.

As a well planned national level partnership between research institutions and industry, the Network creates research, training, and transfer opportunities that would be impossible otherwise.

Overview of the network

The Network is a partnership between ten research institutions and seven provincial producers' organisations, two national producers' organisations, and six allied organisations. The research program comprises a core research platform (CRP) to which is linked two research themes: Mastitis Monitoring and Mastitis Control. A board of directors provides oversight and accountability. The research and training programs are planned and implemented by a scientific committee. A transfer committee guides knowledge and technology transfer. The industry has roles in the three decision-making bodies and participates in the field research.

Funding model

The Research Network Grants program of the Natural Sciences and Engineering Research Council of Canada was one stimulus to explore developing a mastitis research network. This program seeks to fund multi-disciplinary national research networks in partnership with Canadian industry. A proposal for five years of funding of the Network was still pending before the Council at the time of this writing. Funding comprises 23% ($2.2 million CAD) from industry, allied groups, agencies, and the host institution, and 77% ($7.5 million CAD) from the Council. The Network budget funds administration, the transfer and training programs, the CRP and research critical to the scientific plan but that is not likely to be funded from other sources. Technological research that will be transferred through

commercial channels is funded up to the proof of concept, after which continued funding from the Network is contingent upon ramped-up financing from commercial enterprises. High risk research is evaluated at a critical point and continued funding allocated if the risks have been surpassed. Starting in the third year, a budget set-aside will be used to fund new research initiatives. Training of undergraduates and post-graduate students, and post-doctoral fellows is an important consideration in planning and funding decisions.

Scientific program

The rationale of the Network's scientific program is to enable the research that takes advantage of the Network's research strengths and resources. Member scientists come both from within and outside of the mastitis research domain. Gaps in expertise are filled by domestic and international collaborations. The planned must be novel, results should be transferable in the near term or provide critical materials for other parts of the research program and must be of interest to dairy producers. Network-funded research should be research that would be unlikely to be performed outside of the Network.

The research program consists of a Core Research Platform (CRP) to which are linked the Mastitis Monitoring and Mastitis Control research themes. The CRP is a national-scale collaborative data collection and archival platform. It includes a national dairy farm cohort and coordinated mastitis diagnostic laboratories. The farm cohort will provide data and material for the mastitis monitoring and control research themes. The diagnostic laboratories at the country's four veterinary faculties will analyse milk samples from the cohort with coordinated protocols for milk bacteriology, quality control and reporting of results. All isolated mammary pathogens will be characterized and archived in a mastitis pathogen culture collection and host DNA will be archived for current and future host genetics research. The archives and epidemiological databases will be linked to one another and will remain available to researchers beyond the Network research program.

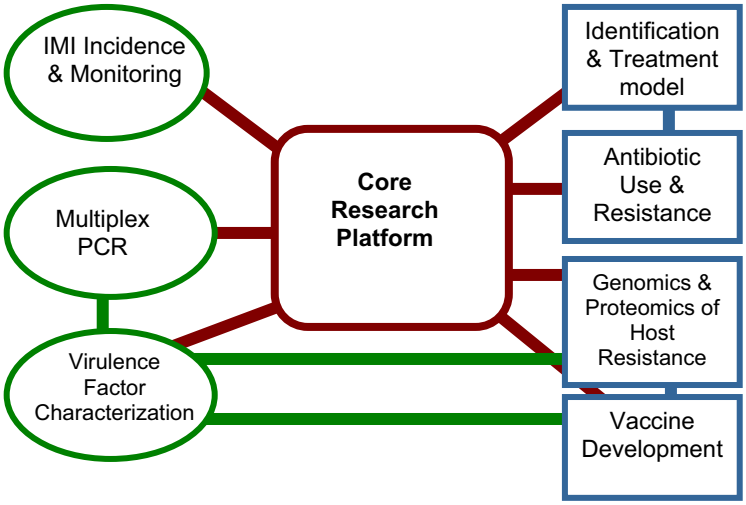

Figure 1. Inter-theme and intra-theme relationships among projects in the CBMRN research program. ○ Mastitis Monitoring, □ Mastitis Control

Each theme's scientific objectives give the theme a specific role in the overall program, but the objectives are also broad enough to permit taking advantage of existing and newly arising opportunities. The Mastitis Monitoring theme aims to develop new knowledge and new technologies for monitoring intramammary infection (IMI) and mastitis. The theme's objectives are 1) to develop novel strategies for utilising easily obtainable data to monitor udder health and milk quality, 2) to characterize genes and gene products associated with virulence of IMI pathogens, and 3) to develop rapid methods of pathogen diagnosis. The Mastitis Control research theme aims to develop knowledge, new practices, products, and technologies to reduce the incidence and prevalence of IMI and clinical mastitis. The research objectives are 1) to discover tools and technologies for enhancing host resistance, 2) to develop novel therapeutic strategies, and 3) to understand more precisely how mastitis treatment influences antimicrobial resistance. New research initiatives will be started in response to new ideas and technologies that advance the Network towards its objectives. The themes' projects will obtain data largely from the CRP, but projects will also extensively collaborate directly with one another.

We have a four-level networking plan to help achieve the full benefits of collaborations, coordination and innovation. Level 1: The Network's website (http://www.medvet.umontreal.ca/reseau_mammite/) and listserver will be used as a platform for members to exchange ideas and information, and to access inventories of infrastructure available at participating laboratories. Research results and minutes of inter-disciplinary research workshops will be accessible too. Level 2: Semi-annual intra-theme videoconferences or teleconferences will be used for problem solving, promoting innovative ideas and preparing theme reports. Level 3: Annual Scientific Meeting will involve all members, trainees, collaborators, partners and invitees in an exchange of knowledge, research approaches, results, and planning. Level 4: Purposeful international networking will be ensured by inviting eminent international scientists as keynote speakers at our Annual Scientific Meeting and funding two international scientists per year to collaborate on-site in one or more of our laboratories.

Training program

Training people with a good grasp of both the fundamental and applied aspects of mastitis research and control is a very durable means of knowledge transfer. The Network's objective is to give trainees an integrated multidisciplinary understanding of bovine mastitis research and mastitis control. Trainees will have a perspective on fundamental research aspects and field research and application regardless of whether the main focus is mostly fundamental or mostly applied. A training program adds value to the participating scientific institutions' training mandate and provides knowledgeable personnel to the serve the industry and society for many years.

The training plan's elements include inter-laboratory exchanges, mastitis control and research training modules, and scientific meeting mentoring. A minimum of 30 trainees will be recruited in connection with Network research projects. Their participation in inter-laboratory exchanges will be promoted by competitively offering up to five scholarships per year for extended inter-laboratory exchanges. The Network will cover expenses for trainees to take part in two training modules per year. Each module will focus on one aspect of mastitis research and control and may be developed specifically for the Network or be part

of an existing workshop or course offered to a non-Network audience by Network members and collaborators. Modules will be chosen so as to ensure the multi-disciplinarity that is at the heart of the Network's training program. All trainees will present their research at the Annual Scientific Meeting where they will receive feedback from Network scientists and guests and will compete for scholarships to present their work at a major international scientific conference. Network scientists will be encouraged to take highly pro-active roles in mentoring their trainees' interactions at scientific meetings to ensure that no opportunities for growth and stimulation are missed.

Transfer program

Transferring knowledge and technology to the dairy industry is a cornerstone of the partnership with the industry. The concept of far reaching research partnership grew in part from the desire of industry leaders to more clearly see the fruits of their research investments. Knowledge and technology transfer is the most important link to industry partners. The input of industry leaders into oversight of the Network and into the scientific program is important, but it is transfer that connects the Network with the widest cross-section of the industry. Moreover, the Network provides dairy producers a sense of ownership of this mastitis research program which may have the added benefit of sensitizing producers to knowledge transferred from the program thereby increasing the likelihood of adoption.

The Network's role in technology transfer is to use the collective contacts and resources of its members to promote up take of technologies by commercial entities. This includes establishing commercial partnerships as early as possible in the development research and providing recommendations and contacts to the research institutions' offices of technology transfer.

The knowledge transfer program has three objectives to help assure that the Network's mastitis monitoring and control knowledge is transferred widely and effectively in all of Canada's dairy regions. 1) to communicate new knowledge and existing knowledge uniformly through the already recognized regionally distributed transfer channels, 2) to increase access of all Canadian dairy producers, regardless of language barriers, to mastitis monitoring and control resources on the internet, and 3) to promote high quality mastitis monitoring and control practices and technologies by supporting education and training.

The Network employs a transfer manager holding a Master of Science degree in veterinary science and possessing strong dairy industry knowledge and experience. The transfer manager executes the transfer program. Knowledge transfer channels to dairy producers have evolved at the provincial and regional levels. The Network will assure that information is uniformly fed into the entire breadth of transfer channels in both English and French. Channels include the Network's website, a bi-monthly bulletin, short communications in regional agriculture print media, and technical articles in Canada's three leading dairy magazines. The Network will collaborate with transfer organizations (NMC for example) to make the best use of existing resources. A consensus conference (anon, 1999; anon, 2001) will be held in year three to explore a controversial topic of mastitis monitoring and control. Both the underlying scientific issues and field application will be explored and particular emphasis placed on recommendations that can be derived for application in the Canadian dairy environment and enumeration of the knowledge gaps that are preventing full application in the field. The latter results will help guide future research and transfer plans.

A Transfer Committee, composed of experts in agricultural knowledge transfer, scientists and users, will guide planning, execution, and evaluation of the transfer program. Evaluation is a fundamental means of assessing segments of the industry not reached and to gain insight into the impact of the program on mastitis monitoring and control decision-making at the producer level.

Partner involvement

For the Network to be a true research partnership with the dairy industry, it is fundamental to have clearly developed means of involving dairy industry members in the Network's activities. The depth and the nature of involvement must enable the industry to truly have influence on the Network and must be active and dynamic. Partner involvement that accomplishes the foregoing will help the industry organizations, and hence their members, to maintain a sense of identify with the Network or a sense of ownership of the program. We think this will optimize their receptivity to transferred mastitis monitoring and control knowledge.

Dairy industry leaders will compose one-half of the Network's Board of Directors. In this role, industry representatives will guarantee accountability of the Network with respect to objectives and financial management. Board members will influence the direction of the Network's programs; interact regularly with leaders from the research institutions and with Network leaders.

The Network's Scientific Committee implements all of the Network's activities except for knowledge and technology transfer. Dairy farmers representing industry organizations and sitting on this committee will be full participants in the Committee's work. They will influence scientific planning and training and ensure that Network scientists keep industry priorities and eventual field application in full view. They will also play a specific role as outsiders in progress-evaluation of projects and in the Annual Partners' Review. The latter will be an annual industry-perspective review of the effectiveness of the entire program. In addition approximately 100 farms nation-wide will intimately participate in the research as part of the cohort in the CRP.

The all important activities of knowledge and technology transfer must be planned and executed from the perspective of understanding dairy producers' views, practices and expectations. For these reasons, the Transfer Committee will include knowledge and technology transfer experts among its members. But dairy producers and industry representatives on the committee will also be instrumental to determining the means of knowledge transfer that will most effectively reach the rank-and-file producer and properly evaluating the success of transfer efforts.

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Mastitis control program in The Netherlands: Goal, tools and conditions

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Introduction

Mastitis is considered as one of the main health issues in the Dutch dairy industry. The Incidence of clinical mastitis is estimated to be at least 25% per year.(Barkema *et al.*, 1998).The prevalence of subclinical mastitis based on a bacterial culture has been studied in the past (Vecht, 1985/86,1987). Subclinical mastitis defined by a certain level of somatic cell count, is not described so far. Since 1971 a considerable decrease is realized in the bulkmilk somatic cell count (BMSCC), (Figure 1)

During the last 5 years, the average BMSCC showed an increasing fluctuation during the year, peaking in the summer months. Although the average BMSCC per year did not increase dramatically, the increased fluctuation indicates a change in SCC patterns and possibly in clinical mastitis incidence (Figure 2).

As in other countries, the Dutch dairy industry puts focus on healthy milk from healthy cows, being the base for all dairy products from the Netherlands. The industry considers good udder health as one of the most critical factors in this. In addition, mastitis is considered as the endemic disease causing the highest economic losses at farm level. De Vos *et al.* (1997) showed an average cost of 255 euro per clinical case, resulting in an overall damage for the Netherlands (1.6 million dairy cows) of a little over 100 million euro per year, assuming an incidence rate of 25%. Losses due to antibiotic violations by farmers,

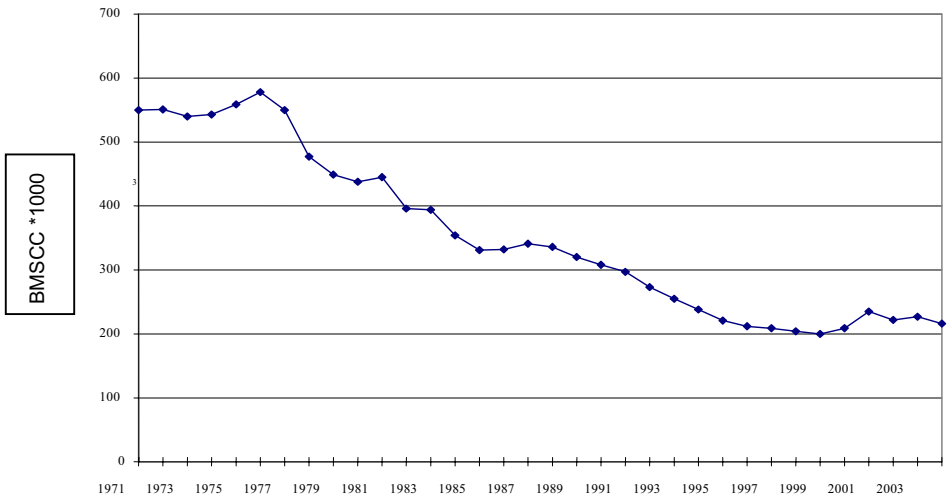

Figure 1. The arithmetic average somatic cell count in bulkmilk of Dutch dairy herds from 1971 to 2004.

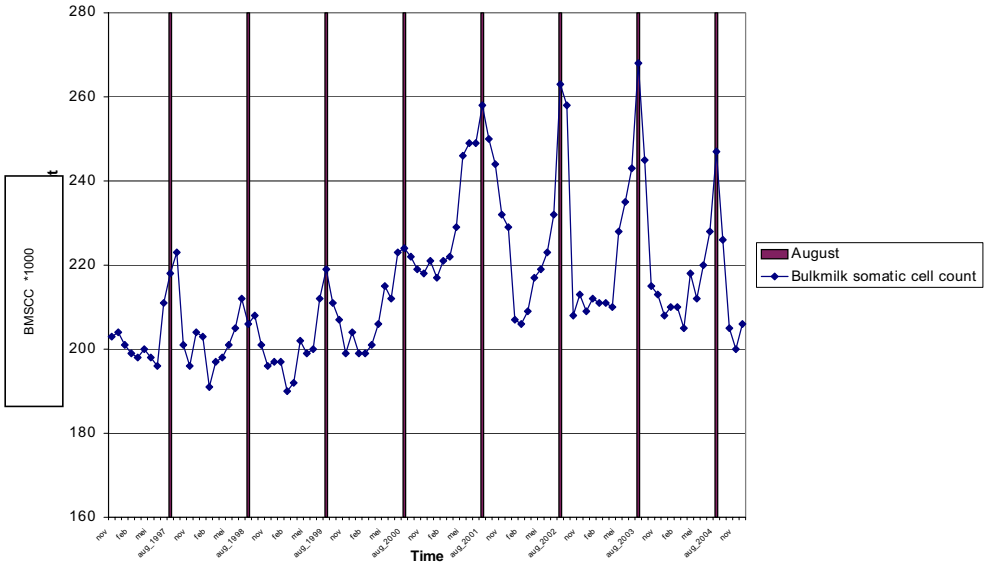

Figure 2. Arithmetic Average BMSCC of Dutch dairy herds from 1997 to 2004.

processing problems because of this, and losses caused by subclinical mastitis are not regarded in this estimate.

All this motivated the Dutch dairy industry (NZO) and the Dutch farmers organisation (LTO) to improve udder health. The Animal Health Service (GD) was asked to develop and execute a program with the objective to decrease the number of cases of (sub)clinical mastitis with 10% absolutely in 5 year, by 2009. The objective of this paper is to describe the current situation in the Netherlands, the organisation of the national program and its critical success factors.

Experiences in different countries

In the Nordic countries, large efforts have been made to reduce mastitis. Østerås *et al.* (2000) showed a decrease of 43% of incidence of mastitis treatments in Norway since 1994. Ekman *et al.* (2003) concludes that reliable records are the foundation of successful control programs. The most important method for a decrease of mastitis, however, were comprehensive programs promoting cattle health, alternate actions to treatment with antibiotics and selective rather then total dry cow therapy . Leslie *et al.* (2002) stated that setting meaningful goals and monitoring the progress towards them are cornerstones of an effective program. Van Schaik *et al.* (2002) concluded that high BMSCC herds more often violate antibiotic residue rules in NY State, and suggested testing programs combined with incentive, education and training focused on these farms.

Sargeant *et al.* (1998) described the results of the ongoing SCC reduction program in Ontario. They concluded that after an initially successful reduction of BMSCC, not only penalizing of farms which exceed thresholds is necessary, but also an incentive to prevent farms from increasing BMSCC. In The Netherlands, Lam *et al.* (1998) developed a mastitis management planner. Farm-level goals are set, a plan is made and parameters are measured

and analyzed, all with the objective to structure, improve and simplify udder health management in dairy herds.

The current situation in The Netherlands

Designing a successful program with achievable goals means that you have to know the current situation. Also knowledge of which instruments will contribute to what extent and the costs to accomplish the goal are essential. To measure the current situation a study was set up in two parts. The first part is a survey of knowledge, attitude and behaviour of dairy farmers with respect to mastitis, the 'knowledge' survey. The second part consists of the estimation of the incidence of clinical mastitis, an estimation of prevalence and incidence of subclinical mastitis, the "mastitis base study".

The knowledge survey

A random sample of 560 dairy farmers were asked to complete a questionaire about knowledge, attitude and behaviour concerning mastitis (Kuipers *et al.*, 2005) The results of this study will be presented during this conference. Restrictions were placed on age of the farmer (< 55), herdsize (> 50 dairy cows) and they had to participate in a regular milktesting program. Of these 560 randomly selected farmers, 380 completed the questionnaire and joined the study. The main conclusions were: (1) Farmers have a high awareness of the importance of mastitis, but don't act in a preventive fashion(2) Not only cost, but also extra labour, work satisfaction and the uncertainty whether a mastitis cow will cure or not are the main issues on the dairyman's mind, (3) Of all advisors, the private practitioner is by far the most influential (4) Information on mastitis is gathered from the practitioner, and the three main farmers magazines; other sources are not consulted that

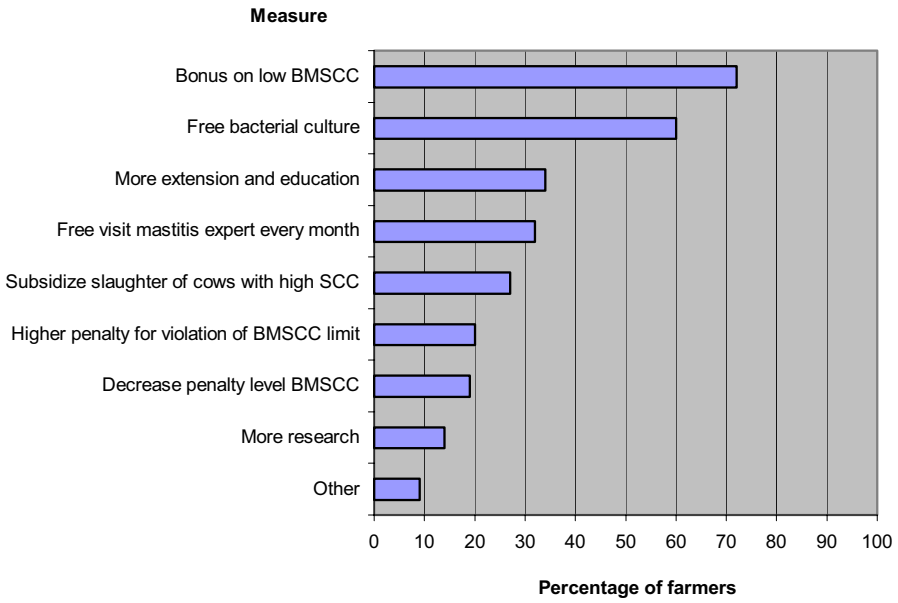

Figure 3. Popularity of various control options to reduce BMSCC in Dutch dairy herds (Kuipers et al., 2005).

much. (5), Farmers prefer positive stimuli in order to reduce mastitis, illustrated by the following figure.

Mastitis base study

All farmers who completed the knowledge survey were asked to register all cows with clinical mastitis from July 1-th 2004 to June 30-th 2005. A case of clinical mastitis was defined as abnormal milk. All individual SCC and BMSCC of all participating herds will be available as well. Data are gathered and validated by NRS, (Dutch Cattle Syndicate). Together with the questionnaire data these data complete the data set of the 'knowledge survey', giving the opportunity to correlate knowledge, attitude and behaviour to the (sub)clinical mastitis data.

The Mastitis Reduction Program 2005-2009

The Mastitis Reduction Program is split up in two parts. In the first part, the research part, technical knowledge will be generated. At the same time research will be done in how existing and yet to generate knowledge will lead to a decrease of mastitis incidence. The principle of this applied research will be the main criterium: What will this project contribute to (10%) less mastitis cases in 2009?

The second part is the applied, the practical part. The 'knowledge' survey shows very clear that veterinary practitioners play a dominant role. They are by far the most influential advisors on the dairy, and farmers tend to follow their advises. Therefore, in 2005 the program started with 10 veterinary practitioners, who serve in total approximately 1500 dairy farms as clients. They are offered a variety of (new) tools, which are communicated and used in (new) ways. The goal is to decrease the mastitis incidence in these herds. Based on the effectiveness of these new tools, on the perception of dairymen and veterinary practitioners, the best tools will be selected to put in practice in the next 50 areas. After that, upscaling will continue to all veterinary practices in The Netherlands.

Mastitis is considered a multi discipline problem. Epidemiology, economy, microbiology, immunology, communication and other disciplines can play an important role in decreasing mastitis incidence. The principle of the program is to integrate the various disciplines in the various research projects and practical applications.

Examples of (new) tools

In the Netherlands, as in other countries, many tools to reduce mastitis are available. Much research has been done to improve them, technically or by means of better communication. Based on this, a selection has been made of tools to be implemented in the first ten veterinary practices. Most likely, not all of these will prove to be equally effective. By the end of the first year, a sharp evaluation will set priorities, and not all tools will continue in this program. Research will qualify the best, based on the perception of the farmer and practitioner, the change of knowledge, attitude and behaviour of the farmer and practitioner by using them and on their effectiveness in reducing mastitis incidence.

- Milk-Mirror: Have a specialist milk together with the farmer once a year. Have her/him fill out a small checklist, talk it over after milking and set points for improvements. This tool was successfully applied in New York State at Cornell University.

- Fast diagnostic tests: Invest in a faster bacteriology, making results of bacterial culture of milk samples from cows with clinical mastitis faster available. Try to get back the first results within 24 hours, then follow up after 48 hours with a final result. The reason for this is that in the knowledge survey, farmers show a great interest in a quick first test result, and seem to improve their management if they know 'something already'. Also this proves to work at Cornell University as well.
- Develop an easy, farm specific, one page scheme for handling a mastitic cow: structured, protocolled, easy an effective. Avoid discussions among professionals about what is the best approach on farm level, since uncertainty will not contribute to mastitis decrease. In Norway this approach has proved to work during the last 20 years.
- Support practitioners on their monthly visit with news about mastitis, and support them in prioritizing mastitis on the working list. The Dutch experience is that practitioners are very eager for news on new methods or new research results.
- Show every farmer every month how much money he/she saved compared to the national average by realizing a lower mastitis incidence. The feed back on results is applied on the Dutch dairy farms on all levels: milk testing, finances, feed analysis, etc. A regular feed back on this completes the management list.
- Segmentation of farmers by high BMSCC or high clinical or subclinical mastitis incidences makes it possible to focus on some topics in a selected group by means of meetings, hand outs, consultancy, etc. In the Dutch breeding industry this approach has proven to attrack many participants, willing to debate their goals and tools.
- The practitioner who realizes most decrease in mastitis incidence in all of his/her practice wins a 2 week all inclusive tour to the Bahama's for him/herself and his/her partner.
- A quick scan on preventive measurements per herd. Look at 10 critical aspects, score each of them on scale 1-10, discuss results with the herdsman, come back one year later, go through it again. Link improvement in points with saving money. This way Jones disease on farm level is addressed in The Netherlands.
- Do a bacterial culture on bulk milk every month, show the results as a management tool. In the US this is applied in several areas, and farmers show interest in this as an awareness-tool.

Critical success factors for the mastitis program

In order to realize the objective of the program, the most critical conditions, which have to be met are:

1. Optimize the use of existing knowledge
2. Invest in applicable new knowledge
3. Make sure board members, practitioners and other key decision makers speak out their commitments
4. Make sure research and other investments are with a good cooperation of all involved institutes, universities and industries. Have everyone involved.
5. A bonus/malus system on milkquality by BMSCC.
6. Strong coordination of the project and its various parts
7. Integrated, multi discipline approach on research as well as in the milking parlor.

8. Measure and report on progress in the course of the program, based on real data, on the level of farm, practice, and country.
9. Budget.

Discussion

A program of this size with a quantitative goal is new and unique for the Dutch dairy industry. The challenge is to integrate science, research and daily operations on farm level, but also on the level of veterinary practice and on the industry level. A good coordination and communication of the program will be critical. The main conclusion of the knowledge survey was that farmers do know how important udder health is but that they do not act likewise. Our challenge will be to change that and increase awareness at this point.

The main focus is on the farmer and his or her private practitioner. Activities of other actors such as the pharmaceutical industry, feed industry, etc, are stimulated. They are however, not our first goal, because of the results in the 'knowledge study'. Then, last but not least, welfare has not been mentioned yet, but a more and more broad politically and socially accepted way of producing milk in the Netherlands, means that welfare items related with udder health have to be solved, which this program will do.

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"FRISKKO - JUVER" ("Healthycow - Udder") A systematic veterinary approach to udder disease in dairy herds

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Abstract

Modern udder health programmes on dairy herds need to integrate different expert functions and at the same time involve the manager and the staff in order to achieve good results. A programme should include several investigatory and advisory visits, analysis of gathered data, set goals and targets, evaluate success and failures and give feedback to farmers and staff, as well as include educational elements. In our experience veterinary work should focus on gathering and analysis of data to be able identify risk factors. Advice should then aim at, in dialog with the staff, prioritize among and stepwise eliminate these risk factors. Another necessary element in any advisory program is that is increases profitability for the farmer.

FRISKKO

"FRISKKO" ("HealthyCow") is a voluntary herd health prevention program and has, in controlled studies in Sweden, been shown to increase the earnings on average by 600 SEK (approximately € 70) per dairy cow and year mainly by reducing costs (Hallén Sandgren, 1999; Hallén Sandgren and Carlsson, 2000). The major parts of costs associated with disease on dairy herds are indirect, such as involuntary culling, wastage of milk and increased labor hours. Direct costs, such as veterinary bills including medicine, account for less than 15 % of total costs (Emanuelsson and Hallén-Sandgren, 1996). FRISKKO aims at improving welfare and health in dairy cattle by helping the farmer focus on what he/she can do to improve management and feeding with as little use of antibiotics or hormones as possible. The program also comprises computerized tools for working with udder health, fertility, claw disorders and various other herd health problems. It also features a computer program for comparing the economic result on a particular farm, based on its production records, with other farms in Sweden.

The veterinarians working with FRISKKO are encouraged to work together with other professionals giving advice to dairy farmers such as feeding specialists, advisors on breeding, milk machine technicians etc, to create a network of advisors around the dairy farmer. Disease levels are evaluated though the national dairy production and disease treatment records (Emanuelson, 1988).

"FRISKKO - Juver" ("HealthyCow - Udder") is special service within FRISKKO designed to deal with udder disease. "FRISKKO Udder" has improved udder health and reduced the incidence of clinical mastitis on most participating farms, thereby also reducing the use of antibiotics. The methodology of FRISKKO Udder is similar to that of FRISKKO and the work

is done in 4 steps: 1) available data and routines are checked and documented, 2) the data is analysed and a herd diagnosis is made, 3) based on the analysis and herd diagnosis a number of things to do or change are arrived at in dialogue with the farmer and a consensus on what needs to be done and goals to achieve are arrived at, 4) a follow-up visit is made where the compliance with and effect of the agreed measures are evaluated, herd data are re-checked, new goals set and new measures to reach those goals are agreed upon. One important tool in the preventive veterinary work in FRISKKO Udder is to use the economic evaluation program mentioned above to be able to estimate the economic losses due to udder disease on a particular farm. It is imperative to be able to demonstrate to the farmer what his/her disease problems cost in order for the farmer to weigh that loss against whatever investments are required in veterinary, other advisory time and/or equipment.

Herd data and routines evaluated in FRISKKO udder

Herd data and routines are evaluated in FRISKKO Udder by:

A. systematic scoring of biological markers such as body fat, cleanness, and teat condition,
B. computer tools that stratify the herd according to lactation number and stage of lactation in order to analyze new infections rates and udder pathogens in different strata of the herd,
C. dynamic testing of the milking machine at teat level during milking, and
D. evaluation of milking technique and milking routines. Please also see fact sheet FRISKKO Udder - at a glance.

A. Evaluation of biological markers

Evaluation of biological markers is a very important part of FRISKKO. By systematically scoring and analysing cleanness of cows, body condition, indicators of lameness and condition of teat skin and teat ends the veterinarian and the farmer get measurements that can be used in the analyses and that can be compared over time to indicate progress or deterioration in the herd.

Cleanness

In FRISKKO cleanness of cows is scored on an ordinate scale of 4 steps, where 1 means very clean (spotless), 2 acceptably dirty - the little dirt there is on the cow may (imaginably) easily be brushed off, 3 unacceptably dirty, the cow is rather dirty and getting her clean requires somewhat more work and 4 dirty bordering on being an animal welfare issue. Current Swedish acceptance level: 80 % or more of the cows should fall in categories 1 and 2.

Body condition

Scoring body condition (BCS) is a universally accepted tool to evaluate feeding regimes in dairy herds. It can be used as a one-time measurement as a screening test and as repeated measurements to follow a herd or group of cows within a herd over time. FRISKKO uses the method of Edmonson *et al.*, (1989) and Brand *et al*. (1996), for Holsteins and a BCS modified by Gillund *et al*. (1999) for Scandinavian red cows.

Stable design and animal behaviour - "Cow-Comfort"

Within FRISKKO an attempt to evaluate the function of the barn and the comfort it provides cows is made by the herd veterinarians. This is done by studying the behaviour of the cows as the stand, walk, try to lay down etc (Anderson, 2003; Gaworski *et al.*, 2003; House *et al.*, 2003). A complete account of everything that goes into an evaluation of cow comfort is not possible within this text but as examples the proportion of cows standing or perching in the cubicles, the position of the cows as they lie in the cubicles, the amount of head room available when trying to get up etc are points of interest. FRISKKO uses repeatable animal based observations to evaluate overall stable function. For the loose barn a Stall Standing Index is calculated. Two hours prior to milking of maximum 15 % cows standing is accepted. The animals rising behaviour and expression of fear are other parameters that are systematically used. In addition to animal observations FRISKKO proposes biological optimums rather than minimums for a number of critical stable dimensions.

Lameness

Lameness is a serious problem in dairy production, in some countries equaling or even surpassing mastitis (Overton *et al.*, 2003). In our experience lameness is easiest evaluated by looking at the gait and the arching of the cow´s back as she stands and/or walks. The stance and gait of as many cows as possible is rated with a locomotion score from 1-5 (Sprecher at al., 1997). Cows that both stand and walk with an arched back are considered lame. The proportion of lame cows should not exceed 5%.

Teat Scoring

Scoring of teat skin and teat end condition is an important part of the evaluation of the interaction between the biology of the cow and the technicalities of milking machines and milkers. Chaps and cracks in the teat skin may indicate exposure to wind and cold or an excess use of water or irritating teat dips (Timms, 2004). Hyperceratosis at the teat end may indicate over-milking and/or vacuum levels that fluctuate or are too high (Neijenhuis, 2004). More short lived symptoms of circulatory challenges to the teat such as blue or red discoloration should be evaluated at take off of the milking unit. Scoring of teat skin and teat end condition and circulatory changes are done in accordance with the suggested 4 step ordinal scale of the Teat Club International (TCI) where 3 or above indicates excessive changes (NMC Short course, 2004 and 2005).

B. The computer tools

The computer tools - soft ware - that is currently used in FRISKKO is called IndividJuver (IndividualUdder) and provides the veterinarian and dairy farmer with a graph of the herd´s production and estimated bulk milk somatic cell count (BMSCC). BMSCC is a herd SCC calculated from the SCC of each cow and her milk production on the monthly test days. IndividualUdder also illustrates the milk production and cow SCC (CSCC) and various treatments as a graph for each cow. This provides an easy over-view of the CSCC variation, milk production and treatments done to each cow. The herd veterinarian can add information about identified pathogens and other relevant observations on teat, udder or cow. An Excel-file with valuable options to graphically illustrate the above mentioned cow related variables and identified udder pathogens from both clinical and subclinical cases of mastitis are

additions to the toolbox of IndividualUdder that further increases the veterinarians possibility to analyse the udder health problems of the herd.

C. Milking techniques and milking routines

Milking techniques and milking routines and is a powerful didactic tool to show milkers where they can improve their milking technique and routines. Working with and evaluating the readings of the VADIM requires the expertise of trained personnel co-operating with the herd veterinarian.

D. Dynamic testing of the milking machine

The milking machine should be tested in two steps. The first is to check that the machine functions and settings are in accordance with ISO standards and manufacturers specification under non-milking conditions. The second test is the dynamic test of the machine during milking. In Sweden this is done with a Norwegian device called the VADIM (Ronningen, 2002). The VADIM is a small, tubelike vacuum registration device, with a memory capacity of up to two hours, that is attached to the teat cup. It measures the vacuum in the mouth piece chamber (MPC) and at the base of the teat cup as close as possible to the teat end. The objective of the VADIM is to be able to see the finer details of milking such as vacuum fluctuations, air admissions, machine on time etc. Apart from being an excellent tool to analyse the function of the milking machine during milking the measurements of the VADIM give very valuable information about the interaction between milker, machine and cow. Thus objective values are obtained for preparation time, milk flow and over-milking as a result of the milking routine.

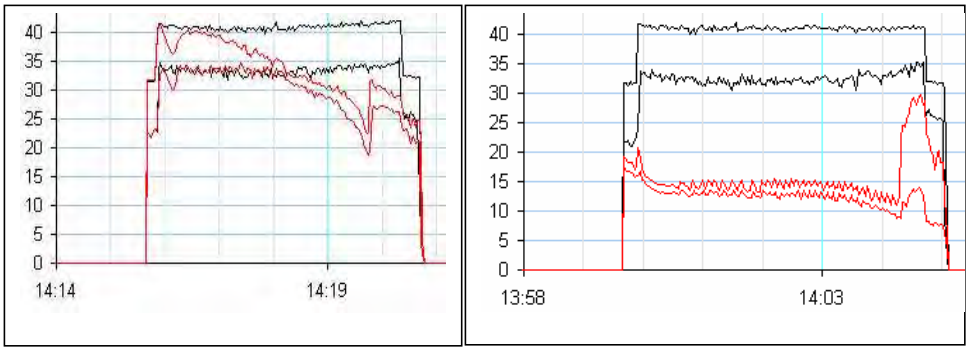

Figure 1. The VADIM-logger measures teat end and mouth piece chamber (MPC) vacuum 100 times per second. The sloping lines on the left and the bottom lines on the right are MPC vacuum registrations.

General advice concerning prevention of mastitis in Sweden:

1. Good housing with clean and dry stalls and sufficient clean and dry bedding.
2. Keep animals clean and well fed and managed.
3. Keep good records of the udder health of dairy cows by being part of the cow control system.
4. Use those records to establish a milking order with healthy cows being milked first and infected cows last.

5. Milk the cows with a good and clean technique and with the same friendly routine by all milkers.
6. Keep the milking equipment in good condition.
7. Use post milking teat dipping if the herd has elevated cell counts.
8. Treat acute clinical mastitis in young or previously untreated cows promptly.
9. Use dry cow therapy selectively.
10. Cull chronically infected cows or cows with recurrent mastitis - clinical or sub-clinical.
11. Breed for increased resistance to mastitis.

FRISKKO udder - at a glance!

- **Collection of data** — usually takes 2-3 visits to the herd
  - Herd check: — Check list FRISKKO Basics; Herd star: Animal health; Analysis of disease costs
  - Important biological markers: — Body condition; Cleanness; Behaviour; Function and design of barn
  - Milking study: — Milking routines; Milking technique; VADIM - dynamic study; Teat score - skin and teat ends; Video recording of milking
  - Tools of analysis: — Mastitis profile - clinical and sub-; New infections - which and when; Follow up check list; Risk factors for mastitis; Udder disease costs in herd
- **Analysis of gathered data**
  The data above are analysed and a *herd diagnosis* made.
- **Setting goals and giving advice**
  Three well-defined advices should be given to improve the situation in the coming six months. An attempt should be made to get the full co-operation of the farmer to take part in the analysis and suggest changes to be made.
- **Follow-up**
  - Book a date for: — FRISKKO Udder Follow-Up; New herd check of vital data and functions; Re-activation of the wheel of process: Analysis-Strategy-Advice-Follow-up

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Monitoring daily measurements of somatic cell count: Automatic and on-line detection of mastitis

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Abstract

Somatic cell count (SCC) is an indicator of mastitis as high values of SCC indicate low udder health status. In this presentation daily measurements of ln-transformed somatic cell count (somatic cell score (SCS)), which are subject to noise and outliers, are modelled with a multiprocess class II mixture model, namely the local linear growth model. The extended Kalman filter is applied to give on-line probabilities of different states of the model, which are associated with different kinds of changes in the pattern of the dynamic development of SCS. High probabilities of biologically relevant changes in the dynamic development of SCS trigger an alarm for mastitis. The data consist of daily records of SCS on cow-level and are from Holsteins, Danish Reds and Jerseys in first, second and third lactation. Model parameters were estimated based on data from half of the Holsteins in first lactation. Next the extended multiprocess Kalman filter was applied on the same data to provide on-line probabilities of the different types of changes. The robustness of the parameters was investigated by applying the multiprocess Kalman filter on datasets consisting of Holsteins, Danish Red and Jerseys in different lactations. In conclusion, the method seems promising for providing on-line probabilities of mastitis - based on indicators on-line available.

Keywords: multiprocess class II mixture model, Kalman filter, somatic cell score, detection model

Introduction

Fast, automatic and reliable detection of mastitis in individual cows is a challenge to producers of milking equipment and managements systems. One aspect is the development of cheap efficient censors. Another aspect is the development of software with algorithms which are efficient in monitoring cow udder health, and especially in early detection of mastitis. This paper deals with the algorithmic aspect of mastitis detection based on a well-established inflammatory marker of the udder health of individual cows, namely SCS in milk (Dohoo and Lesley, 1991).

For a mastitis infected cow, SCS will first increase and then decrease in the case of a successful treatment or in the case of selfcure. The level of SCS after infection is usually higher than the level before infection and furthermore measurements of SCS are subject to noise and outliers. Smith and West (1983) successfully modelled timeseries of an indicator

of kidney functioning (with similar characteristics) with the local linear growth model with outliers, in patients who had recently received transplants. With the extended Kalman filter they provided on-line probabilities of serious changes in the indicator. In this paper the local linear growth model with outliers is used for modelling timeseries of SCS, and the extended Kalman filter is applied to provide on-line probabilities of changes in the pattern of SCS. High probabilities of changes associated with mastitis will be used for on-line detection of mastitis. The performance of the method is quantified in terms of sensitivity and specificity.

Material and methods

Data

Data are from an experiment carried out from 1996 to 2001 at the research farm Ammitsbøl Skovgaard, Denmark, having 110 cows per year distributed on Holstein, Red Dane and Jersey cows in the first three parities. (For detailed information about the experiment see Nielsen *et al.*, 2003). Cows were milked twice daily and milk samples assayed for content and SCC. In this study we used daily measurements of SCS in morning milkings onwards from 14 days after calving (dfc).

Udder health status was obtained through disease registrations and the udder health surveillance scheme in which quarter foremilk samples were taken every 8th week starting at the first week after calving and ending at drying off. These days are called mastitis-testdays. Samples obtained on mastitis-testdays were analysed to obtain bacteriological diagnosis of possible intramammary infections. Udder health status of a cow was defined to be 'clinical mastitis' on days where the cow was treated against mastitis, and 'healthy' on mastitis-testdays where the cow had negative bacteriological quarter foremilk samples and no veterinarian treatment against mastitis. All other days had undefined status with regard to this definition of health status. Among the days with defined health status we focus on days with non-missing values of SCS three days before and three days after. This leaves us with 120 'clinical mastitis' days and 486 'healthy' days.

Model and method

Model

We used the local linear growth model with outliers and with fixed selection probabilities (e.g. Smith and West, 1983) as a model for daily measurements of SCS. The model deals with timeseries and is briefly described in the following: For the timeseries $\{y_t\}_{t=1,\ldots n}$ consisting of n observations of SCS (of a given animal in a given lactation), it is assumed the observation at each time, t, is the outcome of one out of four different models, $M_t(j)$, j=1,2,3,4. Model 1 (j=1) is the steady state model (normal evolution), Model 2 (j=2), is the model for change in level, Model 3 (j=3) is the model for change in slope and Model 4 (j=4) is the model for an outlier. The local linear growth model used in this paper, is conditional on $M_t(j)$ and θ_t, given by observation equation:

$$Y_t = F\theta_t + \upsilon_t$$

with system matrix $F = (0,1)$; θ_t is a two dimensional state vector, consisting of a level parameter, μ_t and a slope parameter β_t, $\theta_t = (\mu_t, \beta_t)'$. The observation error, υ_t, is conditional on the model at time t, $M_t(j)$, and θ_t, normally distributed, $\upsilon_t \mid (M_t(j), \theta_t) \sim N(0, V(j))$. The dynamic development in the state vector is described by the system equation(s), which conditional on the model at time t, $M_t(j)$, and the state vector at time t-1, θ_{t-1}, is given by

$$\mu_t = \mu_{t-1} + \beta_t + \varepsilon_{\mu t}$$
$$\beta_t = \beta_{t-1} + \varepsilon_{\beta t}$$

with $\varepsilon_{\mu t} \mid (M_t(j), \theta_{t-1}) \sim N(0, \omega_\mu(j))$ and $\varepsilon_{\beta t} \mid (M_t(j), \theta_{t-1}) \sim N(0, \omega_\beta(j))$, $j = 1,2,3,4$ for $t = 1,\ldots n$. Furthermore all of the observation errors, υ_t's, system errors $\varepsilon_{\mu t}$'s and $\varepsilon_{\mu t}$'s are assumed to be mutually independent. Note, that the system equation(s), conditional on $M_t(j)$, and θ_{t-1} can be rewritten as $\theta_t = G\theta_{t-1} + \omega_t$ with system matrix

$$G = \begin{pmatrix} 1 & 1 \\ 0 & 1 \end{pmatrix} \text{ and } \omega_t \mid (M_t(j), \theta_{t-1}) \sim N_2(0, W(j)), \text{ where } W(j) = \begin{pmatrix} \omega_\mu(j) + \omega_\beta(j) & \omega_\beta(j) \\ \omega_\beta(j) & \omega_\beta(j) \end{pmatrix}.$$

A priori θ_0 is assumed to be normally distributed, $\theta_0 \sim N(m_0, C_0)$, $m_0 = (m_0(1), m_0(2))$ and $C_0 = diag(C_0(1,1), C_0(2,2))$. Finally the prir probabilities of being in state j at time t is $P(M_t(j)|E) = P(M_t(j)) = p_0(j)$ independent of time t, and independent of any event E defined in terms of the history of the system prior to time t. In Model 1 $\omega_\mu(1) = \omega_\beta(1) = 0$, therefore the state vector at time t is completely determined by the deterministic relationship $\theta_t = G\theta_{t-1}$. In Model 2 a level change can be generated because $\omega_\mu(2) \neq 0$ (and $\omega_\beta(2) = 0$) and in Model 3 a slope change can be generated because $\omega_\mu(3) \neq 0$ (and $\omega_\mu(3) = 0$). Model 4 is similar to the model for normal evolution, except for the variance of the observation error being larger, $V(4) > V(1)$. The variance of the observation error in Model 1, 2 and 3 are assumed to be equal, $V(1) = V(2) = V(3)$. The vector of parameters
$\psi = (m_0(1), m_0(2), C_0(1,1), C_0(2,2), p_0(1), p_0(2), p_0(3), V(1), V(4), \omega_\beta(2), \omega_\beta(3))$ was estimated based on an approximate likelihood function and half of the data on Holsteins in first lactation.

Extended Kalman filter

Based on estimated parameters, and data for a given animal up to and including time t, D_t the extended multiprocess Kalman filter (Harrison and Stevens, 1976) provides e.g. posterior probabilities of y_t being an observation from each of the four models in the mixture, $P(M_t(j)|D_t)$, one (two) step backsmoothed probabilities of $y_{t-1}(y_{t-2})$ being an observation from each of the four models in the mixture $P(M_{t-1}(j)|D_t)$ $(P(M_{t-2}(j)|D_t))$, $j = 1,2,3,4$. Posterior mean estimates of the state vector, $E(\mu_t \mid D_t)$ and $E(\beta_t \mid D_t)$ can also be obtained from the Kalman filter.

Validation of the method

In this study, the sum of the probabilities of level change and of slope change in SCS was taken as a warning of mastitis. Three different alarms were defined based on posterior, one and two step backsmoothed probabilities: On day t an alarm was triggered if $\alpha_0(t) = P(M_t(2)|D_t) + P(M_t(3)|D_t) \geq \tau_0$. Second, on day t, an alarm was triggered for day t-1 if $\alpha_{+1}(t\text{-}1) = P(M_{t-1}(2)|D_t) + P(M_{t-1}(3)|D_t) \geq \tau_1$ and thirdly, on day t an alarm was triggered for day t-2 if $\alpha_{+2}(t\text{-}2) = P(M_{t-2}(2)|D_t) + P(M_{t-2}(3)|D_t) \geq \tau_2$, where τ_0, τ_1 and τ_2 are fixed

threshold values. It is expected that alarms triggered based on one and two step backsmoothed probabilities are more reliable (because more information is available) compared with alarms triggered based on posterior probabilities, of course, at the expense of being available with a delay of one and two days respectively.

The performance of the extended Kalman filter is quantified in terms of sensitivity and specificity: An alarm triggered (a missing alarm) within the detection window ±3 days of a 'clinical mastitis' day is considered as a true positive (false negative). An alarm triggered (a missing alarm) within the detection window ±3 days of a 'healthy' day is considered as a false positive (true negative).

Results, discussion and conclusion

For eight days with clinical mastitis, the actual date of treatment against mastitis together with the days triggering an alarm based on posterior, one and two step backsmoothed probabilities are given in Table 1 (with $\tau_0=\tau_1=\tau_2=0.2$). It is observed that days triggering an alarm based on one step backsmoothed probabilities are confirmed based on two step backsmoothed probabilities. The corresponding graphs for three of the days with clinical mastitis are shown in Figure 1. Note, cow no 813 was treated against mastitis 226 dfc, however, this case was not detected since there is hardly any increase in SCS in the period around treatment (Table 1, Figure 1). Cow no 467 (Table 1, Figure 1) was treated against mastitis 234 dfc without triggering any alarm within the detection window ±3 days from treatment against mastitis. For this cow an alarm was triggered on day 230 based on a_0 values, and on day 229 and 230 based on a_{+1} and a_{+2} values, i.e. net four days before treatment a critical change in SCS was detected. Note, sensitivity will increase and specificity will decrease by enlarging the detection window.

In Figure 2, sensitivity and specificity based on , and values are shown as functions of the threshold value. Sensitivity based on and values are almost identical and higher than sensitivity based on values, for any value of the threshold. Specificity based on , and values are almost identical. By definition sensitivity will decrease and specificity will increase by increasing the threshold values. The decision on threshold value will involve economic and ethical considerations.

Table 1. Days with defined health status='clinical mastitis' together with days triggering an alarm (within the detection window ±3 days) based on a_0, a_{+1} and a_{+2} values.

Cow no.	Day in lactation	a_0	a_{+1}	a_{+2}
357	95		94	94
360	122	122	121,122	121, 122
454	195	195	194, 195	194, 195
454	229		227, 231	227, 231
456	241	241	240, 241	240, 241
467	234			
813	179		178, 179	178, 179
813	226			

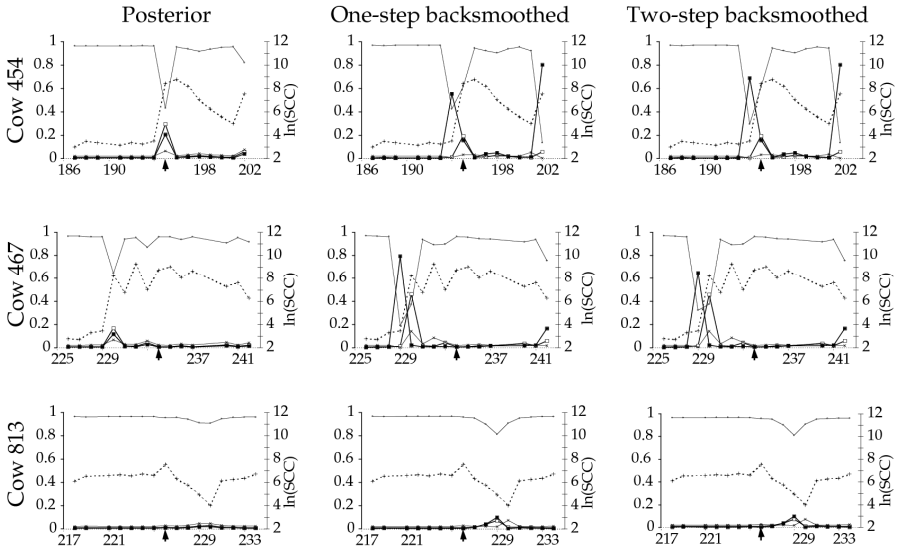

Figure 1. The development in ln(SCC) around days with defined health status equal to 'clinical mastitis' for 3 cows. For each case three graphs are presented. In column no 1, 2 and 3 ln(SCC) together with posterior, one and two step backsmoothed probabilities of the four different models are shown. +++ ln(SCC), · · · model for normal evolution, □ model for change in level, ■ model for change in slope and xxx model for outlier.

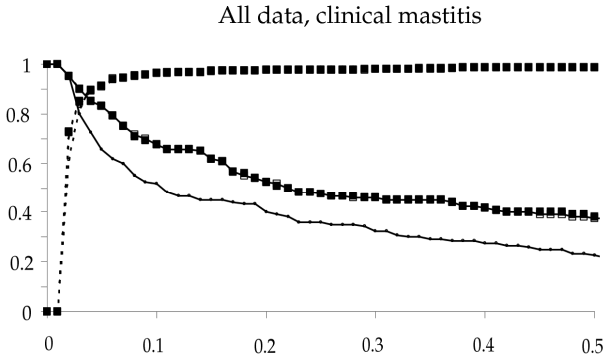

Figure 2: Sensitivity and specificity for detection of clinical mastitis. Full (dashed) line with · · ·, □ and ■ is sensitivity (specificity) based on a_0, a_{+1} and a_{+2} values, respectively.

In conclusion, we have demonstrated, based on regular measurements of SCS, that the method presented by Smith and West (1983) is suitable for detection of mastitis, if measurements of SCS were on-line available. However, measurements of SCS are expensive and the delay caused by measurements not being on-line available should be added to the days triggering an alarm. For implementation on dairy farms, therefore the cost of SCS should be lowered and SCS should be on-line available. An alternative would be to search for other indicator traits of mastitis.

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A biological model for detecting individual cow mastitis risk based on lactate dehydrogenase

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Introduction

Setbacks associated with detection of mastitis by the use of traditional indicators have led several workers to propose the use of enzymes like N-acetyl-β-D-glucosaminase (NAGase), and lactate dehydrogenase (LDH) (e.g. Pyörälä and Pyörälä, 1997). Enzymes are released as a result of the animal's immune response against infection and changes in cellular membrane chemistry. However, the use of enzymes to detect mastitis has so far not been applicable as a cow-side method. Recent advances in biosensor technology together with the advent of in-line automated milk sampling and processing systems have made on-farm automated real-time analysis of milk constituents a realistic proposition. However, a biological model is required to extract useful information from a time series measures of the indicator to detect disease. Other models for detecting mastitis have previously been developed, for example the models based on electrical conductivity, milk yield, and milk temperature (de Mol and Ouweltjes, 2001). However, a time-series model for detecting mastitis based on milk enzymes together with some biological factors of direct physiological relevance to the cow's health status throughout the lactation is currently lacking. The objective of the current study was to develop a biological model for early detection of mastitis for individual cows in a dairy herd based on real time measurements of an enzyme (in this case LDH) and milk yield.

Model description

The main input to the model is LDH although in principle the model could apply to any mastitis indicator measured in milk. LDH activity is increased during to mastitis. Data from several experiments indicate the potential of LDH activity to detect mastitis in different species (Bogin *et al.*, 1977, Batavani *et al.*, 2003; Sommer *et al.*, 1986). High precision, accuracy, and easy handling have made use for analysing LDH in milk even more recommendable (Lipperheide *et al.*, 1995). The model is dynamic and deterministic, designed to run each time a new trigger input occurs. A product of LDH activity and milk yield (LDH amount) are used to generate an Indicator Based Risk (IBR). The LDH amount values are first smoothed using an extended Kalman filter (Korsgaard and Løvendahl, 2002) before being processed in the biological component of the model. Other animal and herd related factors with direct physiological relevance to the cow's health status generate an Additional Risk Factor (ARF). The Additional Risk Factors included in the model are, milk yield acceleration, milking duration, udder characteristics, herd mastitis level, current lactation disease history, and quarter level electric conductivity. Together, IBR and ARF are used to generate an overall risk of mastitis. The structural separation between IBR and ARF reflects

the underlying logic that additional risk factors are only those factors whose effects are not acting on LDH activity. The outputs of the model are; an overall risk of acute mastitis (*OutRisk*), the relative degree of chronic mastitis (*ChronDeg*) and a calculation of when to take the next sample. Days to next sample (*DNS*) is designed to make best use of the opportunities afforded by automated, real-time, in-line sampling technology. It is designed to feedback to the sampling system so that the frequency of milk sampling (i.e. next analysis of LDH for a particular cow) can be varied according to the calculated risk of mastitis. In this scenario, it is desirable for sampling frequency to be increased, for a given cow, when the risk of mastitis is high, and visa versa. Consequently, in the model, an increased risk of (acute) mastitis causes the days to next sample to be reduced from a default value. Apart from the inputs, outputs, and the equations, which go with them, the model has a set of default and constant values for the parameters. The schematic representation of the biological model is presented in Figure 1.

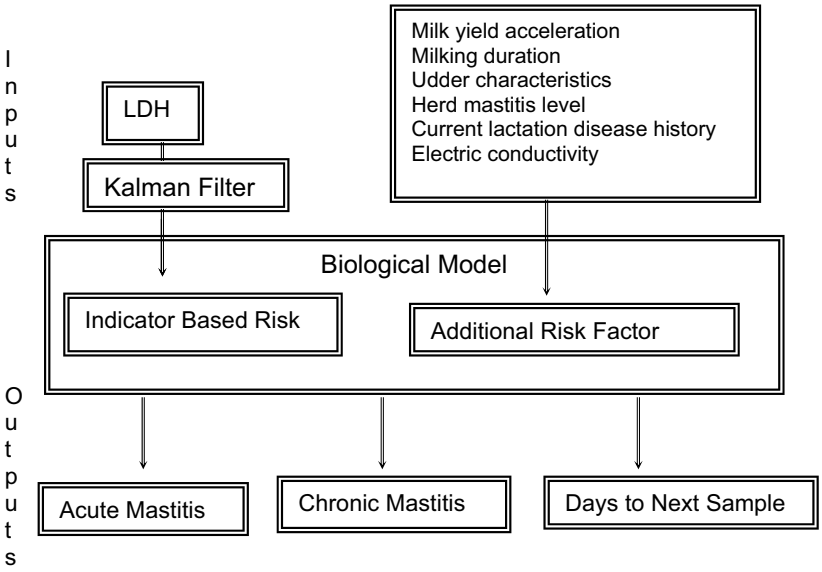

Figure 1. Schematic representation of the biological model for detecting individual cow mastitis.

Model evaluation

Testing of the model functionality was done in two steps. The first step was to test the model logic and robustness. This was performed using simulated data (Figure2). Testing the model logic concerned the running of the model through a dataset with defined characteristics, which included two mastitis episodes. In an investigation aimed at examining the robustness of the model to the accuracy of the indicator measurements random 'noise' was added to the original LDH values. Random values scaled to be in the range ± 1, 2, and 3 residual standard deviations (SD) were used. The residual standard deviation was calculated as the standard deviation of the difference between raw LDH amounts and *Level*. For each level of noise, the model was run 10 times and the average performance was calculated in terms of the model identifying the defined mastitis

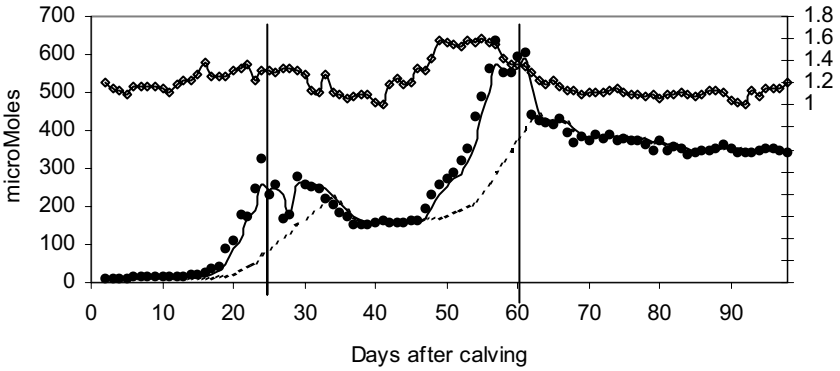

Figure 2. Illustration of an acute mastitis case with two episodes of increasing LDH amount generated from simulated dataset. LDH amount (dots) represents raw values of LDH while Level (solid line) are the smoothed values of LDH calculated using an extended Kalman filter and Stable (broken line) is the average value of the smoothed LDH level (Level) for each cow calculated over a time interval of 7 days. The vertical lines indicate the two incidences of mastitis that were simulated in the dataset on days 25 and 60 after calving. The solid line with diamonds indicates the conductivity values calculated as inter quarter ratio.

incidences. An arbitrary threshold value of 0.7 for *OutRisk* was used as an indicator for the onset of high mastitis risk period. The second step was model validation using real data of naturally occurring mastitis cases from a research herd.

In the model evaluation using real data from naturally occurring mastitis, a dataset from a research farm at the Danish Cattle Research Centre, Foulum, Denmark was used. In the study a healthy cow was defined as a cow with low SCC (less than 200 000 cells/ml) and no veterinary treatment (Dohoo and Leslie, 1991). A clinically infected cow was defined as a cow that received veterinary treatment after showing clinical symptoms of mastitis and high SCC (more than 800 000cells/ml). A subclinically infected cow was defined as a cow with SCC of 500 000 but without veterinary mastitis treatment. All medical treatments were done by a veterinarian. Records from 100 cows, of which fifty were healthy, were used. The cows were from three breeds of Danish Holstein, Danish Red and Jersey and provided a total of 25680 test-day records from September 2003 to February 2005. Three main criteria for testing the mastitis model were applied and these are: how early does the model detect mastitis, the proportion of clinical mastitis detected, and the proportion of mastitis alarms generated by the model that were false. Again, a threshold of 0.7 for the *OutRisk* was used. This culminated into the calculation of model sensitivity and specificity.

Results

Test based on simulated data

The model responds in a biologically logical way. As shown in Figure 3, risk for mastitis increased with increasing LDH and decreased with decreasing and low LDH values. Number of days to next sampling reduced when the risk of mastitis increased and increased with reducing risk. Using the days to next sample function, the model identified the same mastitis cases as the full data set but using only 68% of the number of 'samples'.

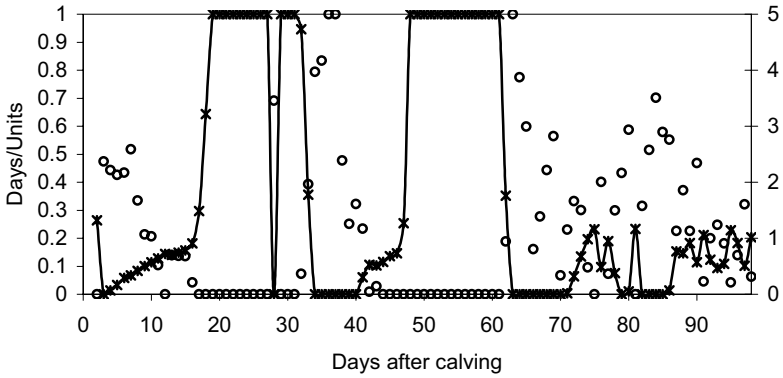

Figure 3. Results after running the biological model based on simulated dataset as illustrated in Figure 1 above. OutRisk (solid line with asterisk plotted on the primary y-axis) indicates the risk of the cow developing mastitis with risk ranging from zero to one. Days to Next Sample (open circles plotted on the secondary y-axis) is the internal feedback of the model on when the next sample of LDH should be drawn. Days to Next Sample drops with increasing OutRisk and increase in conductivity values (as is the case on day 36 after calving.

The consequences of adding random variation (noise) to the LDH values are presented in Table 1.

As the level of noise increased from 1SD to 3SD the model indicated extra mastitis risk periods. With 1SD level of noise, the model indicated an average of 1.1 extra risk periods. The extra mastitis risk periods increased to an average of 4.7 and 8.8 with noise levels of 2SD and 3SD, respectively. However, the model did not miss any of the two 'true' mastitis incidences with noise levels of 1SD and 2SD. When 3SD noise level as added to the LDH amount, the model missed on average 0.1 of the 'true' mastitis cases. The deviation in days to the onset of high mastitis risk period ($OutRisk \geq 0.7$) before a defined mastitis case relative to the base, 0SD, case increased from 0.29 days to 0.36 days when the noise level increased from 1SD to 2SD but the deviation decreased to -0.79 days when noise level was 3SD. With increasing noise in the LDH values there was a relatively small delay in detection of mastitis up to 2SD added noise. At the highest level of noise addition, 3SD, there was significant overlap between the 'true' and noise generated, false, high risks such that the delays in detection of true mastitis cases was no longer reliably measurable.

Table 1. Consequences of adding random variation (noise) to the raw LDH amount on the performance of the biological model.

Criteria of evaluation	Range of random variation (no. of SD)		
	1	2	3
Extra risk periods detected (OutRisk (>0.7)	1.1	4.7	8.8
Number of Mastitis cases missed	0	0	0.1
Deviation* in days to onset of high risk before a defined mastitis case	0.29	0.36	-0.79

*Relative to the original dataset with no added noise

Test based on naturally occurring real mastitis cases

The results indicate that the model is able to detect mastitis on average 4.56 (SD = 4.11) days earlier with respect to the recorded diagnoses. The results also indicated the ability of the model to detect subclinical mastitis (sensitivity) of 71.8% and 76.5% ability to detect clinical mastitis. Further, results showed the ability of the model to avoid misclassifying "healthy" cows as mastitic (specificity) of 97.7%.

Conclusion

A model for early detection of individual cow mastitis based on milk LDH measurements and incorporating known biological effectors of mastitis susceptibility has been described. The results of runs with simulated data suggest that the model has the potential to provide the basis for a useful decision support tool for mastitis management. This conclusion is heavily supported by analysis of real time data where sensitivity was more than 70% and specificity was more than 95%.

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Risk of clinical mastitis within lactation in Dutch dairy cattle

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Abstract

The number of clinical mastitis cases dairy cows may experience over a lifetime has been a subject of several studies. Some of them analysed repeated events by different types of statistical models and used different approaches such as retaining only cases after certain time or considering only the first case within lactation. No studies included the two types of heterogeneity that is often observed in field. One derived from lack of independence of individuals from the same herd and the second, from intra-subject correlation of repeat events. Our objectives were to estimate the risk of clinical mastitis within-lactation for Dutch dairy cattle accounting for conditional dependence between cases and to assess risk factors associated with a changing risk. Data were obtained from farmers' records of 248 farms participating in a voluntary programme. Data included all lactations present during 01/1999 to 04/ 2004 (n=33,105) of 22,603 cows. Data were analysed with a survival analysis conditional model for repeated events using a sojourn time-scale between cases corresponding to time since last event and a frailty term. Higher hazard rates are positively associated with parity number, length of lactation, 305-days milk production, total number of clinical cases in the herd, year of calving and the interaction herd size-305-days milk production. Herd size and the rest of the interactions were negatively associated. However, although number of days open and the lactation value should be incorporated, their effect is not significant. Our estimations serve as guidelines to farmers for improving udder health. In addition, our results and the methodology employed are useful for exploring other aspects of associations between genetic resistance of cattle and occurrence of clinical mastitis.

Keywords: recurrence clinical cases, risk factors

Introduction

Mastitis is still the most frequent and costly health disorder in dairy cattle. Dairy cows may experience any number of clinical mastitis cases within or across lactations.

In the Netherlands, on average 20% of all the cows are experiencing at least one case of clinical mastitis per lactation (Schukken *et al.* (1997). In addition, the range of percentage of cows having at least one case of clinical mastitis shows a large variation between herds.

Models for clinical mastitis occurrence had been used for assisting farmers in herd management but also to help breeders in taking selection decisions.

The association between clinical mastitis in dairy cows and several risk factors has been a subject of several studies (Barkema *et al.*, 1999; Waage *et al.*, 1998). Some of them analysed repeated events by different types of statistical models (Morse *et al.*, 1987) and

used different approaches such as retaining only cases after certain time (Barkema *et al.*, 1998; Elbers *et al.*, 1998), or considered only the first case within lactation (Waage *et al.*, 1998). In fact this is more likely to be an attempt to elude the problem of possible dependence than the opposite. No studies included the analysis the two types of heterogeneity that is often observed in field. One source of heterogeneity is the lack of independence of cows from the same herd (Andersen *et al.*, 1997; O'Quigley and Stare, 2002) and we refer this as heterogeneity and the second, is the intra-cow correlation of repeat events (Therneau and Grambsch, 2000) and we refer this as event dependence.

Our objectives were to estimate the risk of clinical mastitis within-lactation for Dutch dairy cattle accounting for conditional dependence between cases and to assess risk factors associated with a changing risk.

Material and methods

Data were obtained from farmers' records of 244 farms participating in a voluntary program. There were 22,603 cows present and were included all lactations present during 01/1999 to 04/ 2004 (n=33,105).

In this project, disease data were collected by farmers using commercial management programs. Lactations that were extended beyond 700 days were truncated at this level.

In this study we have access to cases of mastitis that had been observed and recorded by farmers and diagnosed on the basis of clinical symptoms.

We defined clinical mastitis as any inflammatory response of the udder that shows visible signs from some flocks to abnormal milk, accompanied or not by other signs such as pain, reddish and swollen quarter, fever, decreased appetite. To identify new mastitis cases the following criteria were used: the lag time between two successive mastitis reports should be at least 8 days within the same quarter. If this lag time was less than 8 days but in another quarter or another pathogen was involved then this second case was deemed as a new case.

We evaluated a certain number of factors to explore their association and impact with recurrence. These factors included: month of calving, season of calving, year of calving, 305-day milk production, length of lactation (days in milk), lactation value, herd size, total number of cases in the herd, number of days open in the lactation where the case(s) occur. Age was evaluated via parity (from 1 to 10) or by a dichotomous variable (primiparous and multiparous cows).

Data were analysed with a survival analysis conditional model for repeated events using a sojourn time-scale between cases corresponding to time since last event. and a frailty term. The multiplicative hazard function for the *ith* cow for the repeated event model is:

$$\lambda_{i\,k}(t) = Y_{ij}(t)\,\lambda_{0j}(t)\exp(X_i(t)\,\beta j + \mu_i) \qquad (1)$$

where $\lambda_{i\,k}$ denotes a cow's risk for event *k,* Y_{ij} *(t)* indicates whether the *jth* event for the *ith* cow is at risk at time *t* when an event occurs (0= is at risk until the j-1 event; 1= otherwise). This risk is a function of λ_{0j}, an event specific baseline hazard, β is a column vector of coefficients related to the variable X_{ij}, which may be constant or time varying and μ_i, a herd specific random effect (as well as any covariates). The former introduces

event dependence, which itself may follow a variety of forms. The latter produces heterogeneity by contributing differently to each cow's risk.

The model selection process involved three steps. In the first step, all single predictors were screened. Only those that had a P-value of less than 0.25 in the univariable analysis were retained. Second, the model building strategy was based on Collet (1994) who suggested a mix of backward and forward procedures using a likelihood ratio test for assessing the goodness-of-the-fit of nested models (for $P \leq 0.05$). We included in the evaluation one-way interactions were tested between the main effects that remained in the model. In the third step, we evaluated the assumption of proportionality of the Cox model. First, assuming time-fixed covariates, the plot of the cumulative hazard against time was evaluated graphically (Collet, 1994). Second, assuming time-dependent coefficients, a chi-square test on the Pearson product-moment correlation between the scaled Schoenfeld residuals and time or some function of time (i.e. log t or rank of event times) for each covariate was performed (Therneau and Grambsch, 2000). The Efron method was used to handle ties.

Results

The average number of cases per lactation for primiparous cows was 1.33 ± 0.79 and for multiparous 1.51 ± 1.04. The distribution of the number of clinical cases per lactation for primiparous and multiparous cows is shown in Figure 1.

Table 1 shows the median times and the 95 % confidence intervals for the time since calving date and the occurrence of clinical mastitis.

After selection process, the final model (Table 2) contains 8 main effects and 4 interactions. Parameter estimated show that parity number, length of lactation, 305-days milk production, total number of clinical cases in the herd, year of calving and the interaction herd size-305-days milk production are positively associated with higher hazard rates. In addition, it was found a negative association with herd size and the rest of the interactions. However, although number of days open and the lactation value should be incorporated, their effect is not significant at P = 0.05. The estimated frailty variance was 0.39.

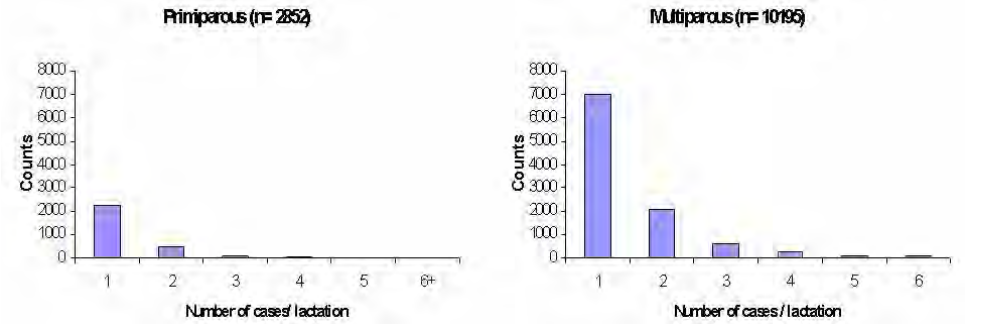

Figure 1. Distribution of the number of clinical cases per lactation for primiparous and multiparous cows.

Table 1. Median times (days) and the 95% confidence intervals of the days after calving and a given clinical case of mastitis, within lactation.

Case	n	Time and 95% CI
1	10373	71 (69; 74)
2	3176	115 (110; 118
3	1014	140 (133; 148)
4	450	158 (146; 170)
5 or more	449	174 (168; 184)

Table 2. Hazard ratios (95% Confidence intervals in parentheses) for Cox conditional frailty model.

Variable	Hazard ratio and 95% CI	P value
Main Effects		
Parity	1.247 (1.147; 1.356)	< 0.001
Days in Milk	1.003 (1.002; 1.003)	< 0.001
305-days Milk Production	1.001 (1.000; 1.002)	< 0.001
Days Open	1.001 (0.999; 1.003)	0.200
Lactation Value	0.996 (0.988; 1.004)	0.360
Total Number of cases in the herd	1.006 (1.005; 1.007)	< 0.001
Herd size	0.994 (0.992; 0.995)	< 0.001
Year of calving	1.034 (1.015; 1.053)	< 0.001
Interactions		
Days in milk* Days open	0.999 (0.999; 0.999)	< 0.001
305-days Milk Production Lactation Value*	0.999 (0.999; 0.999)	0.030
305-days Milk Production Parity*	0.999 (0.999; 0.999)	0.018
305-days Milk Production Herd size*	1.000 (1.000; 1.001)	< 0.001

Discussion and conclusions

We obtained population-average estimations of risk of repeated cases of clinical mastitis within-lactation considering some risk factors. A previous study (Morse *et al.*, 1987) reported higher probabilities of recurrence with increased parity number (31 to 42%) in comparison with our study (24.7%). Moreover, the study of Morse *et al.*, 1987 used other statistical method (least square regression) and they did only considered parity, breed and moth of calving as covariates. However, the risk factors that were associated with recurrence of clinical mastitis were in agreement with literature (Morse *et al.*, 1987; Waage *et al.*, 1998). Our estimations serve as guidelines to farmers for improving udder health in their herds. In addition, our results and the methodology employed are useful for exploring other aspects of associations between genetic resistance of cattle and occurrence of clinical mastitis.

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Environmental control

Feeding factors associated with clinical mastitis of first parity cows

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Abstract

Few studies have investigated associations between clinical mastitis and feeding. However, two Swedish observational studies included feeding factors among risk factors for veterinary treated clinical mastitis (VTCM). The first study comprised 158 high yielding herds with low somatic cell count (SCC) and a high (HI) or low (LO) incidence of VTCM. The farmers were interviewed about management factors and animals and feed storages were inspected. Health and milk production data was collected from the Swedish official milk- and animal disease recording systems. The material was analysed using logistic regression analyses, where herds were the study unit and the dependent variable was type of herd (HI or LO). In the second study, data from birth to first lactation on 2 126 heifers in 107 herds were analysed. The farmers were interviewed about management factors. This material was analysed using a multilevel logistic regression analysis with animals being the study unit. High SCC (≥200,000 at first test milking) and incidence of VTCM in the period 7 days before till 30 days after parturition were the dependent variables. The first study showed that feedings factors associated with VTCM of first parity cows were amounts of concentrate given in the period around parturition and feeding related diseases. In the second study, several feeding factors, e.g. amount of concentrate and type of roughage given, were associated with the outcomes on a univariable level. However, these associations were not significant in the final multivariable analysis.

Keywords: risk factors, first parity cows, feeding, management

Introduction

Many epidemiological studies have studied risk factors for clinical mastitis (CM), but most have focused on risk factors for cows as a group with the majority of animals being second parity cows or older (Schukken *et al.*, 1990; Elbers *et al.*, 1998; Barkema *et al.*, 1999; Peeler *et al.*, 2000). Few studies have looked solely at risk factors associated with CM of first parity cows, but there are some (Waage *et al.*, 1998; Waage *et al.*, 2001). However, a high prevalence of CM during the first lactation, especially in the first weeks after

parturition, has been reported from several studies. According to Valde *et al*. (2004) first parity cows have a higher incidence of veterinary treated clinical mastitis (VTCM) than second and third parity cows during the first month after parturition. Barkema *et al*. (1998) also found a higher incidence of CM in first parity cows than in older cows during the first weeks after parturition. CM in early lactation will have a severe negative effect on the future of the heifer, as first parity cows with mastitis in the period around parturition have an increased risk of additional cases of mastitis, culling and reduced milk yield (Waage *et al*., 2000). Thus, mastitis in first lactation heifers will have considerable economic importance for the dairy producer. The aim of the two studies reported in this manuscript was to identify important risk factors for CM for first parity cows. Potential risk factors in the areas of general health, treatment-, culling- and preventive strategies, housing, pasture, feed, feeding, and milking were studied, with emphasis on feeding-associated risk factors.

Material and methods

Study I

The selection of farms was based on the arithmetic mean values of three years production and udder health data obtained from the Swedish Dairy Association's cow database. There were three selection criteria: the herds should belong to the top third in yearly milk yield, the lowest fourth concerning bulk milk SCC, and the lowest or highest fifth concerning incidence of VTCM. The incidence of VTCM was calculated as number of veterinary treated cases of CM divided by cow-days at risk. The selection was done twice, once in 1997 and once in 1998. The first data selection was based on the arithmetic mean of the production and udder health data of the years 1994 to 1997 and the second of the years 1995 to 1997. Of the 360 farms that fulfilled the selection criteria's 158 farmers agreed to participate: 79 low incidence (LO) herds and 79 high incidence (HI) herds. The study was conducted during two one-year periods, 1997/98 and 1998/99. All herds were visited twice, with an interval of three to five months, the year they participated. During one of the visits, the farmer was questioned about management. Further, housing conditions, storage areas for feedstuff, and milking routines were observed and evaluated as well as the cleanliness and body condition of heifers close to calving. During the other visit, the function of the milking facilities was tested. At both visits, the hygiene of the silage was analysed, and samples of blood and bulk milk were collected. Univariable and multivariable logistic models were used to determine the association of recorded factors with the incidence of CM.

Study II

In 1997, all dairy farms in the county of Skaraborg in the south western part of Sweden, which were enrolled in the Swedish official milk- and animal disease recording systems and had 28-94 cows, were identified. All farms were sent a questionnaire about housing of the calves and replacement heifers and a request to participate in the study. Three hundred and fifty-five (73%) farmers responded and 136 (38%) expressed willingness to enroll in the study. The 122 farms which housed their young calves in single pens or in groups on litter, and their older calves and replacement cattle in group pens with slatted floors, deep-litter boxes or deep-bedded pack system boxes, were selected. All heifer calves born on these farms in 1998 were monitored by research staff from birth to first calving (or alternatively, the day of their removal from the study). The farmers were requested to record all cases of

disease in heifer calves and replacement heifers. They also measured the heart girth of the animals at different ages. Project veterinary surgeons visited the farms every second month to check the records, and upon the visits they also recorded the housing system used for each animal, made a brief physical examination of the calves, auscultated their lungs, and recorded prevalent diseases not detected by the farmers in the health records. In addition they interviewed the farmers about management. In total, records from 2 126 animals from 107 herds were used in the statistical analyses. The material was analysed looking at two outcomes. The two outcomes (both binary) was SCC at first test milking, categorised as <200,000 or >200,000 cells/ml, and mastitis as the presence or absence of VTCM including cases of teat-treads associated with mastitis between 7 days before and 30 days after calving. Associations between each of the two binary outcome variables and the health of the calves and their housing, feeding and management in the period around parturition were investigated using a two-level (calf; herd) variance-components logistic model.

Results

Study I

Feeding and/or feeding related factors associated with a high or low incidence of VTCM in the univariable models ($P \leq 0.10$) were amounts of concentrates given at calving and at peak milk yield, if the heifers had access to salt on pasture, and feeding related diseases (ketosis, laminitis, and udder-thigh dermatitis caused by udder edema). Factors significantly ($P<0.05$) associated with a high or low incidence of VTCM in the final multivariable model were breed, retained placenta, udder-thigh dermatitis caused by udder edema, and amount of concentrates given at calving (Table 1). To have heifers of the Swedish Red and White (SRB) breed compared to having heifers of the Swedish Holstein (SH) breed or mixed breeds, was associated with a low incidence of VTCM. Increasing incidence rates of retained placenta and having a higher proportion of heifers with udder-thigh dermatitis at calving was

Table 1. Final multivariable logistic regression model of risk factors associated with first parity cows being in a herd with high incidence of veterinary treated clinical mastitis (VTCM) compared with being in a herd with low incidence of VTCM (Data from 147 Swedish dairy herds were used).

Variable	Odds ratio	95% CI	P
Breed			
1: Swedish Red and White	1.00[1]		
2: Swedish Holstein	4.62	1.75 - 12.17	0.002
3: Mixed breed	2.91	1.15 - 7.31	0.023
Incidence rate of heifers with retained placenta			
1: 0	1.00[1]		
2: >0	4.58	1.69 - 12.44	0.003
Proportion of heifers with udder-thigh dermatitis at calving			
1: 0	1.00[1]		
2: >0	3.36	1.52 - 7.38	0.003
Amount of concentrates given at the day of calving			
1: <2.5kg	1.00[1]		
2: ≥2.5kg	3.36	1.42 - 7.95	0.006

[1]Reference category

associated with a high incidence of VTCM. To give a higher amount of concentrates at calving (≥2.5kg/day vs. <2.5kg/day) was also associated with a high incidence of VTCM.

Study II

Feeding and/or feeding related factors associated with SCC <200000 or ≥200000 at first test milking in the univariable models (P≤0.10) were amount of concentrates given to calves (<3 months of age), type and amount of roughage given at weaning, time when accustoming to concentrates starts before calving, and heart girth at calving. Factors significantly (P<0.05) associated with SCC <200000 or ≥200000 at first test milking in the final multivariable analysis were concentrate feeding, grazing routines, udder health in the herd and use of restraining methods at milking (Table 2). Increasing amounts of concentrate fed to 11-16 months-old heifers were associated with an increased risk of having ≥200,000 cells/ml as was housing first parity cows on pasture at the same day as calving compared to before calving. To use a stretcher, to tie-up a leg, or to use other methods (than those mentioned) as a way to make the first parity cow stand still at milking was also associated an increased risk of having ≥ 200,000 cells/ml compared to not using any restraining methods. A higher proportion of cows in the herd that some time during the year of participation in the study, had received udder disease score (UDS) 6-9 increased the risk of having ≥200,000 cells/ml at first test milking (UDS is a measure of the udder health of an individual cow, based upon three consecutive months of test milking results of the individual SCC (Funke, 1989), and expresses the probability that a cow has an infection in one or more udder quarters at a given test milking).

Feeding and/or feeding related factors associated with VTCM in first parity cows in the period of -7 - 30 days after calving in the univariable models (P≤0.10) were origin of

Table 2. Final multivariable logistic regression model of risk factors associated with first parity cows having a high SCC (≥200,000 cells/ml) at first test milking compared with first parity cows having a low SCC (<200,000 cells/ml) (Data from 2 068 first parity cow in 105 herds in southwest Sweden were used)

Variable	Odds ratio	95% CI	P
Calving at pasture			
1: Housed before calving	1.00[1]		
2: Housed the same day as calving	2.21	1.18-4.15	0.013
3: Housed after calving	0.91	0.71-1.17	0.48
Restraining methods			
1: None	1.00[1]		
2: Anti-kicking clamp	1.35	0.89-2.05	0.16
3: Other	2.41	1.06-5.51	0.037
4: Leg tied up	1.85	1.03-3.33	0.040
5: Stretcher	2.93	1.58-5.42	<0.001
6: Combinations	1.29	0.83-2.01	0.25
Daily amount of concentrates to heifers 11-16 months (kg)	1.18[2]	1.04-1.35	0.014
Proportion of cows with an UDS of 6-9 at least once during the year	1.35[2]	1.12-1.62	0.002

[1]Reference category

[2]Odds ratio is calculated for an increase corresponding to the interquartile range (0.9kg of concentrates and 16.7 % increase in proportion of cows with an UDS of 6-9, respectively)

colostrum (from mother - first parity cow or older, or from other cow than the mother), amount of concentrates given to calves (<3 months of age), amount of roughage given at weaning, proportion of grain in the total amount of concentrates as well as type of concentrates given to heifers 11-16 months of age, proportion of grain in the total amount of concentrates as well as type and amount of concentrates given to heifers 17-23 months of age, and proportion of grain in the total amount of concentrates as well as type of concentrates given to heifers >4 months pregnant. Factors significantly (<0.05) associated with VTCM in heifers and/or first parity cows in the period of -7 - 30 days after calving in the final multivariable model were mastitis incidence in the herd and reproductive diseases in the first month of lactation (Table 3). Milk yield was considered as a confounder and remained in the model as it influenced the variables in the model. An increasing incidence of mastitis in the herd increased the odds of VTCM in the period around parturition. The odds also increased if the first parity cow had a reproductive disease in the same period.

Table 3. Final multivariable logistic regression model of risk factors associated with fist parity cows being treated for clinical mastitis in the period -7 and 30 after calving (data from 1963 first parity cows in 102 herds in southwest Sweden were used).

Variable	Odds ratio	95% CI	P
Reproductive disease			
1: No	1.00[1]		
2: Yes	81.7	28.7-232.4	<0.0001
Mastitis incidence in the herd	1.72[2]	1.43-2.07	<0.001
Milk yield	1.00[3]		

[1]Reference Category

[2]Odds ratio is calculated for an increase corresponding to the interquartile range (an incidence of 13.2)

[3]Included in the model as confounder

Discussion

The results from both study I and II shows that several feeding variables were associated with VTCM and cow-SCC. However, only two feeding variables remained in the final multivariable models of risk factors for VTCM/high SCC. An increased amount of concentrate at calving increased the odds of being in a herd with a high incidence of VTCM (study I), while increasing amounts of concentrates to heifers 11-16 months of age increased the risk of having SCC ≥200,000 cells/ml (study II). In accordance with our findings, Fraser and Leaver (1988) showed that feeding with higher amounts of concentrates increased the incidence of CM. Other studies have also found associations between concentrate feeding and CM (Schukken *et al.*, 1991; Waage *et al.*, 1998; Barkema *et al.*, 1999). However, the results from these studies and studies I and II are somewhat contradictory as both increasing and decreasing amounts of concentrate have been associated with an increased risk of CM. Further studies are needed before final conclusions about the associations between concentrate feeding and CM can be made.

Of the other factors associated with CM and/or cow-SCC is noteworthy that reproductive diseases, such as retained placenta, were found to be associated with the risk of VTCM in the multivariable analyses of both studies I and II. According to study I, having had retained

placenta increased the odds of being in a herd with high incidence of VTCM nearly five fold. In study II reproductive diseases in the period around parturition were associated with increasing odds of VTCM with 81.7! A strong association between reproductive diseases and CM have also been shown in other studies (Gröhn *et al.*, 1988; Bendixen *et al.*, 1988; Correa *et al.*, 1990). If reproductive diseases in the period around parturition are prevented, the risk of CM would probably be reduced considerably.

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Isolation of *Streptococcus uberis* from different sites of the dairy cow

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Abstract

Streptococcus uberis is the leading cause of clinical mastitis in New Zealand but the primary source of the bacterium in a pasture-based system is unknown. Identifying the niches in the dairy cow's environment that are colonised by *S. uberis* will enable the identification of risk factors and development of effective management strategies that prevent mastitis. Identification of such niches has been hampered by a lack of simple methods to selectively isolate *S. uberis*, but using a selective media, the bacterium has been successfully isolated from heavily contaminated samples derived from faecal material and teat skin. Isolates confirmed to be *S. uberis* by 16-23S PCR were then strain-typed using Rep-PCR typing.

Over 16 months, a herd of approximately 100 cows were monitored for the presence of *S. uberis* from specific anatomical sites of the cow. On any single occasion, the bacteria could be isolated from faeces of an average of 5.6% of cows sampled but some cows (7) yielded the bacteria repeatedly whilst others (6) proved to be positive for the bacteria on only one occasion. The bacteria could be isolated only intermittently from teat skin but more frequent isolations were observed in the dry period, which coincided with the suspension of post-milking teat disinfection.

Cows with *S. uberis* mastitis were sampled anatomically for the presence of the same infective strain. This strain was not detected in faecal material but was detected on feet and on the skin of the infected teat. It is suggested that these body sites became contaminated following establishment of the intramammary infection. Identifying sites that become colonised during an infection event may aid elucidation of risk factors associated with development of mastitis.

Keywords: *Streptococcus uberis*, body sites, faeces, isolation, niches

Introduction

The bacterium *Streptococcus uberis* is a leading cause of mastitis in New Zealand and is common in countries with a predominantly pasture-based dairying system. Heifers are particularly prone to infection with *S. uberis* during the first few days after calving (Pankey *et al.*, 1996) and *S. uberis* is a common cause of mastitis around calving and drying off in older cows (Woolford *et al.*, 1998). During a 6-week study in spring, approximately 10% of calved cows were diagnosed with clinical mastitis (McDougall, 1999) with a higher prevalence observed in herds that treated a low proportion of the herd with prophylactic

dry cow antibiotic treatments. Reducing the incidence of *S. uberis* mastitis requires an improved understanding of the infection mechanisms and the processes by which *S. uberis* enters the mammary gland.

The bacterium has been isolated from a number of sites around the cow and her surroundings but the primary source of infective strains, in a pasture-based system, is still unknown. Isolation of *S. uberis* has been reported from the lips (Cullen, 1966), rumen (Cullen and Little, 1969), rectum and vulva (Bramley *et al*., 1979) and faeces (Bramley, 1982), which suggest that the bacterium can survive for some periods of time on body sites that are distinct from the mammary gland. Cullen (1966) proposed that the lips were a primary reservoir and that isolation in rectal swabs reflected occasional passage through the alimentary tract. Later studies confirmed the presence of *S. uberis* on the lips and in the rumen but fewer isolations were observed from the rectum (Cullen and Little, 1969). In the same study large numbers of *S. uberis* were found on soil collected from where cows congregated at pasture. Kruze and Bramley (1982) suggested that the digestive tract provides the primary reservoir or niche for the bacterium.

This study aimed to describe the presence of *S. uberis* on anatomical sites of cows maintained on pasture, and identify sites that repeatedly yielded the bacteria in appreciable numbers, which could indicate the existence of a resident population. At the same time, cows known to have an existing intramammary infection with *S. uberis* were examined more intensively to identify body sites from which the same, infective, strain of bacteria could be isolated.

Materials and methods

Between July 2003 and November 2004, a herd of approximately 100 cows were sampled at regular intervals to determine the presence or absence of *S. uberis* in the milk from individual quarters, on teat skin and in faecal material. Milk samples were collected aseptically from individual quarters on four occasions during lactation, at calving, in mid and late lactation, and at drying off as well as from any quarters that developed clinical mastitis. Bacteriological analysis of these milk samples was carried out using standard techniques (NMC, 1999) to determine the presence of mastitis pathogens.

Teats (2 per cow) of up to 25 of the 100 cows were swabbed approximately monthly using sterile cotton-tipped swabs moistened in 0.1% sterile peptone diluent. The teat orifice and teat ends were scrubbed rigorously with the swab, which was then placed into 1ml of 0.1% peptone diluent. Swabs were shaken vigorously and 100µl spread-plated onto a selective media and incubated at 37^{o}C for 48 hours. A confirmatory media was used to support preliminary visual identification of *S. uberis* colonies, and from each selective plate, up to 5 colonies were submitted for confirmation of *S. uberis* using 16-23S PCR primers (Forsman *et al.* 1997).

On a monthly basis, faecal isolates were obtained by inserting a swab into the rectum of all cows using sterile cotton-tipped swabs moistened in 0.1% sterile peptone diluent. For cows from which *S. uberis* was isolated, additional swabs were collected weekly for a period of up to 2 weeks after the monthly screening sample. Bacteriology was performed on rectal swabs in the same way as teat skin swabs.

Some cows that developed clinical mastitis (CM) or subclinical (SCM) mastitis, caused by *S. uberis* were subjected to a more intensive sampling procedure. The CM cases were those

that displayed visible clinical signs of mastitis (clots, discoloured milk, etc) at milking time, whilst the SCM cases were selected from cows that were positive for *S. uberis* at the mid-lactation bacteriological foremilk sample, in addition to an elevated foremilk somatic cell count (SCC). Sampling procedures involved swabbing 15 body sites, these being the teat orifice and teat barrel of the infected teat and all other teats, udder skin, inside surface of the hocks, feet (coronet band of both back feet), lips, nostrils, tail swish, and rectum. Foremilk samples were also collected aseptically, after collecting the teat swab samples. The Ruakura Animal Ethics Committee approved all animal manipulations.

Rectal swabs were placed into 1ml 0.1% peptone diluent whilst all other swabs were placed into 1ml sterile milk and then mixed, before spread plating 100µl onto the selective media. Significantly greater colony forming units were achieved when swabs were diluted in sterile milk immediately before plating. Up to 5 colonies were submitted for confirmation of *S. uberis* by 16-23S PCR and then strain typed using Repetitive Extragenic Palindrome (REP) sequence-based PCR methodology. The REP-PCR was modified from Wieliczko *et al.* (2002) and used the BoxA1R and Eric1R primers (Versalovic *et al.*, 1994). Band patterns for all isolates were analysed with GelCompar II software (Version 4.1, 1998, Applied Maths, Kortijk, Belgium), using Pearson's correlation coefficient. Dendograms were created using the UPGMA algorithm and a 90% similarity cut-off point was used to classify isolates as the same strain and to generate strain-type groups.

Results

During the 16-month period, *S. uberis* was isolated consistently from the rectum of 5.6% (range 1.2% to 9.7%) of cows at the monthly samples (Table 1). Those cows (n=13) that yielded *S. uberis* were termed "shedders" and were sampled again one and two weeks after

Table 1. Number of cows (or teats) sampled per month, and number and percentage of cows from which S. uberis could be isolated from the rectum and teat skin. Rectal swabbing occurred between Dec-03 and Oct-04 and teat swabbing between Jul-03 and Jun-04.

		Rectal Samples			Teat Skin	
Date	Lactating status	N cows sampled	N positive for SU	% positive for SU	N teats sampled	% positive for SU
Jul-03	Lactating	-	-	-	36	0
Aug-03	Lactating	-	-	-	40	0
Sep-03	Lactating	-	-	-	50	4.0
Dec-03	Lactating	148	7	4.7	10	0
Jan-04	Lactating	82	1	1.2	10	0
Feb-04	Lactating	-	-	-	40	10.0
Mar-04	Lactating	86	6	7.0	-	-
Apr-04	Lactating	86	4	4.7	34	2.9
May-04	Dry	73	4	5.5	26	42.3
Jun-04	Dry	72	7	9.7	26	19.2
Sep-04	Lactating	144	8	5.6	-	-
Oct-04	Lactating	135	9	6.7	-	-
Average	Lactating	114	6	5.0	31	2.4
Average	Dry	73	6	7.6	26	30.8

the monthly sampling. In contrast, bacteria were isolated only intermittently from teat skin when cows were lactating although more frequent isolations were observed in the dry period (Table 1). No clinical mastitis was detected when *S. uberis* were isolated on teat skin.

Among the 13 "shedder" cows, different patterns of *S. uberis* isolation were observed. Some cows were found to repeatedly yield *S. uberis* and were termed regular shedders (Table 2) whilst others, the intermittent shedders, only yielded the bacterium at a single monthly sample and rarely tested positive at the follow-up samples. From regular shedders, the bacterium could be isolated for periods in excess of one month and usually for at least four months. One cow tested positive throughout a 10-month period but three of the regular shedders were culled at drying off in May-04, which compromised long term profiling. The results suggest that *S. uberis* is not solely a transient contaminant of faecal material. Only one of the intermittent shedder cows developed clinical mastitis with *S. uberis*, which was detected following calving in July-04, approximately one month after a single rectal isolation of *S. uberis*.

Nine cows with a *S. uberis* intramammary infection were subjected to the more comprehensive swabbing. Of these cows, two cows were examined on two separate occasions so a total of seven CM and four SCM cases were examined (Table 3). Single strains of *S. uberis* were identified in the milk from all cases of CM, and from two of the SCM cases, whilst two different strains of *S. uberis* were differentiated in the milk from the remaining two SCM cases. The same strain of *S. uberis* was isolated from cow 1033 during both CM episodes; however the similarity in strains between the CM and SCM episodes for cow 1034 has not yet been determined. No single common strain was observed between different cows.

The feet of infected cows yielded the bacterium in 10 of the 11 cases, whilst other sites yielded the bacterium less frequently. Teat skin of the infected and adjacent teats, the lips, and tail were the next most common sites from which *S. uberis* could be isolated. No *S. uberis* was isolated from the udder skin, and the rectum yielded the bacterium from only two CM cows.

The numbers of strains of *S. uberis* detected at different body sites were found to vary widely when up to 5 colonies per site (3 for SCM cases) were strain typed. Multiple strains were found on teat skin from adjacent teats, feet and tail whilst the infective strain was only found consistently on the feet (4/7) of cows with CM (Table 3). For cows with SCM, no specific sites proved consistently positive for the infective strain. Faecal matter was not positive for the infective strain for any case of mastitis.

Table 2. Number and % of monthly samples, and period of days, for which S. uberis could be positively isolated from faecal swabs of cows. Samples obtained monthly for a minimum of 3 months, maximum of 8 months per cow, during the 10-month period. Cows were defined as regular shedders if S. uberis was isolated from 2 or more monthly samples.

Shedder Group	n cows	N samples/cow positive for SU		Period (days) when positive for SU*	
		n	% of monthly samples	mean	range
Intermittent	6	1	16	5	0 - 13
Regular	7	2, 3 or 4	56	122	34 -228
Total	13				

*Period (days) for when *S. uberis* could be isolated was calculated from dates of the monthly samples plus additional samples collected for 1-2 weeks after a positive isolation at a monthly sample.

Table 3. Number of S. uberis colonies submitted for strain typing from each body site and the strain types found at each site, with different strains, within a cow, denoted by different superscripts. Strains isolated from milk were denoted as strain "a" and were considered to be the infective strain. No comparisons were made between cows. Cows presented with either clinical mastitis (A) or subclinical mastitis (B).

A. Clinical mastitis cases								
Cow no		1033	1033	1034	2031	8138	8212	9123
Date		12-Aug	17-Sep	17-Sep	17-Aug	6-Sep	24-Oct	19-Aug
Sample site	Quarter							
Milk	Infected	5^{a}	3^{a}	3^{a}	5^{a}	5^{a}	5^{a}	5^{a}
Teat Barrel	Infected	5^{a}	-	-	-	3^{cg}	-	5^{a}
	Other	-	-	-	-	-	-	5^{adfi}
Teat End	Infected	5^{a}	-	-	-	5^{a}	-	-
	Other	-	-	-	-	4^{bde}	-	5^{acgh}
	Other	-	-	-	-	-	-	4^{b}
Udder Skin		-	-	-	-	-	-	-
Lips		-	-	3^{b}	-	-	5^{b}	5^{ae}
Nostrils		-	-	-	3^{abc}	-	-	5^{a}
Rectum		5^{ghij}	3^{def}	2^{cd}	-	-	-	-
Feet		1^{a}	2^{bc}	-	3^{ad}	5^{abf}	4^{cd}	4^{ac}
Hocks		5^{bc}	-	-	-	-	-	-
Tail		5^{def}	-	-	-	-	5^{efghi}	2^{a}

B. Subclinical mastitis cases					
Cow no		680	1034	1058	9139
Date		16-Nov	16-Nov	16-Nov	16-Nov
Sample site	Quarter				
Milk	Infected	3^{a}	3^{ab}	3^{ab}	3^{a}
Teat Barrel	Infected	3^{bc}	-	3^{cgi}	3^{gj}
	Other	-	-	3^{fno}	3^{be}
	Other	-	-	-	3^{f}
Teat End	Infected	-		1^{m}	3^{a}
	Other	-	2^{cd}	3^{bfh}	3^{bcd}
	Other	-	2^{ef}	-	-
Udder Skin		-	-	-	-
Lips		1^{a}	1^{c}	3^{cd}	3^{i}
Nostrils		-	-	-	-
Rectum		-	-	-	-
Feet[1]		6^{def}	$6^{cghijkl}$	3^{ej}	3^{eh}
Hocks		-	-	2^{f}	-
Tail		3^{gkl}	-	3^{hk}	-

[1]Feet - both feet were sampled separately but results presented as pooled data.

Discussion

Microbiological studies report *Streptococcus uberis* to be widely distributed on the cow (Cullen, 1966; Sharma and Packer, 1970; Bramley *et al.*, 1979) and detectable on bedding (Bramley *et al.*, 1979; Hogan *et al.*, 1989) and pasture (Cullen and Little, 1969) but the identification of the route of infection by these pathogens remains unknown. This study examined specific anatomical sites for information that might distinguish the primary sites or reservoirs of infection, from fomites, or sites of secondary contamination, that could infect cows when maintained in totally pasture-based systems.

On any one occasion, the bacterium could be isolated from the rectum of approximately 5% of the herd of 100 cows. Kruze and Bramley (1982) repeatedly isolated *S. uberis* from the rectum of 2 of 14 cows (15%) over a period of seven weeks and suggested that, whilst certain cows behaved as intermittent shedders, these two cows repeatedly shed the bacterium over a period of time and were probably colonised in the intestine. We observed that approximately 7/100 cows could be considered intestinally colonised. Faecal excretion of *S. uberis* by such cows may provide the inoculum on bedding (Kruze and Bramley, 1982) or on races and pasture (Cullen and Little, 1969; Lopez-Benavides *et al.,* 2005), where further multiplication is allowed to take place, before contamination of teats and udders. However our results found no evidence that strains derived from faecal material were identical to any clinical isolates. Furthermore we observed infrequent isolation of *S. uberis* on cow's teats, especially on lactating teats.

Cullen (1966) suggested that the teat skin was a relatively unfavourable site for *S. uberis* although a later study observed more frequent isolation of streptococci on teat skin (Cullen and Hebert, 1967). In that study however, no teat dipping was used and no distinction was made between different streptococcal species. The absence of teat disinfection in the dry period may account for the dramatic rise in number of teats from which *S. uberis* could be isolated. This was observed during the months of May and June when the numbers of *S. uberis* isolated from farm race material also showed dramatic increases (Lopez-Benavides *et al.*, 2005).

The infective strain of *S. uberis* was rarely isolated from other body sites of cows harbouring an intramammary infection. Sites such as the teat skin, feet, rectum and tail yielded multiple strains whilst single, or at most two strains, were observed in milk. Previous reports attribute intramammary infections to one strain, with most assessments made by comparing isolations over time (Wieliczko *et al.*, 2002; McDougall *et al.*, 2004). It is rare to strain type more than one colony from a single infection event so further investigation is required to confirm this finding.

The reasonably frequent detection of the infective strain on the feet of a clinically infected cow may be confounded by the practice of stripping the inflamed quarter during manual detection of clinical mastitis, which can then contaminate the feet. Subclinical cases are rarely foremilk stripped so the chances of contaminating the feet would be much reduced. Presence of the infective strain on teat skin could be attributed to contamination with infected milk via the milker's hands or the milking cluster, but detection of the infective strain on lips and nostrils cannot be explained easily.

In conclusion, only a small proportion of the herd were found to be colonised intestinally but *S. uberis* was isolated from a number of other sites on the cow. Cows with an intramammary infection were not necessarily infected intestinally and the infective strain was rarely isolated from other body sites. The presence of the infective strain on other body

sites was more likely to represent contamination by infected milk, rather than a potential source of the infective strain.

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Risk factors contributing to udder health depression during alpine summer pasturing in Swiss dairy herds

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Abstract

One third of Swiss arable land is high altitude grassland. 15% of all Swiss dairy cows feed there for about 2 to 5 months during the summer. About 30 % of all alpine bulk milk samples of the Swiss province "Graubünden" exceeded the somatic cell count (SCC) of 350'000/ml. The objective of this project was to evaluate factors effecting increasing SCC on alpine pastures. Foremilk quarter samples of all lactating cows of 3 high-altitude sites (n= 128 cows) were taken once on the 12 farm valley sites (v) and three times during the summer on the alpine sites (a1 to a3). Quarter samples were investigated for microbial pathogens and SCC. Lactation number and lactation days of the cow were recorded. In the statistical models for the three alpine control dates the factors somatic cell count in the valley, infection status in the valley, number of lactation and state of lactation are showing a significant influence on the linear somatic cell score (LSCS). First lactating cows and cows in earlier lactation are showing least square means (LSQ) for LSCS. The same could be found for sterile quarters in the valley and quarters with a low SCC in the valley site. The microbial pathogens which could be found in the valley and in each alp sample are showing a high repeatability of the valley findings. New infections with major pathogens could be avoided. The biggest potential for improving the milk quality in alpine sites lies in the measures to reduce infected cows in the valley sites. It can be recommended to avoid especially the delivery of cows suffering from subclinical mastitis caused by *staphyloccus aureus* and streptococci to alpine sites.

Keywords*:* high alpine pasturing, somatic cell counts, pathogens

Introduction

1.6 million hectares out of a total of 4.1 million are utilized for agriculture in Switzerland. More than one third (560'000 hectares) is represented by high-altitude grassland, located at altitudes of up to 3 000 m above sea level. Some 460 000 cattle (including 105.000 of 700.000 Swiss dairy cows), feed on this meager vegetation for about 2 to 5 months, depending on altitude and climate. Currently, about 8 % of the total Swiss hard cheese is produced at these sites.

The quality of the milk produced on high-altitude pastures is in discussion frequently, as the somatic cell count (SCC) is significantly higher as compared to milk produced on-farm in the valleys (Regi, 1986). Between 25% and 30% of all bulk milk samples taken on high-altitude sites of "Graubünden" exceeded the critical somatic cell count (SCC) of 350'000 per ml, at which milk is rejected (Walkenhorst, 2002). On the other hand, milk quality differs

widely between different alpine sites. The question rises if the reason for this difference is based mainly on natural circumstances of the alp (climate, feedstuff, new social herd order, etc.), human depending factors (milking technology and hygiene, human cow interaction) or more on alp independent factors (persistent intramammary infections from valley, number and state of lactation). The presented investigation aimed on partial answers to these questions.

Material and methods

The project was conducted in the high altitude region of Engadin, which is part of the Swiss province of Graubünden. The farms are located at an altitude between 1400 and 1700 m above sea level, the alpine sites at an altitude up to 2500 m. On average, less than 20 dairy cows are kept on each farm, 30 to 50 cows on each alpine site. The climatic conditions shorten the vegetation period in the valley and require large amounts of forage to be conserved for the winter period. This implies that grass from relatively large units of steep land have to be cut during one or two harvests. Bringing the cattle to high-altitude pastures, owned by a community of farmers and managed by employees, while the grass is harvested on the farms, is the traditional strategy for coping with this potentially difficult situation. Therefore, roughly 90 % of dairy cows and almost all calves and heifers of this region spend the summer months on high-altitude-pastures. The cows are mostly in the second half of the lactation period, 50 % of the cows are dry towards the end of the summer.

The investigation included three alpine pasture sites (B, P, G). Data were generated in the pasturing season of the year 2001. The employees of these three alps were well educated in milking procedure, following a strict hygiene management to protect new intramammary infections (change of cleaning material after each cow, postdipping). The milking equipment shows also a good condition, so a big part of human depending factors were sound and the same on all three sites. A total of 128 cows of 12 farm valley sites were examined as a maximum five times during the evening milking, once in the valley (v; year 2001, week 20 and 21) and four times during the alpine pasturing period (a1 - a4; year 2001, week 26, 29, 32 and 35). All cows from one farm were going to one and the same alp. During the summer, the number of lactating cows was reduced by the common dry off process. As a consequence of this, 128 (a1), 119 (a2), 103 (a3) and 71 (a4) cows, respectively, were examined during the control dates. The last alpine control findings (a4) are not integrated in the results because of the low number of still lactating cows.

Foremilk samples of all lactating quarters were taken at each control date. Quarter samples were investigated for microbial pathogens and somatic cell counts (SCC) at Agroscope Liebefeld-Posieux, the national reference laboratory of Switzerland. For transformation the SCC into a normal distribution they are converted into the linear somatic cell score (LSCS). Diagnostic findings of the microbiological examination were differentiated into sterile, minor pathogens (Corynebacterium bovis [C.bovis] and coagulase-negative Staphylococci [CNS]), major pathogens (Staphylococcus aureus, and all species of Streptococcaceae [Sc. ssp]) and mixed cultures (mix) if more than one species was growing. Number of lactation (LN) with three levels (first [1], second [2] and more than second lactation [>2]) and state of lactation (CALF) also with three levels (calving date earlier than november [before nov], November and December [nov-dec] and later than December [after dec]) of each cow were recorded. Additionally, the factors SCC in the valley milk sample

(<100.000/ml or ≥100.000/ml) and udder quarter position (front quarters, hind quarters) were included.

The above mentioned factors were set in relation to the linear somatic cell score values (LSCS) calculated during the three control dates (a1-a3) by using standard least square procedure (JMP software, SAS-Institute inc.) taking account the different factors as fixed effects (formula 1).

$$LSCSijklmno = \mu + LN + CALF_j + ALP_k + FARM[ALP]_l + BACTV_m + SCCV_n + QPOS_o + ei_{jklmno} \quad (1)$$

with m = population mean of SCS, and e as the intercept. The least square means for the different levels of factors were compared. The significance level was fixed with a = 0.05.

Furthermore the bacteriological profiles of the valley samples were compared to those during the alpine control dates.

Results and discussion

In spite of the lack of effects of human dependent factors on the three alpine sites a difference between the three alpine sites and a clear difference between the valley and the first alpine sample could be found (Table1).

Udder quarters of first lactating cows (LN=1) and of an early state of lactation (CALF=after dec) are showing a significant lower level of LSCS than older or later lactating quarters respectively, both in valley and through the three alpine times. The LSCS differs also between the twelve farms from 0.1 to 2.3. A clear difference could be shown according to the status of infection in the valley (BACTV). Quarter samples with no infection in the valley (sterile) have the lowest LSCS (v=0.1, a1=0.8, a2=1.2, a3=1.9) followed by infections with minor pathogens and major pathogens (Table 1). During the third sample primary infections with minor pathogens have a LSCS of 3.4, major pathogens of 4.8.

Quarters with a somatic cell count in the valley (SCCV) lower than 100'000/ml are showing through all alpine samples an obvious lower LSCS than quarters with an primary high LSCS. There is no significant difference in LSCS between fore- or hindquarters (Table 1).

In all alpine control date associated fixed effect models the factors SCCV, BAKTV, CALF and LN are significant (Table 2-4). The impact of the levels of these four factors is nearly identical in all models. First lactating cows and cows in earlier lactation (CALF=after dec) are showing least square means (LSQ) for LSCS. The same could be found for sterile quarters in the valley and quarters with a low SCC in the valley site. Only the last both levels of CALF (nov-dec; before nov) and of LN (2, >2) showed different relative LSQ during investigation time. The factor FARM nested in ALP was furthermore significant for two models (LSCS in a1 and LSCS in a2), the factor position of the quarter (QPOS) for one (LSCS in a2).

The alp shows no significant influence on LSCS in all three models. Consequently, the clear difference in-between the alpine sites (Table 1) is obviously not influenced by the alpine site itself but more by other factors.

The microbial pathogens (BACTDIFF) which could be found in the valley and in each alp sample are showing a high repeatability of the valley findings except for the mixed cultures. These findings differ during the alpine period more or less into sterile quarters and infections with C. bovis (together 76% - 86%). Sterile quarters, C. bovis infections, and the major pathogen infections were repeatable between 73% and 86%. CNS findings were only in 56%

Table 1. Descriptive statistic of variables with possible influence on SCS of quarter milk samples on alp.

variable	level	Control sample date				
			v	a1	a2	a3
all	±	n	510	510	474	411
		LSCS (±sd)	0.9 (±1.9)	1.6 (±2.1)	2.0 (±2.3)	2.5 (±2.3)
LN	1	n	159	159	155	136
		LSCS (±sd)	0.4 (±1.8)	1.0 (±2.1)	1.4 (±2.5)	1.4 (±2.1)
	2	n	160	160	144	116
		LSCS (±sd)	1.2 (±1.8)	1.9 (±1.9)	2.3 (±2.3)	2.5 (±2.1)
	>2	n	191	191	175	159
		LSCS (±sd)	1.0 (±2.0)	1.9 (±2.2)	2.2 (±2.1)	3.3 (±2.2)
CALF	after dec	n	119	119	115	115
		LSCS (±sd)	0.5 (±2.4)	0.8 (±2.0)	0.6 (±2.0)	1.8 (±2.2)
	nov-dec	n	148	148	140	128
		LSCS (±sd)	1.0 (±1.9)	1.8 (±2.2)	2.5 (±2.4)	3.1 (±2.6)
	before nov	n	243	243	219	168
		LSCS (±sd)	1.0 (±1.6)	2.0 (±2.0)	2.3 (±2.2)	2.5 (±2.0)
ALP	G	n	191	191	183	167
		LSCS (±sd)	0.4 (±1.7)	0.9 (±1.9)	1.4 (±2.2)	1.9 (±2.3)
	P	n	187	187	175	148
		LSCS (±sd)	1.2 (±2.0)	2.0 (±2.2)	2.3 (±2.5)	3.0 (±2.3)
	B	n	132	132	116	96
		LSCS (±sd)	1.3 (±2.0)	2.2 (±2.1)	2.4 (±2.0)	2.6 (±2.1)
BACTV	steril	n	290	290	278	241
		LSCS (±sd)	0.1 (±1.56)	0.8 (±1.8)	1.2 (±2.2)	1.9 (±2.4)
	mix	n	64	64	55	51
		LSCS (±sd)	1.5 (±2.2)	2.1 (±2.0)	2.2 (±1.9)	2.5 (±1.9)
	minor path.	n	127	127	114	95
		LSCS (±sd)	1.9 (±1.3)	2.7 (±1.9)	3.1 (±2.0)	3.4 (±1.8)
	major path.	n	29	29	27	24
		LSCS (±sd)	3.1 (±2.1)	4.4 (±1.4)	4.6 (±1.4)	4.8 (±1.3)
SCCV	< 100'000	n	435	435	408	358
		LSCS (±sd)	0.4 (±1.4)	1.2 (±1.9)	1.6 (±2.1)	2.2 (±2.3)
	>100'000	n	75	75	66	53
		LSCS (±sd)	4.1 (±1.0)	4.1 (±1.6)	4.4 (±2.0)	4.2 (±1.8)
QPOS	fore	n	254	254	236	205
		LSCS (±sd)	0.9 (±1.8)	1.6 (±2.1)	1.8 (±2.2)	2.3 (±2.2)
	hind	n	256	256	238	206
		LSCS (±sd)	0.9 (±2.0)	1.7 (±2.2)	2.1 (±2.4)	2.6 (±2.4)

- 63% repeatable (Table 5). Quarters with two equal microbial findings in the vally and the alpine sample are showing very typical profiles of LSCS in a similar way as described by Lamarche (2000). Sterile quarters in two samples risen from LSCS 0.6 (a1) to 1.4(a3), whereas continuous infections with C. bovis risen from 2.7(a1) to 3.6, CNS from 3.5 (a1)

Table 2. Model parameters of LSCS in a1.

Variable of LSCS a1	F Ratio	p	Relation between level least square means
SCCV	98	<0.0001	"<100'000" < ">100'000"
BAKTV	32	<0.0001	steril < mix < minor < major
CALF	26	<0.0001	after dec < nov-dec < before nov
LN	13	<0.0001	"1" < ">2" < "2"
FARM nested ALP	5	<0.0001	
QPOS	3	0.08	not significant
ALP	1	0.18	not significant

Table 3 Model parameters of LSCS in a2

Variable of LSCS a2	F Ratio	p	Relation between level least square means
SCCV	64	<0.0001	"<100'000" < ">100'000"
CALF	39	<0.0001	after dec < before nov < nov-dec
BAKTV	18	<0.0001	steril < mix < minor < major
LN	8	0.0006	"1" < ">2" < "2"
QPOS	4	0.0355	fore < hind
FARM nested ALP	2	0.0305	
ALP	3	0.0508	not significant

Table 4. Model parameters of LSCS in a3.

Variable of LSCS a1a3	F Ratio	p	Relation between level least square means
LN	32	<0.0001	"1" < "2" < ">2"
SCCV	22	<0.0001	"<100'000" < ">100'000"
CALF	17	<0.0001	after dec < before nov < nov-dec
BAKTV	10	<0.0001	steril < mix < minor < major
QPOS	4	0.059	not significant
ALP	2	0.1087	not significant
FARM nested ALP	2	0.1279	not significant

to 4.0 (a3) and major pathogens from 4.3 (a1) to 4.6 (a3). In the valley there were already clear differences noticeable between sterile and infected quarters.

New infections with major pathogens could be avoided very successfull. Not more than 3% (valley site finding: sterile and C. bovis) to 5% (valley site finding: CNS) of such infections could be found during the alpine session.

Because it is not possible to prohibit all potential udder health influencing factors during the high alpine grazing period, an increase of SCC could not be completely avoided. Nevertheless, with good preconditions it is possible to produce a high milk quality on the alpine sites. Number and state of lactation are unchangeable factors of alpine cows and can not be influenced by management. The biggest potential for improving the milk quality

Table 5. bacteriological profiles and LSCS of the valley milk samples in relation to alp milk samples.

BACTDIFF(VALLEY)		BACTDIFF (ALP)		Control sample date		
				a1	a2	a3
steril	n=290;	steril	n(%)	249 (86%)	233 (84%)	198 (82%)
	LSCS=		LSCS	0.6 (±1.8)	0.9 (±1.9)	1.4 (2.1±)
	0.1(±1.6)	C.bovis	n(%)	22 (8%)	30 (11%)	30 (12%)
			LSCS	2.5 (±1.4)	2.7 (±2.7)	4.0 (±2.6)
		major	n(%)	6 (2%)	7 (3%)	7 (3%)
			LSCS	1.5 (±2.2)	2.2 (±4.8)	3.8 (±3.7)
		others	n(%)	13 (5%)	8 (3%)	6 (2%)
			LSCS	0.5 (±2.3)	2.5 (±3.3)	2.5 (±1.6)
C. bovis	n=89;	steril	n(%)	11 (12%)	9 (12%)	14 (22%)
	LSCS=		LSCS	2.5 (±2.6)	2.0 (±1.7)	3.5 (±2.2)
	1.8(±1.3)	C.bovis	n(%)	75 (84%)	63 (82%)	46 (73%)
			LSCS	2.7 (±1.8)	3.2 (±2.0)	3.6 (±1.4)
		major	n(%)	1 (1%)	1 (1%)	1 (2%)
			LSCS	3.2	5.4	6.5
		others	n(%)	2 (2%)	4 (5%)	2 (3%)
			LSCS	5.0 (±1.5)	2.6 (±3.1)	3.6 (±0.8)
CNS	n=38;	steril	n(%)	10 (26%)	10 (27%)	10 (31%)
	LSCS=	LSCS	1.2 (±2.1)	1.0 (±2.0)	1.1 (±1.7)	
	2.1(±1.5)	CNS	n(%)	24 (63%)	22 (59%)	18 (56%)
			scs	3.5 (±1.4)	3.8 (±1.2)	4.0 (1.3±)
		major	n(%)	1 (3%)	2 (5%)	0 (0%)
			LSCS	3.8	3.1 (±2.1)	
		others	n(%)	3 (8%)	3 (8%)	4 (12%)
			LSCS	2.8 (±0.4)	2.9 (±0.4)	2.6 (±1.1)
Sc.ssp	n=8;	steril	n(%)	0 (0%)	1 (13%)	1 (14%)
	LSCS=		LSCS		3.1	3.5
	3.3(±2.0)	Sc.ssp	n(%)	6 (75%)	7 (88%)	5 (71%)
			scs	4.4 (±1.5)	4.0 (±2.0)	4.6 (±1.1)
		others	n(%)	2 (25%)	0 (0%)	1 (14%)
			LSCS	5.8 (±1.5)		6.1
S. aureus	n=21;	S.aureus	n(%)	17 (80%)	16 (84%)	13 (76%)
	LSCS=		LSCS	4.2 (±1.5)	5.0 (±1.0)	4.6 (1.2±)
	3.1(±2.2)	others	n(%)	4 (20%)	3 (16%)	4 (24%)
			LSCS	4.2 (±1.1)	4.7 (±1.0)	5.7 (±1.7)
mix	n=64;	steril	n(%)	29 (45%)	25 (45%)	25 (49%)
	LSCS=		LSCS	0.9 (±1.8)	1.0 (±1.9)	1.0 (±1.4)
	1.5(±2.2)	C.bovis	n(%)	20 (31%)	20 (36%)	19 (37%)
			scs	3.0 (±1.5)	3.1 (±1.3)	3.7 (±0.9)
		major	n(%)	7 (11%)	4 (7%)	4 (8%)
			LSCS	3.8 (±2.0)	3.4 (±0.6)	4.3 (±1.9)
		others	n(%)	8 (12%)	6 (11%)	3 (6%)
			LSCS	2.8 (±1.4)	3.3 (±1.5)	4.3 (±1.9)

in alpine sites lies in the measures to reduce infected cows in the valley sites. These especially by major pathogens infected cows had the greatest contribution to bulk milk somatic cell count. It can be recommended to avoid the delivery of cows suffering from subclinical mastitis caused by staphyloccus aureus and streptococci.

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Streptococcus uberis population dynamics in the New Zealand pastoral dairy farm

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Abstract

Few management strategies to control *Streptococcus uberis* mastitis for dairy cows managed under pastoral conditions have been developed. Cows may become contaminated with the bacterium when lying down in the paddock or when in contact with muddy races. To examine potential *S. uberis* niches on the dairy farm, cow races were identified as being of high or low cow traffic, and monitored every fortnight for contamination by *S. uberis* for a 12-month period. Similarly, paddocks were monitored for contamination by *S. uberis* at mid lactation, late lactation and during the dry period. Average contamination levels in high and low-traffic races were 160 cfu/g wet material and 6 cfu/g wet material ($p<0.001$), respectively. Continuous and heavy *S. uberis* contamination was always observed in the high-traffic race proximal to the farm dairy, whilst in low-traffic races *S. uberis* only reached high levels when there was cow traffic in previous days. On the paddocks, *S. uberis* contamination was only detectable after grazing. Higher post-grazing *S. uberis* contamination was observed in the dry period, during the winter months, when wet conditions prevailed and when cow grazing density was higher. Results show that high levels of *S. uberis* are found in farm races of high traffic and in paddocks during the winter months. It is suggested that these environmental niches are risk factors associated with increased exposure of bacteria onto cow teats. Information on the population dynamics of this bacterium within the farm are necessary to develop herd and environmental management strategies that reduce exposure of cow teats to *S. uberis*.

Keywords: *Streptococcus uberis,* environment, soil, grazing

Introduction

The development of management strategies aimed to control *S. uberis* mastitis for dairy cows managed under pastoral conditions is limited. This environmental pathogen is especially important in New Zealand, where cows spend all their time in the paddock. *Streptococcus uberis* causes around 70% of clinical mastitis (CM) cases and is more frequently isolated from CM quarters in the dry and calving periods (Williamson *et al.*, 1995; Pankey *et al.*, 1996; McDougall, 1998). Although this bacterium has been isolated from different sites around the dairy environment and the cow (Sweeney, 1964; Cullen, 1966; Sharma and Packer, 1970; Bramley, 1982; Bramley and Kruze, 1982; Lacy-Hulbert *et al.*, 2005; Lopez-Benavides *et al.*, 2005), little information exists on the level of *S. uberis* contamination in

environmental niches and the consequences of increased bacterial populations for intramammary infection.

Early studies by Cullen and Little (1969) reported that *S. uberis* was found only in wet and muddy soil where cows congregated. Bramley (1982) isolated *S. uberis* in 35% of faecal samples and in varying frequencies (0-40%) from straw bedding. More information regarding the ecology and population dynamics of *S. uberis* in the pastoral system are necessary to define risk factors associated with increased susceptibility to intramammary infection and a better understanding of the infection process.

Materials and methods

Races selection and sampling

Races were selected from the farm manager's assessment of daily cow traffic. Initially, a total of six farm races at the Dexcel Lye research farm were selected for the study. Each 5 m wide race comprised of a 400 mm base of compacted layer of 'rotten' rock, overlayed with a 50 mm layer of compacted pit sand. One race was subdivided into 'Main A' and 'Main B' because of its length, giving a total of seven races; four from high-traffic and three from low-traffic. Three permanent sampling points were selected per race. On a fortnightly basis, surface material from an area of around 25 cm^2 was scooped with a sterile plastic vial (approx. 10 g) at the centre of each race sampling point. Individual race material samples were analysed quantitatively for the presence of *S. uberis*. Race sampling started in mid November 2003 and finished in mid November 2004.

Paddock sampling

Three paddocks were selected for monitoring *S. uberis* contamination at three stages of lactation: mid lactation (November 2003), late lactation (April 2004) and during the dry period (August 2004). Each paddock was sampled at nine locations at each of the following times: one day prior to grazing, one day post-grazing, one week post grazing, and at approximately two weeks post-grazing. On each sampling occasion, a sample of grass, surface soil and sub soil (obtained at 5 cm depth) were collected in a clean plastic bag or sterile plastic container. At each stage of lactation, a single paddock generated 108 samples for analysis.

Leaves of grass were cut aseptically from a 200 cm^2 area, leaving a 5 cm grass residual. To obtain the soil surface material, a 100 cm^2 area was cleared of grass, and soil was scooped (approx. 10 g) into a sterile plastic vial. A similar approach was conducted when obtaining the sub soil sample, except that a layer of 5 cm soil was first discarded before scooping the sample.

Cow presence in races

The presence of cow traffic near the sampled races (due to routine milking or paddock rotation) was recorded for each of the three days prior to sampling. During the study period, approximately 340 cows were milked off a 90 ha area. Planned start of calving was July 7, 2004. The rotation length of paddocks during the lactation period was about 17 d and increased to 120 d in the dry period (April to June).

Bacteriology of race and paddock material

One gram of wet weight material from each race or paddock sample was added to nine ml of 0.1% peptone diluent. Samples were mixed vigorously for 2 min and 100 µl was spread plated onto a selective *S. uberis* medium. Plates were incubated at 37ºC for 72 h and *S. uberis* colonies were identified on the basis of colony morphology, esculin reaction, inulin fermentation and β-glucuronidase activity. Colony forming units were expressed as cfu/g wet weight and $\log_{10}$ transformed for analysis.

Data analysis

Comparison of average $\log_{10}$ *S. uberis* cfu contamination levels between high and low-traffic races, or between levels of *S. uberis* in paddocks were conducted using ANOVA (STATISTICA 6.1, StatSoft, Inc., Tulsa, OK, USA). Averages are expressed with their respective standard error (SEM) and where appropriate, with the standard deviation (SD).

Results

Races and *S. uberis* contamination

The overall $\log_{10}$ *S. uberis* cfu average for high- or low-traffic races was $\log_{10}$ 2.20 ± 0.10 cfu and $\log_{10}$ 0.75 ± 0.09 cfu, respectively ($p<0.001$). In high-traffic races, an average of $\log_{10}$ 3.0 ± 0.1 cfu (SD = 1.6) was observed in the months of April to September, whilst in the low-traffic races it was $\log_{10}$ 1.1 ± 0.1 cfu (SD = 1.5) ($p<0.001$). These higher contamination levels coincided with the dry and calving periods. The lowest bacterial contamination in the high-traffic races occurred in the month of January (<$\log_{10}$ 1.0 cfu), which was similar to *S. uberis* levels in low-traffic races throughout the year (Figure 1).

Closer examination of individual high-traffic races revealed a pattern in *S. uberis* levels throughout the year. *S. uberis* levels rose above $\log_{10}$ 4.0 cfu in the months of May to August in the two races nearest the milking shed. In the high-traffic 'Shed' race (closest to the farm dairy), the recovery of *S. uberis* was usually above $\log_{10}$ 3.0 cfu for the majority of the sampling period. However, contamination levels below $\log_{10}$ 2.0 cfu were observed in the summer months (i.e. January and February) (Figure 2).

In contrast, higher *S. uberis* contamination levels (>$\log_{10}$ 2.0 cfu) in individual low-traffic races were only observed in the winter months (i.e. July and August). For example, in the 'Low-West' race, contamination levels were normally below $\log_{10}$ 1.0 cfu during the sampling period. However, a sporadic peak was observed in the month of August, which coincided with calving and cow presence (Figure 2).

Associations between cow presence and *S. uberis* contamination in races

In general, cow presence was associated with higher *S. uberis* contamination levels in farm races (Figure 3). In high-traffic races, cow presence was usually observed in two out of three days before sampling occurred. In the race next to the farm dairy (i.e. High-Shed), cow presence was constant and we also observed the highest average *S. uberis* contamination ($\log_{10}$ 3.6 cfu). In the other two races near the farm dairy (i.e. High-Main A and High-Main B), cow presence was observed for at least two out of three days before sampling. The opposite trend was observed in low-traffic races, where cow presence was usually less than one day in the three days before sampling, and the average *S. uberis* contamination levels were around $\log_{10}$ 1.0 cfu.

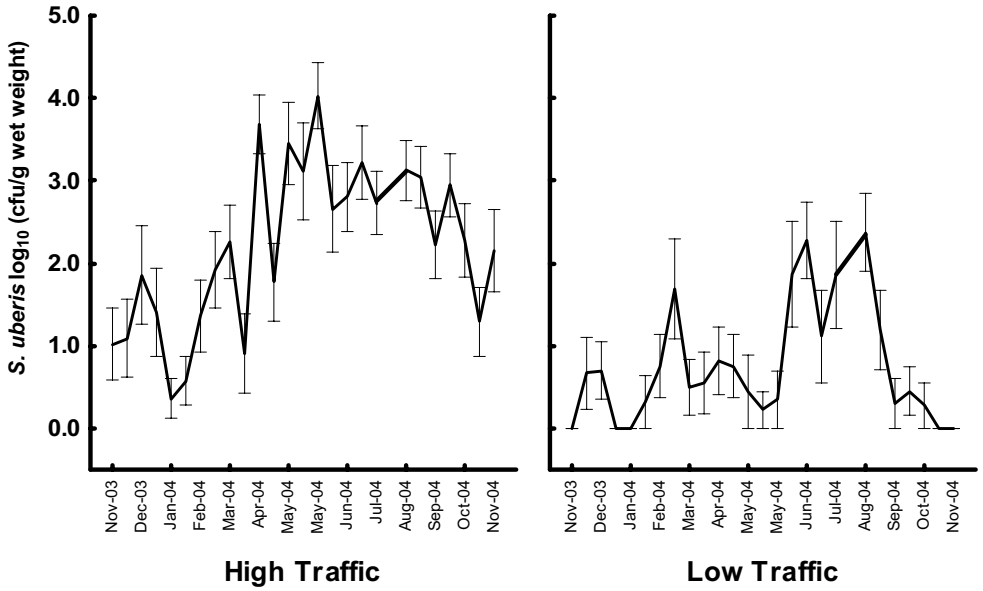

Figure 1. Average S. uberis log_{10} cfu/g wet weight (± SEM) contamination levels in races of high and low cow traffic during a 12-month period

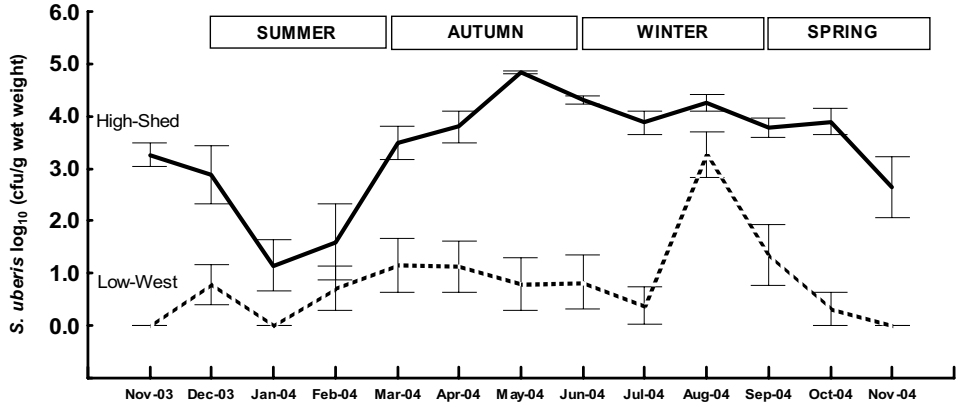

Figure 2. Average S. uberis log_{10} cfu/g wet weight (± SEM) contamination levels during the 12-month period for the race next to the farm dairy and a low traffic race

Streptococcus uberis contamination in paddocks: before and after grazing

Contamination levels of *S. uberis* during mid and late lactation, and during the dry period are shown in Table 1. No *S. uberis* was isolated in any of the paddocks prior to cows grazing. *S. uberis* was detected one day after and up to two weeks after cows grazed the paddock. Isolation of the bacterium was always achieved from the soil surface the day after grazing in all stages of lactation.

During the lactation period, *S. uberis* was only detected in grass and the soil surface the day after cows had grazed. In the dry period, *S. uberis* was isolated from the three

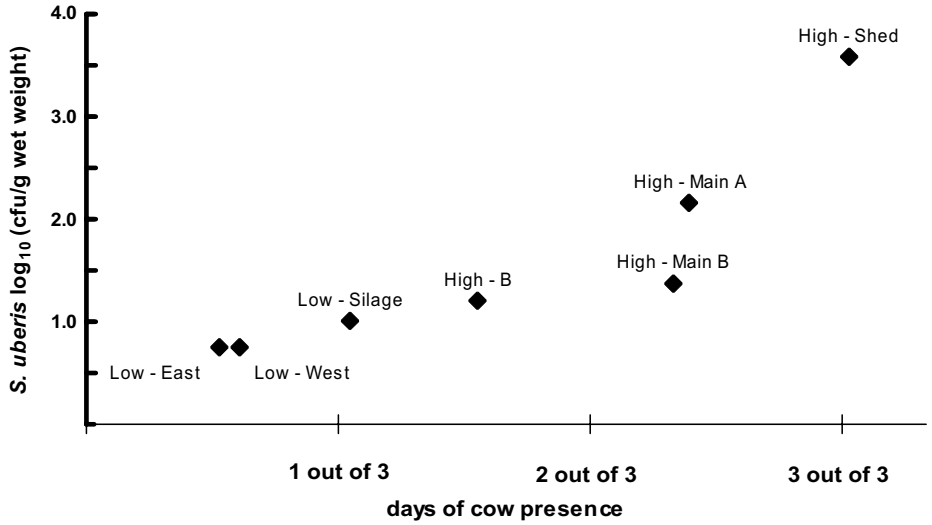

Figure 3. Average annual number of days of cow presence three days before sampling and S. uberis contamination levels in farm races (High = high traffic; Low = low traffic).

Table 1. Average contamination levels of S. uberis log_{10} cfu/g wet weight (max. values in parentheses) in ungrazed and grazed paddocks during three stages of lactation

Lactation stage	Source	Pre-grazing		Post-Grazing	
		-1 d	+1 d	+1 wk	+2 wk
Mid lactation	Grass	0	0	0	0
	Soil surface	0	0.1 (1.1)	0	0
	Sub-soil	0	0	0	0
Late lactation	Grass	0	0.2 (0.8)	0	0
	Soil surface	0	0.1 (0.5)	0	0
	Sub-soil	0	0	0	0
Dry period	Grass	0	0.3 (1.4)	0	0
	Soil surface	0	0.6 (1.4)	1.0 (1.9)	0.8 (2.1)
	Sub-soil	0	0.1 (1.8)	0	0.3 (1.6)

sampling sources the day after grazing, and in the soil surface it was detected up to two weeks after grazing. It was only in the dry period that *S. uberis* was isolated from the sub-soil, and persisted until two weeks after grazing.

Discussion

This study investigated the levels of *S. uberis* occurrence in dairy farm races and paddocks during a 12-month period. There was a three-fold difference between the high and low-traffic race contamination levels. In high and low-traffic races, an increased recovery of *S. uberis* was observed in the months of April to September. This coincided with low solar

radiation levels, low temperatures and higher soil moisture levels that are known to enhance the survival of bacteria (Lopez-Benavides *et al.,* 2005). It was observed that the 'Shed' race was normally muddy and faecal matter was always present. However, in the summer months (January and February), the 'Shed' race was drier and bacterial recovery was consistently lower.

Based on a 10 g race sample of the 'Shed' race, we calculate that on average cows may be exposed to muddy race material of 4 X 10^6 cfu/m^2 *S. uberis* inoculum at least four times per day. Exposure of cows to such muddy and faecally-contaminated material may increase the risk factor of teat contamination with *S. uberis.* Pankey *et al.* (1996) suggested that practices such as minimising exposure to muddy conditions prior to calving may decrease the chances of cows suffering from mastitis at calving. Future experiments that explore cause-effect situations are necessary to support these hypotheses.

Our data support the hypothesis that faeces play a significant role in contaminating the races with *S. uberis*, even though the primary reservoir of *S. uberis* in the cow is yet undefined. Bramley isolated low numbers of *S. uberis* from around 35% of faecal samples and suggested that faeces acted as an inoculum of *S. uberis* on bedding material. Cullen (1966) proposed that the lips were the primary reservoir of the bacteria and that the isolation in rectal swabs reflected the occasional passage of the bacteria through the digestive system. Sweeney (1964) argued that the udder surface was the most important *S. uberis* reservoir. Our experience from monthly monitoring of around 100 cows (Lacy-Hulbert *et al.*, 2005) shows that around 5-10% of cows shed *S. uberis* in their faeces. It is very likely that sites of cow congregation in the farm provide an opportunity for the bacteria to transfer from environmental fomites onto the cow udder and teats by contact with contaminated material. Cows walking through highly contaminated races may increase their possibilities of acquiring a *S. uberis* intramammary infection. In low-traffic races, higher *S. uberis* levels were observed when cows walked on the races, but in the following sampling the bacterial levels usually decreased to very low levels. Management of races (e.g. alternative races or rotation of highly used races) that allow the natural decrease in bacterial populations may help to reduce the chances of the bacteria gaining entry into the mammary gland and causing infection.

Streptococcus uberis is an environmental bacterium that can be isolated from the dairy environment, although it requires cow presence to provide the initial inoculum. Isolation of *S. uberis* from paddocks was positive only after cows grazed the paddock. In the lactation period, it was only recovered the day after cows left the paddock, but in the dry period (winter months) *S. uberis* could be recovered for up to two weeks following grazing. In New Zealand it is common practice to increase the grazing interval and stock density during the winter period to meet herd feed requirements (Bryant and L'Huillier, 1986; MacDonald and Penno, 1998). At the Dexcel Lye Farm, the grazing interval was 120 d during the dry period. It is probable that the increased number of days that cows spent grazing the paddock also raised the total bacterial inoculum. A higher bacterial concentration and adequate weather conditions (low temperatures, higher soil moisture and low solar radiation) (Lopez-Benavides *et al.,* 2005) during this critical time may increase the exposure of cow teats to *S. uberis* while lying on the paddock. Experiments that explore more closely the relationship between soil contamination and exposure of cow teats to bacterial inoculum during the winter months would be necessary to confirm this hypothesis.

The observations from this study show that high *S. uberis* contamination levels are found in farm races of high traffic and in paddocks during the winter months. It is suggested that these environmental niches are risk factors associated with increased exposure of bacteria onto cow teats. In periods of high susceptibility to intramammary infection, such as the dry and calving periods, cows should be managed in such way that contact of cow teats to *S. uberis* inoculum is minimal.

Acknowledgements

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A methodology to quantitatively identify effective measures to control environmental mastitis

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Abstract

One of the important micro-organisms causing environmental mastitis is *E. coli*

This paper describes a case study to illustrate the use of the microbial process risk modeling methodology (MPRM) to estimate the exposure udder teats to an infectious microorganism, the occurrence of mastitis in a specific situation and to quantitatively identify effective control measures.

Keywords: E. coli, risk assessment, modeling, Monte-Carlo simulations

Introduction

Objective

Mastitis causing micro-organisms such as *E. coli* and *Streptococcus uberis* are present in various reservoirs in the farm environment. Measures to control mastitis related to these micro-organism should therefore aim at the farm environment. In order to identify the most effective control measure microbial process risk models (MPRM) applied in food safety risk assessment can be applied to environmental mastitis (Cassin *et al.*, 1998). Objective of this paper was to illustrate this approach using *E. coli* as a case study.

Risk assessment using MPRM

In risk assessment four stages can be distinguished. (1) hazard identification; (2) exposure assessment, in which the exposure of in this case udder teats to *E. coli* is estimated; (3) Dose-response assessment, in which it is determined whether the magnitude of exposure results in an infection; and (4) risk characterization, the results of the exposure and dose response assessment are used to estimate the effects likely to occur in a given population or herd.

Using the MPRM methodology stages 2 to 4 can be quantified. Advantage of the MPRM methodology is the description of health risks (in this case animal health) as a function of interpretable and measurable variables. This enables the identification of a effective control strategy based on model simulations.

Materials and methods

Infection scheme

In order to illustrate the principle of the approach, the MPRM methodology was applied to a simplified infection scheme of udder teats with infectious *E. coli*, (Figure 1). The

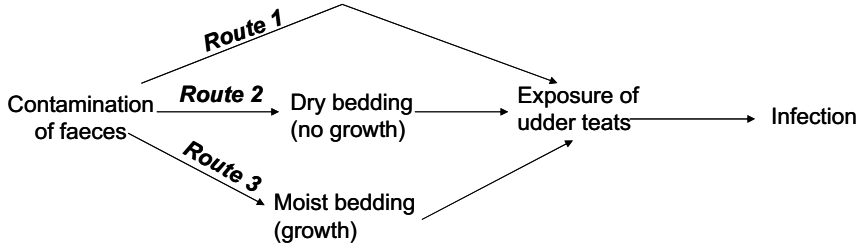

Figure 1.Simplified infection scheme for mastitis caused by E.coli as applied in the case study

infection system starts with contaminated faeces. *E. coli* present in the faeces can infect the udder teats of a single cow via three routes.

1. Direct contamination of the udder teats during excretion of faeces;
2. Faeces is dropped on dry bedding and after a cow lies down additional contamination takes place;
3. Faeces is dropped on moist bedding. In moist bedding growth can occur and as a result the contamination level of faeces in moist boxes will exceed the level in dry boxes.

Model simulations

The models used to perform the exposure and dose response assessment were programmed in Excel. Monte Carlo simulations were performed using @Risk and Latin-Hypercube sampling. Each simulation consisted of 5000 iterations. Variable and parameter values or distributions were estimated based on literature experimental data and expert knowledge.

Risk assessment

Before the MPRM methodology was applied the following assumptions were made:

- All cows excrete faeces with the same amount of infectious *E. coli*;
- Each cows only lies down in his own specific box that is either dry of moist;
- All *E. coli* are evenly spread over the four teats;
- All *E. coli* transferred to the udder teat surface have the ability to be transferred to the inside of the teats and cause an infection;
- The probability of infection is independent of the time passed since the last milking.

Again these assumptions reflect a simplification, but the assumptions were considered to be in line with the defined objective. Besides MPRM models have a very open structure and can be made more detailed when necessary.

Exposure assessment

Model set-up

The contamination pathway of raw milk with bacteria, e.g. butyric acid bacteria, at dairy farms can be considered as a chain of unit-operations (Vissers *et al.*, 2005). In this case, the same approach was followed for the exposure model. For example, faeces was considered as a contamination source, presence of *E coli* in the bedding material was considered to be

a storage step and contamination of the teats was (for route 2 and 3) the result of cross contamination between bedding and teats.

Also in line with (Vissers *et al.*, 2005) the dairy herd was divided into three groups to differentiate in farm hygiene. These three groups are:

1. Hardly contaminated cows with no visible dirt attached to the teats ≡ clean cows. Clean cows are only contaminated via the direct route (1).
2. Moderately contaminated cows with visible dirt attached to the teats ≡ dirty cows. Dirty cows are contaminated via the direct route (1) or one of the two indirect routes (2 or 3). The number of dirty cows is an indication of farm hygiene.
3. Highly contaminated cows with large amounts of dirt attached ≡ filthy cows. Filthy cows are also contaminated with the direct route (1) or one of the two indirect routes. Filthy cows are cows that prefer to lie down on dirty patches, a preference independent of farm hygiene. The udder teats of filthy cows are more contaminated than the teats of dirty cows.

Each cow group relates to a specific mass of faeces attached to the udder teats. For dirty and filthy cows it was assumed that the amount of faeces transferred to the teats via route 1 was equal to the total contamination of clean cows.

Calculation procedure

The exposure model calculates the exposure per teat for each cow separately. Based on the fraction boxes with moist bedding first each cow of the herd was assigned randomly either a dry or moist box. For each cow-bedding combination different equations were used. To estimate growth in moist bedding an exponential microbial growth model was used (equation 1). In this equation the time for growth (t) was assumed to equal the time between two box cleanings (a worst case estimate).

The specific growth rate ($\mu_{specific}$) in equation 2 depends on temperature (equation 3) and pH (equation 4) of the faeces and was estimated using the gamma concept(Zwietering *et al.*, 1996). The growth rate under optimal conditions (μ_{opt}), minimum, optimum growth temperature (T_{min}, T_{opt}) and minimum, optimum and maximum growth pH (pH_{min}, pH_{opt}, pH_{max}) are model parameters and have a specific value for each micro-organism. The values of these parameters for *E. coli* are shown in Table 1.

$$C_{faeces\_after\_growth} = C_{faeces} * \exp(\mu_{specific} * t) \quad (1)$$

$$\mu_{specific} = \mu_{opt} * \gamma(T) * \gamma(pH) \quad (2)$$

with

$$\gamma(T) = ((Temperature - T_{min})/(T_{opt} - T_{min}))^2 \quad (3)$$

$$\gamma(pH) = ((pH - pH_{min}) * (pH_{max} - pH))/((pH_{opt} - pH_{min}) * (pH_{max} - pH_{min})) \quad (4)$$

Variable values

Variables in the model were represented with either a single value or a distribution. Variables represented with a single value are input variables and relate to factors a farmer can control via management. The model contained 6 input variables: (1) herd size; (2) number of dirty cows; (3) number of filthy cows; (4) number of moist boxes; (5) cattle house temperature and (6) time between two cleanings of the boxes.

Table 1. Growth characteristics E. coli (Retrieved from Gothier et al., 2001).

Description	*Variable name*	*Unit*	*Value*
Growh rate under optimal conditions	μ_{opt}	hr^{-1}	1,8
Minimum growth temperature	T_{min}	°C	11,2
Optimum temperature for growth	T_{opt}	°C	41,3
Minimum pH needed for growth	pH_{min}	-	4,0
Optimum pH for growth	pH_{opt}	-	6,5
Maximum pH for growth	pH_{max}	-	9,0

Table 2. Uncontrollable variables in the exposure model and the applied distributions.

Description	*Variable name*	*Unit*	*Distribution*
Contamination level faeces	C_{faeces}	CFU/g	10^pert(1;2;3)[1]
pH faeces	pH	-	normal(6,9;0,4)[2]
Faeces attached to clean cow	M_{clean}	g	pert(0,01;0,04;0,5)[3]
Faeces attached to dirty cow	M_{dirty}	g	pert(1;3;10)[3]
Faeces attached to filthy cow	M_{filthy}	g	pert(15;25;40)[3]

[1]*Estimation*

[2]*Retrieved from experimental data*

[3]*Retrieved from expert opinion and (Stadhouders and Jorgensen, 1990)*

Distributions relate to factors a farmer can not control through his management but that can affect the exposure. Applied distributions are listed in Table 2.

Dose response assessment

A dose-response relationship describes the probability of infection ($P_{infection}$) given a specific exposure ($Exposure_{teat}$). Provisionally equation 5 was applied as a hypothetical dose response relation. This equation gives a 1% probability of infection in case a teat is exposed to 100 infectious *E. coli*.

$$P_{infection} = 0.0001\ Exposure_{teat} \quad (5)$$

Risk characterization

The risk for a specific dairy herd on environmental mastitis caused by *E.coli* can be simulated through combination of the exposure and dose response models. In the developed MPRM the number of infected udder teats ($N_{teats\_infected}$) was calculated using equation 6. The model also calculated the number of infected cows within the herd.

$$N_{teats\_infected} = binomial(4;P_{infection}) \quad (6)$$

Results

To illustrate the use of the MPRM approach to environmental mastitis two simulations were performed for a hypothetical farm with the following values for the input variables:

- Number of cows: 65
- Number of dirty cows: 8
- Number of filthy cows: 2
- Number of moist boxes: 4
- Temperature of faeces in boxes: 15 °C
- Time between two cleanings of the boxes: 8 hr.

In the first simulation the number of infected cows for this farm was simulated, results in Figure 2. This figure indicates that in this specific situation there is a probability of about 40% that no cows will get mastitis due to *E. coli* infection and the number of infected cows will be below 4 with a probability of 95%.

In the second simulation the effect of changes of the different input variables on the probability of 0 infected cows in the herd was determined. In this simulation the probability of 0 infected cows was determined for each variable (except the herd size) at a value 50% less than the initial value (thus for example 4 dirty cows in stead of the initial 8). The

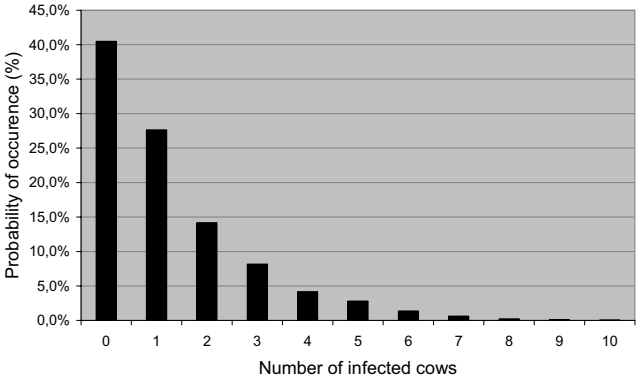

Figure 2. Probability of different number of infected cows for a dairy herd with 65 cows including 8 dirty and 2 filthy cows, 4 moist boxes, a temperature of 15 °C and 8 hours between two cleanings of the boxes.

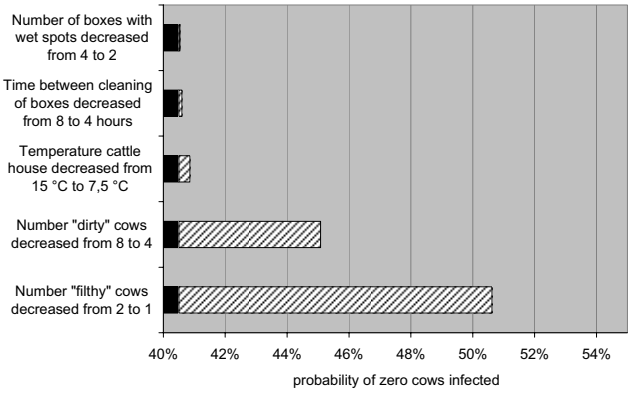

Figure 3. Effect of different control measures on the probability of zero infections, black bar is current level, striped bar indicates effect of measure.

results of this calculation are plotted in Figure 3. This figure shows that the most effective way to increase the probability of a mastitis free herd is to decrease the number of filthy cows. It is also effective to improve farm hygiene and consequently decrease the number of dirty cows. Measures related to decreasing the growth in moist bedding will not result in the desired effect.

Conclusion

This case study demonstrates that MPRM methodology can be applied to quantify the risk of occurrence of environmental mastitis at a specific farm, and to identify effective control measures quantitatively. The simplified case clearly showed that farm hygiene has more effect on the occurrence mastitis caused by *E. coli* than the presence of moist bedding in the cattle house.

As in food safety risk assessment the most difficult stage will be to derive the dose-response relationship, due to absence of relevant data. But even without a proper dose-response relationship, the development of an exposure model with this methodology is useful to and to compare various options for control of environmental mastitis via scenario analysis.

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Food safety issues related to mastitis

On-Farm sources of foodborne pathogens: Isolation from the dairy farm environment

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Abstract

The objective was to determine on-farm sources of *Campylobacter jejuni*, *Salmonella* spp., *Listeria monocytogenes*, and Shiga toxin-producing *Escherichia coli* (STEC) which might serve as reservoirs for transmission of pathogens. Six visits were made to 4 dairy farms to collect swab, liquid and solid dairy farm environmental samples. *Campylobacter jejuni*, *Salmonella* spp., *L. monocytogenes*, Sorbitol-negative (SN)-STEC O157:H7 and sorbitol-positive (SP)-STEC were isolated from 5.06%, 3.76%, 6.51%, 0.72%, and 17.3%, respectively, of samples evaluated. SN-STEC O157:H7 was isolated from only two farms, whereas other pathogens were isolated from all 4 farms. Diverse serotypes of SP-STEC including O157:H7, O26:H11, O111, and O103 were isolated. None of the five pathogen groups studied were isolated from bulk tank milk (BTM). Most pathogens (44.2%) were isolated directly from fecal samples; bovine fecal samples, lagoon water, and bedding constituted areas of major concern on dairy farms. Although in-line milk filters from two farms tested positive for *Salmonella* or *L. monocytogenes*, these pathogens were not detected in the corresponding BTM samples. Good manure management practices are critical in assuring dairy farm hygiene. Identification of on-farm pathogen reservoirs could aid with implementation of farm-specific pathogen reduction programs.

Keywords: Shiga toxin-producing *Escherichia coli, Listeria monocytogenes, Campylobacter jejuni, Salmonella* spp., dairy farm environmental samples

Introduction

More than 200 known diseases are transmitted through food by a variety of agents that include bacteria, fungi, viruses, and parasites. According to public health and food safety experts, each year millions of illnesses in the United States and throughout the world can be traced to foodborne pathogens. While the food supply in the United States is one of the safest in the world, the Centers for Disease Control and Prevention (CDC, 2003; CDC, 2004) estimates that 76 million people get sick, more than 300,000 are hospitalized, and 5,000 die each year from foodborne illness. STEC (O157 and non-O157 serotypes), *L. monocytogenes*, *C. jejuni,* and *Salmonella* spp generally have low infective doses (Mortimore and Wallace, 1994), can cause diseases with extensive debilitating effect, and are a major public health concern worldwide (Mead *et al.*, 1999). Reducing bacterial pathogen contamination of our food supply could save both lives and billions of dollars in costs annually.

Dairy farms are an important reservoir of foodborne pathogens. The presence of foodborne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and in some cases to excretion from the udder of an infected animal. Most foodborne pathogens inhabit the ruminant intestinal tract, and therefore, dairy cattle are considered a major reservoir of *Salmonella*, *Campylobacter,* and STEC. *Listeria monocytogenes* is found widespread in nature and lives naturally in plants and soil environments. Epidemiological studies have shown that cattle probably become infected through consumption of water and feedstuffs contaminated with feces and other cattle secretions/excretions. Previous studies from our laboratory on 30 dairy farms confirmed that BTM and culled dairy cow feces were potential sources of *E. coli* O157:H7 (Murinda *et al.*, 2002a) and *Salmonella* spp. (Murinda *et al.*, 2002b). Four farms (A, B, C and D) that represented the diversity of farms in stockmanship, management styles and types of pathogens isolated were selected for the current study. Farms A and D were positive for both *Salmonella* and *E. coli* O157:H7. Farm C was negative for *E. coli* O157:H7, but positive for *Salmonella*, whereas farm B was negative for both pathogens. The present study was conducted to investigate the major habitats of pathogens on dairy farms that could act as reservoirs or transient carriers of foodborne pathogens.

Materials and methods

Six visits (January/February, April, June, and September 2002, and March/April and June 2003) were made to 4 dairy farms to collect swab, liquid and solid environmental samples. In addition, BTM and in-line milk filters were collected. The profile of samples analyzed is in Table 1. Samples were collected using aseptic techniques into sterile containers and were transported to the lab, stored overnight at 5°C and analyzed the next day, or at most, within a week of collection. Swab samples were placed in screw-cap tubes containing 6 ml buffered peptone water (0.1%, vol/vol). Fecal slurry/pats (100 g) were collected from at least 6 locations using spatulas and composited. Liquid samples were collected using ladles equipped with screw caps. Bulk samples (100 g), such as feed and bedding (sand, wood shavings or straw) were collected into zip-loc bags using sterile gloves from at least 6 locations, and composited. Rats and flies were collected using live traps and sticky tape, respectively. Rat and bird intestinal contents, and whole flies were blended (1:9 ratio) in enrichment media. Solid samples were comminuted, then stomacher- or hand-blended (for sand) for 60 sec in enrichment media. In-line milk filters were collected after milking, placed in a sterile zip-loc bag, and prepared for bacteriological analysis by stomaching in 400 ml buffered peptone water. The resulting suspensions were added to enrichment broths (11 ml:99 ml).

Isolation of *L. monocytogenes*, *Salmonella,* and STEC: Solid samples (11 g) or 11 ml of liquid samples were transferred to 99 ml (pre)enrichment broth. Solid samples were blended for 60 sec. For small sample volumes, such as swabs (made of soluble calcium alginate tips), 1 ml of vortexed (60 sec) sample was transferred to 9 ml enrichment broth. Pathogens were isolated using the US Food and Drug Administration's Bacteriological Analytical Manual (2001 http://vm.cfsan.fda.gov/ ~ebam/bam-toc.html). However, the primary enrichment medium of each pathogen was substituted with universal pre-enrichment broth (UPB; Difco, Sparks, MD; Nam *et al.*, 2004). STEC were isolated without secondary enrichment, however, *Salmonella* and *Listeria* were isolated following secondary enrichment in tetrathionate broth

and Fraser broth, respectively. SN-STEC O157:H7 were isolated and confirmed using the protocol described by Murinda *et al*. (2002a). Other STEC were isolated from 104 samples enriched in UPB using acid-treatment to select acid-resistant, tellurite-resistant strains. Reference strains from the American Type Culture Collection (ATCC, Manassas, VA) including *L. monocytogenes* (ATCC 19115 and ATCC 15313), *Salmonella* Typhimurium (ATCC 14028 and ATCC 9706), *E. coli* O157:H7 (ATCC 43889 and ATCC 43888), were used for quality control.

Isolation of *C. jejuni*

Samples were prepared as described above using Bolton broth (BB) with *Campylobacter* growth supplement, SR183E (Oxoid Inc., Napean, Ontario, Canada). Test tubes were loosely capped and incubated at 42°C for 48 h under microaerophilic conditions (O_2:CO_2:N_2 = 5%:10%:85%; vol/vol) in a gassed incubator. Before entering the incubator, gases were prefiltered via 0.2 µm-pore-size filters. Cultures were streaked onto blood-free charcoal cefoperazone deoxycholate agar, CCDA, (Oxoid Inc.) containing CCDA selective supplement, SR155 (Oxoid Inc.). Alternatively, 0.1 ml of BB culture was passed through 0.45 µm-pore-size nitrocellulose filters (Millipore Corp., Bedford, MA) resting on CCDA, and after 30 min contact, filters were removed. Plates were incubated at 42°C for 48 h after which 5 presumptive *Campylobacter* colonies were picked per duplicate plate, and streaked for isolation on antibiotic-free Abeyta-Hunt-Bark agar plates. Plates were incubated as before, and presumptive *Campylobacter* colonies were examined microscopically for vibrioid morphology, and typed biochemically using oxidase, catalase and hippurate tests. *Campylobacter jejuni* strains ATCC 43457 and ATCC 43430 were used for quality control.

Detection of pathogens by Multiplex PCR

Presumptive positive isolates of all pathogens were confirmed by multiplex PCR targeting mostly toxin-encoding or serotype specific genes. Reference ATCC strains described above carrying relevant characteristics were used for quality control. Primers were obtained from a commercial source (Integrated DNA Technologies, Inc., Coralville, IA). Concentrations of primers for all multiplex PCR protocols were: 0.05 µM - 0.5 µM per 50 µl reaction mixture. Other components were: 3 - 4 mM $MgCl_2$, 200 µM dNTPs, 1X buffer, 0.05 U *Taq* polymerase, and 5 µl DNA (balance; distilled water). Common multiplex PCR annealing temperatures for mixed primer pairs were derived by gradient PCR. Primer concentrations and $MgCl_2$ were adjusted empirically to optimize PCR reactions. DNA extraction and preparation of reaction mixtures was conducted as reported previously (Murinda *et al.*, 2002a). Initial denaturation was conducted at 94°C for 4 min, followed by 30 amplification cycles each of denaturation at 94°C for 30 sec, optimum annealing at 56°C-57°C for 30-45 sec, and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 5-7 min. Gel electrophoresis and documentation were conducted as described (Murinda *et al.*, 2002a). For *Salmonella* detection, a multiplex PCR incorporating hemolysin A gene (*himA*) and INVA-1/INVA-2 (invasive gene; *invA*) described by Chen *et al*. (2000) and Chiu *et al*. (1996), respectively, were used. *Listeria monocytogenes* was confirmed using HlyF/HlyR (listeriolysin O gene, *hlyA*) and LAI/LB1 primers described, respectively, by Nogva *et al*. (2000) and Makino *et al*. (1995). The protocol used for *C. jejuni* detection was developed recently by our group (Murinda *et al.*, 2004). Briefly, hippuricase gene sequences, HIP400F/HIP1134R, described by Linton *et al*. (1997) and universal thermophilic *Campylobacter* 23S rRNA gene sequences (THERM1/THERM4) described by Fermer and Engvall (1999) were used in multiplex format

to confirm *C. jejuni*. The multiplex PCR technique described by Fratamico *et al*. (2000) was used to confirm presence of Shiga toxin-encoding genes (stx_1 and stx_2) and ancillary virulence genes in STEC isolates.

Serological characterization of STEC

SN-STEC O157:H7 were tested for serology as described (Murinda *et al*., 2002a). Acid-resistant, tellurite-resistant isolates identified as STEC using multiplex PCR were sent to the *E. coli* Reference Center (Penn State University, University Park, PA) for O:H serological characterization.

Results and discussion

A total of 16%, 10%, 6%, 3.4%, 43.6% and 20.5 % of total pathogens, excluding the SP-STEC, were obtained over the 6 sampling visits to dairy farms conducted in January/February, April, June, and September 2002, and March/April and June 2003 (data not shown). A total of 84%, 33%, 40% and 73 % of *L. monocytogenes*, *C. jejuni*, SN-STEC O157:H7 and *Salmonella* spp. were isolated from samples collected in the last 2 farm sampling visits. *Salmonella* spp. were the most ubiquitous followed by *L. monocytogenes* and *C. jejuni*. The pathogens were, respectively, isolated from 9, and 8 each, of the 15 sample types that were examined (Table 1). SN-STEC O157:H7 and SP-STEC were isolated from 3 and 5 sample types, respectively. Some SP-STEC serotypes (O145, O103, O26, O136 and O84) possessed only stx_1 gene sequences; others had stx_2 alone (O?:21, O2 and SP-STEC O157:H7), whereas O111 had only stx_1 or both stx_1 and stx_2. SN-STEC O157:H7 had both gene sequences

Table 1. Detection of foodborne pathogens in dairy farm environmental samples.

Sample type	Number samples	Pathogens isolated[1]			
		L. monocytogenes	*Salmonella*	*C. jejuni*	*SN-STEC O157:H7*[1]
Feed/silage	97	6	3	1	0
Trough water	92	1	3	0	0
Lagoon water	94	7	2	13	0
Fecal slurry	98	14	4	7	2
Calf fecal swab	86	1	2	4	1
Heifer fecal swab	4	0	0	1	0
Bedding	90	13	6	5	0
Parlor floor	10	0	0	3	0
Bulk tank milk	49	0	0	0	0
In-linemilk filters	24	1	1	0	0
Hose water	17	0	0	0	0
Bird droppings	20	2	2	1	2
Flies	5	0	0	0	0
Rats	4	0	3	0	0
Birds	1	0	0	0	0
TOTAL	691	45 (6.51%)	26 (3.76%)	35 (5.06%)	5 (0.72%)

[1]Pathogens were generally isolated using protocols outlined in the US Food and Drug Administration's Bacteriological Analytical Manual (2001: Available online: http://vm. cfsan.fda.gov/ ~ebam/bam-toc.html).

and the usual ancillary virulence factors (Murinda *et al.*, 2002a) associated with this serogroup (data not shown). Ancillary virulence factors were encountered at a lower frequency among SP-STEC (data not shown). A total of 72.2% of the SP-STEC isolates were of fecal origin; 27.2% were from bedding, trough water and feed/silage. *Campylobacter jejuni*, *Salmonella* spp., *L. monocytogenes* and SN-STEC O157:H7 were isolated from 5.06%, 3.76%, 6.51%, and 0.72% of samples tested, respectively (Table 1). SP-STEC were isolated from 17.3% of samples tested.

SN-STEC O157:H7 were isolated from only two farms, whereas other pathogens were isolated from all 4 farms. SP-STEC, including an O157:H7 isolate, were found on all four farms. Farm A had the least number of pathogens isolated; 59.4% - 61.5% less isolates than farm B, C and D. None of the 5 pathogen groups studied were isolated from BTM, however, one isolate each of *L. monocytogenes* and *Salmonella* were found on in-line milk filters from two farms.

Most pathogens (44.2%) in the present study were isolated directly from fecal samples. Bovine fecal samples, lagoon water, bedding, bird droppings and rats constituted areas of major concern on dairy farms. Some of these targets can be defined as critical control points on the dairy farm, where pathogen reduction interventions can be introduced. Although in-line milk filters from two farms tested positive for *Salmonella* or *L. monocytogenes*, these pathogens were not detected in the corresponding BTM samples.

Among the four farms studied, farm A had the best rodent and manure-management practices; this appeared to be reflected in the fewer number of pathogens isolated (59.4%-61.5% less) than farm B, C and D. As in our earlier studies (Murinda *et al.*, 2002a, Murinda *et al.*, 2002b), Farm D was positive for both STEC O157:H7 and *Salmonella* spp., while farm C was negative for STEC O157:H7 and positive for *Salmonella*. These data appear to indicate persistence of the pathogens at both farms or presence of locally cycling populations. Farm A was positive for both pathogens in the first study, although in the current study, it was O157:H7 STEC-negative, but *Salmonella*-positive. Farm A had the most improved manure management practices and had eradicated rats which were rampant in the first study. On the other hand, farm B was negative for both pathogens in the first study, but was *Salmonella* and SN-STEC O157:H7-positive in the current study; this might suggest a deterioration of management conditions at this farm. Our observations at farm B support these inferences. The retrogression of farm B from negative to positive was correlated by certain poor management practices, visually, there were infrequent changes of heavily manure-laden straw bedding and frequent changes in milking staff. Furthermore, due to off-farm commitments, the farm owner spent less time on the farm than during the first study.

Conclusions

Dairy farms constitute a major source of pathogens that could be transmitted to humans. Good manure management practices are critical in assuring hygiene. Our data indicate that there could be many environmental habitats of pathogens outside cattle on dairy farms. It is not known whether these habitats are merely transient carriers, or are true reservoirs that sustain pathogens for long durations in the absence of cattle. Further studies, genetic or otherwise, are required to establish the reservoir status of habitats, including if or how these habitats are responsible for cycling pathogens on the farms, and to verify whether the same subtypes are persistent on farms. These data are relevant for purposes of pathogen

control or eradication. The current hypothesis is that the presence of pathogens on the farm depends on ingestion of contaminated feed followed by amplification in bovine hosts and fecal dissemination in the farm environment. The final outcome of this cycle is a constantly maintained reservoir of foodborne pathogens that can reach the human population by direct contact, ingestion of raw contaminated food (raw milk or cheese made with raw milk), or contamination during the processing of milk.

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Shiga Toxin Genes from *Escherichia coli* strains isolated from mastitic milk

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Abstract

The bacterial species *Escherichia coli* includes a variety of different types that range from avirulent, commensal strains to virulent strains that cause a variety of severe infections. Over 700 antigenic types of *E. coli* have been recognized. In dairy cows *E. coli* are regarded as opportunistic and environmental pathogens that can cause mastitis.

Recent advances in molecular biology have facilitated the investigation of virulence factors in bacteria. We used DNA amplification to investigate the occurrence of 10 virulence factors in 80 isolates of *E. coli* from mastitis milk. The most common detected virulence gene was *stx*1 (31%), *cnf*2 (7.5%), *stx*2e (6.25%) and *eae*A (4%). The possession of such virulence genes by bacteria had no discernable influence on the severity of both the localized and systemic symptoms of infected cows. However the possession of toxin genes in mastitis isolates of *E. coli* is problematical, particularly for both the public health and animal husbandry. Infection of man by shiga toxin *E. coli* (STEC) can range from mild diarrhoea to severe disease such as haemorrhagic colitis and the haemolytic uraemic syndrome. Furthermore the practice of feeding mastitis milk to calves should be discouraged both from an animal health perspective and as a preventative measure to reduce colonization of cattle by these strains and ultimately carriage rates. Over 200 serotypes of *E. coli* can produce shiga toxins and rates of colonization of STEC of any serotype in cattle herds can range from 10-25%.

Keywords: virulence genes, shiga toxins, bacteriophages

Introduction

The bacterial species *Escherichia coli* includes a variety of different types that range from avirulent, commensal strains that are present in the normal intestinal flora to highly virulent strains that cause a variety of severe infections in both humans and animals. Over 700 antigenic types or serotypes of *E. coli* have been recognized based on O, H, and K antigens (Kaper *et al.*, 2004).

In dairy cows *E. coli* are regarded as opportunistic and environmental pathogens that not uncommonly can cause infection and inflammation of the mammary gland. The incidence of *E. coli* mastitis varies with country. Whereas the prevalence of this environmental pathogen is low in New Zealand, it is the main causative agent of environmental mastitis in the United Kingdom (Bradley and Green, 2001). There has been a substantial rise in the incidence of *E. coli* mastitis in some countries since 1960 and it is accepted to be the most common microbial cause of fatal mastitis (Wenz *et al.*, 2001). In contrast to the

enteropathogenic and bacteraemia strains which are caused by a relatively low number of *E. coli* serotypes, the isolates from bovine coliform mastitis belong to a very large number of serological groups and are similar to faecal isolates (Burvenich *et al.*, 2003).

The pathogenicity of a specific *E. coli* strain is considered to be mainly determined by specific virulence factors (such as adhesins, invasins, toxins and capsules) which are frequently encoded on genetic elements that can be mobilised into different strains of *E. coli* to create novel combinations of virulence factors. These virulence factors are often organized in large blocks called pathogenicity islands, and are located on either the chromosome or large plasmids, or are transmitted by bacteriophages. Genomic comparisons between the sequenced *E. coli* strains have revealed that the pathogenic strains contain approximately 25% extra genomic DNA as compared to the non-pathogenic type strain of K-12 (Kaper *et al.*, 2004). Only the most successful combinations of virulence factors have persisted to become specific "pathotypes" of *E. coli* that are capable of causing diseases in healthy individuals. Three general clinical syndromes can result from infections with one of these pathotypes: enteric/diarrhoeagenic, urinary tract infections and sepsis/meningitis. All diarrhoeagenic strains of *E. coli* were initially termed enteropathogenic *E. coli* (EPEC) but as more is learnt about their pathogenic mechanisms, they are now grouped into seven classes depending on the virulence factors they possess: enteropathogenic *E coli* (EPEC), enterohaemorrhagic *E.coli* (EHEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E.coli* (EAEC), enteroinvasive *E.coli* (EVEC), diarrhoea-associated haemolytic *E.coli* (DHEC) and cytolethal distending toxin (CDT) producing *E.coli* (Smith, 1992).

Recent advances in molecular biology have facilitated the investigation of virulence factors in bacteria (Kuhnert *et al.*, 2000, and Pass *et al.*, 2000). The genes encoding virulence factors are tightly regulated by a variety of factors and their expression in bacteria is often repressed under laboratory conditions. This makes both the phenotypic characterization of virulence and the determination of the virulence in isolates from clinical specimens often difficult and unreliable (Kuhnert *et al.*, 2000). Previous studies have documented the occurrence of a variety of virulence factors in *E.coli* isolated from bovine clinical mastitis (Kaipainen *et al.*, 2002). This presumably reflects the mobile nature of these genetic elements and, not surprisingly, no single or group of virulence factors has been associated exclusively with bovine mastitis. Furthermore the possession of different combinations of virulence factors appear to have little influence on the severity of mastitis per se, as the severity of *E.coli* mastitis in the bovine is mainly mediated by host factors rather than by bacterial virulence factors (Burvenich *et al.*, 2003). In this respect, the pathogenesis of coliform mastitis differs from other diseases such as colibacillosis of calves and haemolytic uraemic syndrome (HUS) in humans, where the pathogenicity of these diseases mainly depends on the expression of particular bacterial virulence factors. Nevertheless the possession of virulence genes by mastitic *E. coli* isolates, especially those that may be potentially expressed during infection or on subsequent transfer to a new host, has important public and veterinary health ramifications. Unpasteurised milk contaminated with shiga toxins can cause adverse health effects in humans or animals that consume such a product, as has been reported by Clark *et al.*, 1997, Gillespie *et al.*, 2003, and Eurosurveillance Weekly, 2004. Furthermore the sporadic isolation of STEC from bulk tank milk reinforces these issues. (Jayarao and Henning, 2001, and Murinda *et al.*, 2002). In addition to these public health risks, coliform mastitis can also severely impact on the well being of affected animals (Mercado *et al.*,2004). Therefore we undertook a pilot study to

investigate the prevalence of 10 virulence factors representative of the 4 major pathotypes (STEC, EHEC, ETEC, and EIEC) that are associated with different diarrhoeagenic strains of *E. coli*.

Materials and methods

Bacterial isolates

Eighty *E.coli* isolates obtained from 3 dairy herds were cultured from the milk of thirty four cows diagnosed with clinical mastitis. Clinical mastitis was defined as any visible abnormalities (clots or blood) in the milk, and which frequently was accompanied by additional signs of pain and swelling of the mammary gland. Foremilk samples for culture were collected using standard aseptic technique by trained field technicians. Primary isolation was on blood agar followed by culture on MacConkey agar and presumptive identification using Gram's staining and IMVIC biochemical tests (Blazevic and Ederer, 1975). All isolates were further identified as *E. coli* by DNA amplification of the glutamate decarboxylase (gad) gene which is an *E. coli*-specific gene (McDaniels *et al.*, 1996).

DNA isolation

A single bacterial colony from the MacConkey agar plate was removed and transferred to brain-heart infusion agar for overnight growth. A loopful of cells was resuspended in sterile TE buffer (10mM Tris pH 8.0; 1mM EDTA) and the DNA isolated as previously described (Sleigh and Cursons, 2000). The DNA concentrations were then estimated by running 2 µl of the bacterial DNA on a 1.2% agarose gel and staining with ethidium bromide (0.5 µg/ml). The DNA concentration was then adjusted to 1-2 µg/µl.

DNA amplification

DNA amplification was performed in a total of 50µl using the buffer and $MgCl_2$ as supplied by the manufacturer of the Taq DNA polymerase (Roche). The final concentration of the deoxyribonucleotides was 200µM, the primers 20pM, the Taq (1.25U) and the DNA 1µg.The primers (with accession numbers in brackets) for the following E. coli genes: *gad* (M84024), *cnf*1 (U42629), *cnf*2 (U01097), *eae*A (X60439), *eagg* (X81423), *einv* (M32063), H7 (L07388), *Rfb* (S83460), *stx*1 (L04539), *stx*2 (AB048233), *lt*1 (S60731), and *vt*2e (M21534) were synthesised by Invitrogen New Zealand using previously published sequences (McDaniels *et al.*, 1996, Hu *et al.*, 1999, Pass *et al.*, 2000). All amplifications were performed using the following parameters: 94°C for 120 secs, then 40 cycles of 94°C for 20 secs, 60°C for 20 secs and 72°C for 40 secs. The amplified products were then analysed by ethidium bromide staining following gel electrophoresis in a 2% agarose gel. All amplifications included a negative control (no template added) and the amplification of the *gad* gene demonstrated that the isolated DNA was able to be amplified.

DNA sequencing

All genes that were successfully amplified from the *E.coli* isolates were sequenced once to confirm the specificity for the sets of primers. The amplified product was gel purified and the DNA cycle-sequenced in both directions using Big-Dye chemistry with the respective forward and reverse primers. The derived sequence was then analysed by inserting the sequence into BLAST at the NCBI site: (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

The DNA from the 80 isolates was amplified for specific *E. coli* and virulence genes. The *gad* gene was uniformly amplified in all 80 isolates whereas the genes for *cnf*2, *eae*A, *stx*1, *vt*2e and for *rfb* and H7 were sporadically detected in 34 of the isolates. The most common detected virulence genes were *stx*1 (31%), *cnf*2 (7.5%), *vt*2e (6.25%) and *eae*A (3.75%). The O157 gene *rfb* was detected in 3.75% and the H7 flagella gene in 1.25%. Two of the 80 isolates had both *cnf*2 and *stx*1 genes and one both *eae*A and *vt*2e. Overall, 30 of the isolates (37.5%) had a detectable virulence gene. There was no amplification for the virulence genes *cnf*1, *eagg*, *einv* or *lt*1 detected in any of the *E. coli* isolates.

All the infected cows displayed symptoms of mastitis such as localized changes in the visible appearance of their milk at the time of sample collection. Twelve cows had multiple infections resulting in 55 isolates of *E. coli* during the 17 month sampling period. In some cows, the infection incident was sporadically associated with additional symptoms of pain and swelling of the udder. In other cows the infection incident was associated with a systemic illness characterized by lethargy and loss of appetite and required administration of systemic antibiotics to resolve symptoms. Most of the recurrent infections were not genetically related to each other, as assessed by repetitive extragenic palindrome-typing (Versalovic *et al.*, 1991), but in one cow two isolates had an identical typing pattern to each other, even though there was a 30 day interval between sample collections. Fourteen of the multiple isolates from the repeatedly infected cows had a *stx*1 gene detected and 2 isolates a *vt*2e gene detected.

Discussion

Twelve cows with recurrent infections were responsible for the majority (68.75%) of the isolates. Bradley and Green, 2001, also reported a relatively high incidence of recurrent infections associated with *E. coli* mastitis. In contrast to our observations that most of the recurrent isolates from repeatedly infected quarters were not genetically related to each other Bradley and Green reported that 20.5% of their *E. coli* isolates from recurrent quarters were the same genotype. The difference in incidence of recurrent genotypes between both investigations may reflect the use of different sets of genotyping primers, or the percentage of identical quarters that became reinfected.

Of the 80 milks cultured, approximately one-third of the *E. coli* isolates contained genes for the synthesis of established virulence factors. The most common microbial toxin detected was *stx*1, followed in descending prevalence by *cnf*2 and *vt*2e. Shiga toxin-producing *E. coli* (STEC or Vero toxin *E. coli*, VTEC) are a heterogeneous group of enteric pathogens that have emerged as new food-borne pathogens of clinical and public health concern (Gavin and Thomson, 2004). Although shiga toxin-producing *Shigella dysenteriae* was first described nearly 100 years ago, the production of shiga toxin by *E. coli* was first described in 1977 by Konowalchuk *et al*. These protein-inhibiting toxins have been associated with a wide spectrum of human disease ranging from mild diarrhoea to severe diseases such as haemorrhagic colitis and the haemolytic-uraemic syndrome. The most important epidemic STEC serogroup has been O157:H7 (so named because it expresses the 157th somatic O and the 7th flagella H antigens), but about 200 other STEC serogroups have been identified. In

addition shiga toxins are sporadically produced by *Citrobacter* and *Enterobacter* spp (Kaper *et al.*, 2004).

Shiga toxin genes are encoded by bacteriophages that infect *E. coli* and cattle are considered to be the main reservoir of these strains. Over 200 serotypes of *E. coli* can produce shiga toxins and rates of colonization of STEC of any serotype in bovine herds range from 10-25%. Two classes of shiga toxin, *stx*1 and *stx*2 are recognized. Shiga toxin 1 is identical to the shiga toxin produced by *S. dysenteriae* and is genetically highly conserved whereas sequence variation exists within shiga toxin 2 (2c, 2d , 2e). The shiga genes are located in similar positions in the late region of the lamboid prophage genome and shiga toxin synthesis is under the control of the phage genes. Therefore, any compounds or physical events that induce the stx-converting prophages triggers increased production of the shiga toxin. These inducing events are variable and include ultra-violet irradiation, low iron containing media or the use of antibiotics such as quinolines, trimethoprim and furazolidine (Kimmitt *et al.*, 2000).

The pathogenesis of STEC infections in humans is not completely understood. However, the capacity of STEC strains to cause disease in man is associated with shiga toxin production, enterohaemolysin production and possession of the locus of enterocyte effacement (LEE). Thus only subsets of STEC are thought to be human pathogens (Beutin *et al.*, 1993). The LEE locus contains the eaeA gene encoding intimin which is a bacterial outermembrane protein involved in the intimate attachment of the bacteria to the gut mucosa of the host. Humans become infected with STEC primarily through ingestion of contaminated meat or other food products or by faecal-oral transmission (Garvin and Thomson, 2004).

Two forms of cytotoxic necrotizing factor, *cnf*1 and *cnf*2 have been described and *E. coli* strains that possess such genes are referred to as necrotoxic *E. coli*. Both forms are large monomeric proteins of 110-115kDa that induce mutinucleation and actin stress fibres in cell cultures. The *cnf*1 gene is found on a pathogenicity island whereas the *cnf*2 gene is found only on plasmids. The *cnf*2 gene has been reported to be present in *E. coli* strains isolated from the normal intestinal flora of health ruminants (De Rycke *et al.*, 1999).

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Influences on excretion of antibiotic residues in milk with special emphasis on milking frequency

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Abstract

The influence of milking frequency on excretion of antibiotic residues in milk was determined in worst case experiments with repeated treatment of all 4 udder quarters. 5 healthy cows per group were milked 3x, 2x or 1.5x per day after treatment with 4 different veterinary drugs containing 6 antibiotics alone or in combination. Shorter excretion periods in milk were observed for cows milked more frequently. For 4 antibiotics significant differences were determined between cows milked 3x and 1.5x per day ($p<0.05$). Similar experiments in cows with clinical mastitis showed no significant difference between cows milked 1.5x and 2x per day, respectively.

In addition to milking frequency influences of the antibiotic substance itself, the dosage and the variation between individual cows had effects on the withdrawal time. Inappropriate storage of udder injectors caused markedly prolonged excretion times in milk leading to violation of the MRL after the end of the withholding period.

Keywords: antibiotic residues, milk, milking frequency, pharmacokinetics, therapy

Introduction

Positive inhibitor tests are sometimes reported from practice after treatment of dairy cows with antibiotics, although the withholding period for milk was regarded. A series of experiments is performed in the institute to evaluate potential reasons for such events. Influences of antibiotic substance, drug formula, dosage and udder health status on excretion time in milk have been reported (Edwards, 1964, Schällibaum *et al.*, 1981, Ziv and Schultze, 1982, Moretain and Boisseau, 1989). Information on the influence of milking frequency on withdrawal times of residues in milk is limited. This aspect is of importance due to the increased numbers of automatic milking (AM) systems installed on dairy farms in recent years. These systems aim for increased milking frequencies but irregular or prolonged milking intervals may occur. The effect is also of interest for farms milking three times per day with conventional milking systems. Previous studies have mainly focused on the influence of frequent milk out on therapeutic concentrations in milk after treatment of dairy cows (Henschelchen and Walser, 1983). With milking frequencies simulated as in AM systems own investigations were performed in healthy cows according to the "worst case" procedure of the EMEA to determine withholding periods in milk. Times until concentrations in milk fell below MRLs as fixed by Regulation 2377/90/EG ff. were determined. These experiments were supplemented by studies in cows with clinical mastitis.

Materials and methods

Dairy cows

Trials in healthy cows: German Holstein, lactation number 1 to 6, days after calving 41-299, somatic cell count (SCC) in composite milk $<10^5$/ml at three samplings in weekly intervals before start of trials, average milk yield 21.4-37.8 kg per day, comparable milk yield of 3 groups tested per drug.

Trials in cows with clinical mastitis: German Holstein, lactation number 1 to 5, days after calving 1-422, SCC in composite milk between 3.2×10^5/ml and 7.0×10^6/ml, average milk yield per day between 10.3 and 37.9 kg. Six cows were included for more than one case of mastitis.

Treatment

Treatment scheme and milking frequency

Healthy cows: one injector per quarter, 4 quarters per cow (worst case), 3 treatments within 24 hours (Cobactan® LC) or 48 hours (Procain-Penicillin G 3 Mio., Nafpenzal® MC and Omnygram®), milking 2 times per day (interval 14/10 hours), 3 times per day (interval 8 h), 1.5 times per day (interval 16 h), 3 groups of 5 (4-6) cows per drug

Cows with clinical mastitis: one injector Cobactan® LC per quarter, only affected quarters (1-2 quarters per cow, additional systemic treatment in one cow), 3 treatments within 24 hours; milking 2 times per day (17 cases in 12 cows), 1.5 times per day (5 cases in 4 cows)

Sampling

Cow composite milk was sampled at every milking time including anamnesis, treatment period, indicated withholding period for milk plus 2 days.

Laboratory analysis

For quantitative detection of residues LC methods were applied (Knappstein *et al.*, 2003).

Analysis of variance

In order to determine factors with a systematic influence on the withdrawal time an analysis of variance was carried out (General Linear Model, procedure of SAS, release 8.01):

Table 1. Drugs used for intramammary treatment of healthy cows.

Drug/ manufacturer	Antibiotics (Abbreviation)	Withholding time [h]	MRL milk [µg/kg]
Cobactan® LC	75 mg cefquinome (CEF)	120	20
Intervet Int., DE			
Procain-Penicillin			
G 3 Mio./WDT, DE	1898 mg penicillin G (PEN)	120	4
Nafpenzal® MC	180 mg penicillin (PEN)	120	4
Intervet Int, NL	100 mg nafcillin (NAF)		30
	100 mg dihydrostreptomycin (DHS)		200
Omnygram®	866 mg ampicillin (AMP)	144	4
Virbac S.A., F	82.5 mg colistin (COL)		50

$$Y_{ijkl} = \mu + mf_i + dac_j + ln_k + b_1(X_{ijkl}) + b_2(X_{ijkl}) + e_{ijkl} \quad (1)$$

where Y_{ijkl} is the dependent variable (first time in hours when the antibiotic content fell below the MRL), μ the overall mean, mf_i the effect of the i^{th} milking frequency (3, 2, 1.5), dac_j the effect of j^{th} days after calving (healthy cows: ≤100 d, >100 d; cows with clinical mastitis: ≤60 d, >60 d), ln_k the effect of the k^{th} number of lactation (1, >1). Milk yield and SCC as continuous variables were used as covariate with b_1 the slope for milk yield, b_2 the slope for SCC; e_{ijkl} the random residual error.

Results and discussion

Milking frequency

Healthy cows

The results of the analysis of variance concerning the excretion time in milk in dependence on milking frequency are summarized for the six different antibiotics in Table 2.

For all antibiotic substances a longer excretion time was observed in cows milked less frequently. For AMP and COL the difference was not significant due to a large variation of excretion time between individual cows which may be due to irritating nature of COL.

Cows with clinical mastitis

For excretion studies in cows with clinical mastitis CEF was chosen, because relatively long excretion times in healthy cows milked 2x per day were determined compared to the indicated withholding period of 120 h (Table 2). The results for diseased cows are shown in Figure 1.

The excretion in cows treated for clinical mastitis was much shorter than in the worst case studies in healthy cows. Prolonged milking intervals did not increase the withdrawal period in milk.

Table 2. Predicted withdrawal time (in hours) for the different antibiotic drugs in healthy cows (Least Square Means (LSQ_M) and standard error), n = 5 cows per group.

Drug/ Antibiotics	Milking frequency per day[1]		
	3x	2x	1.5x
Cobactan® LC/			
CEF	71.8 ± 6.5 [a]	111.6 ± 4.8 [b]	99.9 ± 5.6 [b]
Procain-Penicillin G 3 Mio./			
PEN[2]	64.7 ± 4.6 [a]	65.7 ± 4.5 [ab]	96.3 ± 4.5 [b]
Nafpenzal® MC/			
PEN[3]	51.1 ± 4.5 [a]	61.4 ± 4.7 [ab]	76.7 ± 5.3 [b]
NAF	31.8 ± 1.6 [a]	38.8 ± 1.7 [b]	48.0 ± 1.9 [c]
DHS	34.8 ± 3.9 [a]	48.9 ± 4.0 [b]	56.7 ± 4.6 [b]
Omnygram®/			
AMP	82.9 ± 27.8 [a]	not applied	123.5 ± 18.5 [a]
COL	62.3 ± 20.3 [a]	not applied	69.5 ± 13.5 [a]

[1]Different letters within the same row show significant differences between milking frequencies ($p<0.05$), [2] 1898 mg PEN per injector, [3] 180 mg PEN per injector in a drug combination

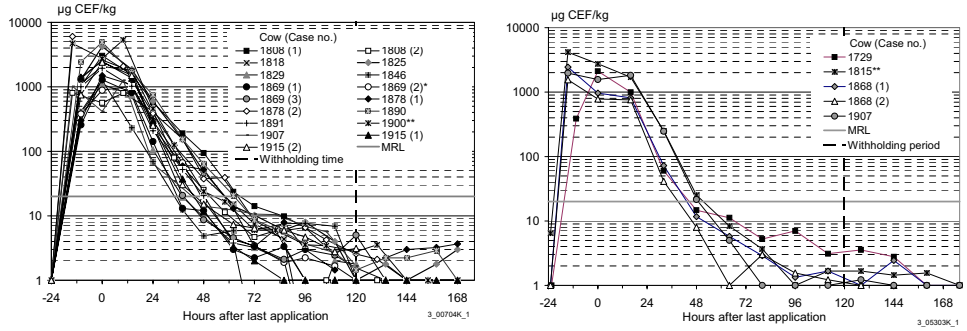

*Figure 1. Excretion of CEF in milk of cows with clinical mastitis, left: milking 2x per day, right: milking 1.5 x per day, * additional application of 50 mg CEF i.m., ** treatment of 2 quarters.*

Antibiotic substance

In Nafpenzal® MC treatments the antibiotics NAF and DHS were applied in the same concentration within one injector and thus have the same drug basis. Nevertheless the excretion time in the same cows varied (Figure 2).

The concentration of NAF was reduced to about 10 % with every milking time whereas the concentration of DHS decreased only to about 25 % with every milking time. These differences are caused by physico-chemical properties of the antibiotics (Ziv, 1975).

Dosage

The average time until the concentration of PEN residues in milk fell below the MRL was dependent on the dosage applied (Table 3), but only when milking frequencies deviating from milking 2x per day were applied.

Part of this effect may be due to the drug formulation because not only the dosage but also the drug vehicle affects the withdrawal time of PEN in milk (Edwards, 1964).

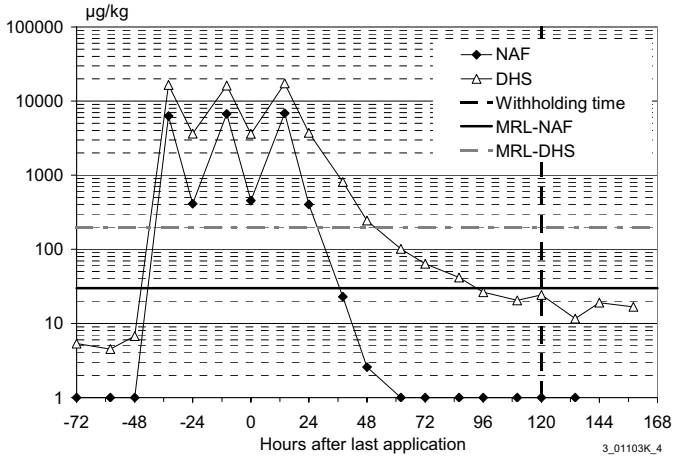

Figure 2. Excretion of NAF and DHS in milk after intramammary treatment with Nafpenzal® MC (100 mg per injector each in combination with penicillin), average of 5 cows milked 2x per day.

Table 3. Time (in hours) until concentration in milk fell below MRL after treatment with PEN in dependence on dosage applied, n=5 cows per group.

	Milking frequency per day		
	3x	2x	1.5x
180 mg PEN	56	72	80
1898 mg PEN	64	72	96

*in combination with NAF and DHS

Nevertheless the potential for longer excretion of antibiotics should be regarded when higher dosages are applied than recommended by the manufacturer.

Individual differences

Individual cows show different excretion patterns of antibiotics in milk, but these differences are also dependent on the antibiotic substance (Figure 3). Whereas for NAF the concentrations in milk of individual cows were almost identical during the whole experimental period, the excretion pattern of COL in milk varied distinctly.

The differences between NAF and COL may be due to the fact that after intramammary application only about 30 % of NAF are excreted via milk whereas about 65 % of COL are excreted in milk. In addition COL is highly irritating and the inflammatory process after udder treatment may also have an effect on the excretion pattern.

Differences between individual cows are taken into account when the withholding period is fixed according to the procedure of EMEA. If large deviations occur, a higher tolerance limit is calculated for the withholding period.

Storage of antibiotics

For veterinary drugs usually a storage temperature is recommended by the manufacturer. In practice drugs may be exposed to higher temperatures for a certain time period. The effect of inappropriate storage on the excretion time in milk is demonstrated in Figure 4.

After treatment with properly stored injectors in none of the cows the withholding time was exceeded whereas the MRL was exceeded in all cows with mishandled injectors. According to an analysis of the mishandled injectors by the manufacturer an increased

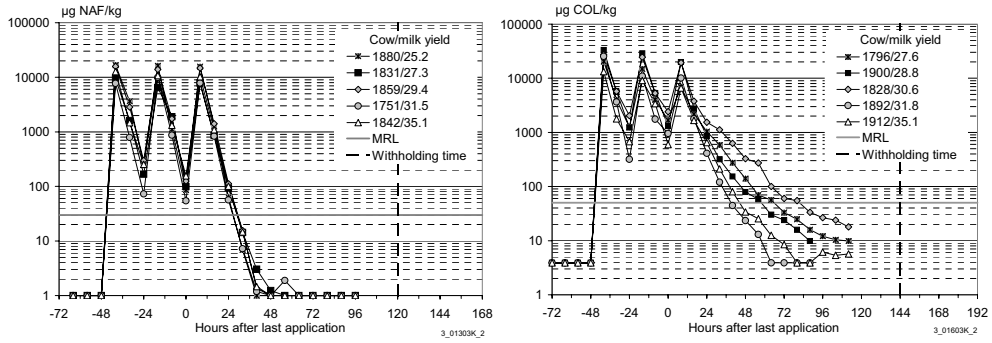

Figure 3. Influence of individual cows on excretion time in dependence on antibiotic substance, 3x milking per day, left: excretion of nafcillin (treatment with Nafpenzal® MC), right: excretion of colistin (treatment with Omnygram®).

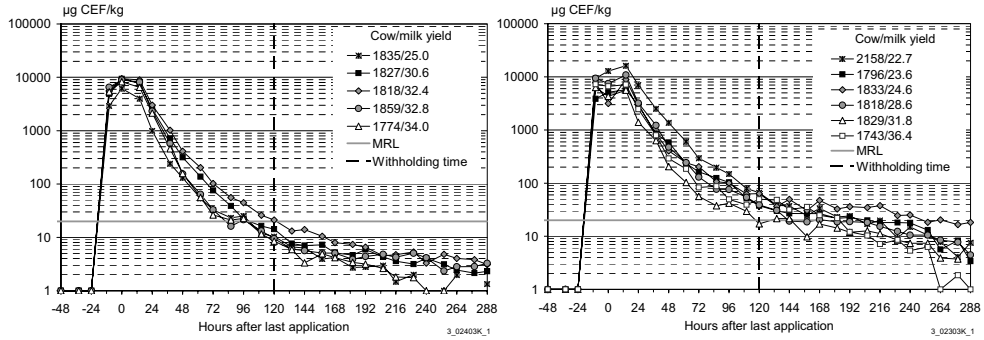

Figure 4. Excretion of CEF in milk, milking frequency 2x per day; left: proper storage of udder injectors, right: heat treated injectors.

viscosity probably lead to a retarded release of CEF and thus to a prolonged excretion in the 6 cows treated.

Conclusions

The milking frequency influences the excretion time of antibiotic residues in milk and leads in tendency to prolonged excretion in cows milked less frequently for all 6 antibiotics studied. The indicated withholding period was sufficient to ensure residue concentrations below the MRL after the end of the withholding period. Studies in cows with clinical mastitis showed no prolonged excretion after treatment with CEF. Nevertheless differences in excretion times are also dependent on the antibiotic applied, the dosage and the drug formulation as well as on the individual cows. Changes in milking frequency may have more pronounced effects on the excretion pattern of other drugs than of those used in this study. Therefore it is recommended to milk cows at least twice daily after intramammary treatment with antibiotics.

Recommendations of the drug manufacturer regarding dosage, treatment interval, withholding period and especially storage temperature should be closely followed to avoid concentrations of residues in milk exceeding MRLs.

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Antimicrobial susceptibility of *Staphylococcus spp*. isolated at dry-off and calving

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Introduction

Antimicrobial resistance of both human and animal pathogens has been an increasing concern worldwide in recent years (WHO, 1997, 2000). Consequently, antimicrobial drug use in animal agriculture has also been under intense scrutiny as a potential source of drug-resistant bacteria. In dairy production, mastitis is the single most common reason for antibiotic use and antimicrobial resistance can affect the cure rates of intramammary infections (Østerås *et al.*, 1999). In many countries the recommendation is to routinely treat all quarters of all cows with antibiotics at dry-off (IDF, 2001), however, it is being questioned whether this blanket use of antibiotics contributes to the development of antimicrobial resistance. The objective of this study was to describe and compare susceptibility patterns of staphylococcal isolates at dry-off and at calving, before and after antibiotic dry cow therapy (DCT).

Materials and methods

The coagulase negative staphylococci and *S. aureus* isolates used in this study were collected as a part of an ongoing study being conducted in Ohio dairy herds. Milk samples were collected aseptically from all quarters of all cows twice before dry-off and twice after calving and examined microbiologically according to the guidelines of National Mastitis Council (NMC, 1999). Antimicrobial susceptibility of the isolates was determined using the Sensititre® system following manufacturer's guidelines (Trek Diagnostics, Cleveland, OH). A commercially available microwell dilution panel, designed for mastitis pathogens, was used to determine the minimum inhibitory concentrations (MIC) for 10 different antimicrobial drugs. The antimicrobial agents and dilution ranges tested for each agent are presented in Table 1. All MIC determinations were performed and isolates categorized as susceptible, intermediate or resistant according to methods and criteria described by National Committee for Clinical Laboratory Standards (NCCLS, 2002). NCCLS recommended quality control strains *S.aureus* ATCC 29213 and *E.coli* ATCC 25922 were included with each batch of organisms tested. The first dilution with no visible bacterial growth was considered the MIC for the strain.

For the data analyses, isolates categorized as intermediate were considered resistant. All data analyses were performed using Statistical Analysis System, SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA). Proportion of bacteria resistant to each antibiotic was calculated. Also, proportion of isolates susceptible to all antimicrobials (or conversely, resistant to at least one antibiotic) was calculated. Data were analyzed separately for each antibiotic and initially, proportion of isolates resistant to these antimicrobials between dry-

Table 1. Antimicrobial agents and their dilution ranges in the susceptibility panel used in the study.

Antimicrobial agent	MIC dilution range (µg/ml)
Penicillin	0.12 - 8.0
Ampicillin	0.12 - 8.0
Oxacillin	2.0 - 4.0
Cephalothin	2.0 - 16.0
Ceftiofur	0.5 - 4.0
Penicillin+novobiocin	1.0/2.0 - 8.0/16.0
Erythromycin	0.25 - 4.0
Pirlimycin	0.5-4.0
Tetracycline	1.0 - 8.0
Sulfadimethoxine	32.0 - 256.0

off and calving was compared using chi-square test of independence. Further analyses were performed with generalized linear models with logit link and binomial error distribution using the susceptibility status of an isolate as the outcome and accounting for the clustered data structure due to potentially several isolates originating from a same cow. The time point (dry-off vs. calving) was included as the main variable of interest in the model.

Results

Approximately 90% of the staphylococcal isolates that were evaluated for antimicrobial susceptibility in this study were coagulase negative staphylococci (CNS) and 10% *S. aureus*. Results indicate that some level of resistance was found to all 10 antimicrobials among these isolates. Most resistance was observed to sulfadimethoxine (40.1% of all isolates resistant), followed by penicillin (23.7%) and penicillin and novobiocin combination (20.8%). Least resistance was observed against ceftiofur (3.4% of isolates resistant). Of all the isolates, the percentages of bacteria that were resistant to at least 1 antimicrobial at dry-off and at calving did not differ and were 68.7 and 70.8%, respectively. When comparing the proportions of resistant bacteria to each antibiotic individually at the two time points, several differences were observed. For each antibiotic, a higher proportion of isolates was resistant at calving than at dry-off, however, all the differences were not numerically large or statistically significant (Table 2). The differences were statistically significant for penicillin and penicillin+ novobiocin combination, and approached significance also for oxacillin and pirlimycin when the correlated data structure was accounted for in the analysis. The CNS isolates tended exhibit more resistance than the *S. aureus* isolates (data not shown).

Discussion

Recent reports over the past 3 years have suggested that there is no evidence for increasing trends in antimicrobial resistance among mastitis pathogens over the years (Erskine *et al.*, 2002, Makovec and Ruegg, 2003; Erskine *et al.*, 2004), while some earlier studies have reported increases in antimicrobial resistance of mastitis pathogens over time

Table 2. Percentage of resistant staphylococcal isolates at dry-off and at calving.

Antimicrobial	Percent resistant		*P*-value
	Dry-off	Calving	
Penicillin	19.3	27.5	0.05
Ampicillin	14.0	18.0	0.37
Oxacillin	11.6	17.8	0.07
Cephalothin	11.7	14.5	0.46
Ceftiofur	3.3	3.4	0.86
Penicillin+novobiocin	14.4	26.3	0.002
Erythomycin	16.3	21.4	0.12
Pirlimycin	3.3	8.4	0.06
Tetracycline	11.8	12.6	0.80
Sulfadimethoxine	39.7	40.4	0.89

(Myllys *et al.*, 1998; Garrison *et al.*, 2000). Our study aimed at looking at potential changes in the susceptibility among mastitis pathogens over a shorter period of time, i.e. the dry period, specifically before and after dry cow therapy. We observed a trend towards an increasing proportion of antimicrobial resistant staphylococcal isolates between dry-off and calving, suggesting that changes in antimicrobial susceptibility occur with exposure to antibiotic dry cow therapy. This agrees with results from the study of Leslie *et al*. (2003) and Schultze (1983). The levels of resistance in our predominantly CNS isolates were much higher, though, than those reported for *S. aureus* by Leslie *et al*. (2003). However, also in our study, the *S. aureus* isolates exhibited less resistance than CNS.

The level of resistance to penicillin among CNS in this study agrees with previous reports (Myllys *et al.*, 1998, Gentilini *et al.*, 2002, Rajala-Schultz *et al.*, 2004). The overall high level of resistance to sulfadimethoxine was surprising in this study and no clear explanation for it is readily available. Significant increases in resistance between dry-off and calving were observed in penicillin, penicillin+ novobiocin and in oxacillin (borderline significance). These all are beta-lactam antimicrobials that are included in the dry cow preparations used in the study herds. The data suggest that changes in antimicrobial susceptibility occur during the dry period and after exposure to antibiotic dry cow therapy.

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Prepartum intramammary antibiotic therapy: Effect on risk of antibiotic residues in milk from dairy heifers

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Abstract

Milk was collected at the third, sixth and tenth milkings following parturition from 136 heifers treated with a commercial β-Lactam intramammary (IM) antibiotic preparation containing cephapirin sodium at 10 to 21 d prior to anticipated parturition to determine the risk of antibiotic residues occurring in milk resulting from prepartum antibiotic therapy. This was part of a study to determine the effects of prepartum IM therapy on milk production, reproductive performance and cure rate of existing intramammary infections in 9 herds in the U.S. and Canada. The mean interval (d) between IM antibiotic therapy and parturition was 15.0 (standard deviation= 9.61 d). Milk was analyzed for β-Lactam residues using a microbial inhibition antibiotic residue screening test. Antibiotic residues were confirmed with β-Lactamase treatment and re-tested for residues. The percentage of antibiotic residues detected was 28.0, 8.82, and 3.68 in the third, sixth, and tenth milkings following parturition, respectively. Antibiotic residues occurred only in the third milking for 26 primiparous heifers (19.1%). Residues persisted until the sixth milking for 7 heifers (5.15%) and until the tenth milking for 5 heifers (3.68%). The mean interval for heifers identified with an antibiotic residue in milk at the sixth milking or persisting to the tenth milking was 7.58 d (range = 1 to 15 d). An increase in interval and milking number was associated with a decrease in risk of antibiotic residues ($P<0.0001$). Prepartum IM antibiotic therapy increases the risk of antibiotic residues occurring in milk in the periparturient period. Increasing the interval between prepartum IM antibiotic treatment and parturition and testing for antibiotic residues in milk postpartum will reduce the risk of antibiotic contamination resulting from this treatment.

Keywords: antibiotic residues, prepartum antibiotic therapy, heifers

Introduction

Mastitis pathogens have been isolated in heifers prior to parturition and these pathogens can persist in milk postpartum (Fox *et al.*, 1995). Several studies have shown a benefit in reduction in the prevalence of mastitis pathogens in heifers following parturition

with prepartum IM antibiotic therapy (Owens *et al.*, 2001; Oliver *et al.*, 2003). However, the economic benefit of this therapy and effect on antibiotic residue contamination of milk postpartum has not been investigated extensively (Oliver *et al.*, 2003).

Avoidance of antibiotic residues in milk is an important focus of the dairy industry. The judicious use of antibiotics, followed by an appropriate milk withholding time and screening for residues are essential aspects of milk quality management for assuring human food safety (Boeckman and Carlson, 2004). There has been very little research to evaluate the risk for antibiotic residue contamination of milk during early lactation following prepartum IM antibiotic treatment in heifers. Oliver *et al.* (1992) determined that inhibitors were detected at d 3 postpartum in 28.2% of quarters from 36 heifers treated with a lactation IM cephapirin sodium product at d 7 before expected parturition. However, in the same study, no inhibitors were found in milk at d 3 subsequent to a IM treatment with cloxacillin at 7 d prepartum. In a subsequent study, heifers were treated at 14 d prior to anticipated parturition with IM cephapirin sodium and residues detected in mammary quarters from 40 heifers were reduced to 3.1% at d 3 postpartum (Oliver *et al.*, 1997). From these studies, it is hypothesized that treating 14 d prior to expected parturition would reduce, although not eliminate, the risk of residue contamination of milk. However, due to the variability in gestation length, it is hypothesized that parturition occurring earlier than anticipated would increase the risk for antibiotic residues in milk during the periparturient period in heifers treated prepartum with an IM antibiotic.

The objective of this study was to determine factors affecting the risk of antibiotic residues occurring in milk resulting from prepartum antibiotic therapy at 10 to 21 d in periparturient heifers. This was part of a study to determine the effect of prepartum IM therapy on milk production, reproductive performance and cure rate of existing IM infections in 9 herds in the U.S. and Canada.

Materials and methods

Heifers from seven dairy herds in seven states (US) and two herds in one province (Canada) were assigned alternately by identification to one of two treatments at 14 d (range=10 to 21 d) prior to anticipated calving date. Immediately preceding treatment, quarter mammary secretions were collected using aseptic techniques for analysis of mastitis pathogens from all heifers. Odd-numbered heifers served as controls. Even numbered heifers were infused in each mammary quarter with a commercial lactating cow antibiotic preparation containing cephapirin sodium (Cefa-Lak, Fort Dodge Animal Health, Fort Dodge, IA). Following antibiotic infusion for treated heifers and sample collection for control heifers, teats from all animals were immersed in a barrier teat disinfectant (Stonghold^{tm}, West Agro, Inc., Kansas Citiy, MO). Milk was collected aseptically from all quarters at approximately 1 to7 d postpartum, 7 to14 d postpartum and 14 to 21 d postpartum and analyzed for mastitis pathogens (Borm *et al.*, 2005 (this publication)). Interval (d) between antibiotic treatment and parturition was recorded for all heifers.

In a sub-set of herds, milk was collected (100mL) at the third, sixth and tenth milking postpartum from 136 heifers in five herds that were treated with the prepartum IM antibiotic infusion. Milk was frozen at -20^{o} C and shipped to the University of Connecticut and stored at -20^{o} C until analyzed. For each day of analysis, the frozen milk samples were held (approximately 1 h) at 25^{o} C in a water bath until thawed and then placed on ice until

analyzed. Subsequently, the milk samples were centrifuged at 1,200 x g for 3 minutes. Skim milk was decanted and analyzed for the presence of cephapirin using the Delvotest P (Gist Brocades Food Ingredients, Inc., Menomonee Falls, WI), a microbial inhibition test that is specific for β-lactam antibiotics (cephalosporins) in milk. For each day of analysis, a standard antibiotic positive milk sample (penicillin G positive control (0.008 IU/ml, Charm Sciences, Inc., Malden, MA) and a standard antibiotic negative control milk sample (Charm Cowside control samples, Charm Sciences, Inc., Malden, MA) were analyzed with the milk samples to verify test accuracy. To confirm the presence of the β-Lactam in milk, all presumptive positive milk samples were treated with cephalosporinase (cefnase) as described by Gilbertson *et al*. (1995) and subsequently re-analyzed for β-Lactams using the Delvotest P. A negative outcome after cefnase treatment confirmed the presence of cephalosporin in milk (Hillerton *et al.*, 1999).

Frequency tables for prepartum IM infection, cure rates, antibiotic positive results by herd and milking number were calculated. Means and standard deviations for interval across herds, within herds, within milking number and positive results were calculated. Using logistic regression (Cox and Snell, 1989; SAS, 1997), factors associated with risk for a positive outcome were evaluated. Odds ratios and 95% confidence intervals (CI) were calculated for each significant factor. The explanatory variables were herd, milking number (milking 3, 6 and 10, postpartum) and interval between treatment and parturition (d). The score statistic was used to evaluate the model. A statistically significant model was determined at $P < 0.05$.

Results

For heifers treated with cephapirin sodium prepartum from the sub-set of heifers and herds, 222 of 522 (42.5%) quarters were infected with a mastitis pathogen immediately prior to treatment. The mean interval between treatment and parturition was 15.0 d (Standard deviation=9.61 d) with a range of 1 to 95 d. Following parturition, 188 mammary quarters of the 222 (84.7%) quarters that were infected before calving were identified as cured.

Antibiotic residues were detected in 38 (28.0%), 12 (8.82%), and 5 (3.68%) milk samples from the third, sixth and tenth milking postpartum for 136 treated heifers (Table 1).

Table 1. Number of antibiotic-positive milk samples at the third, sixth and tenth ilking postpartum for heifers treated with a commercial intramammary lactation antibiotic (cephapirin sodium) at 14 d prior to expected parturition.

Herd	n	Antibiotic positive results		
		Milking number detected positive		
		3	6	10
1	20	8	5	3
2	12	4	0	0
3	49	11	3	0
4	44	7	2	0
5	11	8	2	2
Total n	136	38	12	5
Percent positive (%)		28.0	8.82	3.68

The persistence of antibiotic residues through ten milkings postpartum within heifers is shown in Table 2. Milk from 26 heifers contained antibiotic residues only in the third milking. Residues persisted to the sixth milking in 7 heifers and to the tenth milking in 5 of the 136 heifers treated prepartum. There were no residues detected the sixth and tenth milkings that were not detected in the previous milkings. The interval between treatment prepartum and parturition decreased as residues persisted from the third milking to the tenth milking from treated heifers (Table 1). Residues were detected in the third milking when the interval between treatment and parturition ranged from 3 to 18 d and the range in interval was reduced to 1 to 8 d for heifers with residues persisting to milking ten.

Using logistic regression, there was a significant decrease in the risk for antibiotic residues in milk from treated heifers with increased milking number postpartum and increased interval (d) between treatment and parturition (P<0.0001).

The odds ratio and 95% CI for the risk of residues associated with increased interval was 0.558 and 0.513 to 0.697, respectively. The risk for antibiotic residues detected in the third milking was 12.1 times greater than the risk for detection of residues in the sixth milking postpartum. The risk of residue contamination was reduced by a factor of 0.195 for the tenth milking versus the sixth milking.

Discussion

The infusion of IM antibiotics prior to parturition for the treatment of, or prevention of, an IM infection must be balanced with the potential for success of treatment and minimization of antibiotic residues occurring in milk subsequent to an appropriate milk withholding time. Intramammary administration of antibiotics at the cessation of lactation (dry cow therapy) has been an established management practice (Sischo *et al.*, 1990). Several studies have reported a low risk for antibiotic residues in milk following dry cow therapy if label directions are followed and the dry period is of normal length (45 to 60 d) (Johnson *et al.*, 1977; Oliver *et al.*, 1984). In the present study, a lactation preparation

Table 2. The persistence of antibiotic residues detected in milk in the third (3^1), the third and sixth (3, 6^2) and the third, sixth and tenth (3, 6, 10^3) milkings postpartum for heifers treated with a commercial intramammary lactation antibiotic (cephapirin sodium) at 14 d prior to expected parturition and mean and range for actual interval (d) between antibiotic treatment and parturition.

Herd	n	Antibiotic positive results		
		Milking number detected positive		
		3^1	3, 6^2	3, 6, 10^3
1	20	3	2	3
2	12	4	0	0
3	49	8	3	0
4	44	5	2	0
5	11	6	0	2
Total n	136	26	7	5
Percent positive (%)		19.1	5.15	3.68
Mean interval (d)		10.8	9.43	5.0
Interval range (d)		3-18	4-15	1-8

containing cephapirin sodium was administered which is a lower concentration of drug compared to the dry cow antibiotic preparations and, therefore, less risk of residues occurring in milk would be expected. However, the drug was administered from 1 to 95 d prior to actual parturition with 48% (n=65) of heifers calving at less than 14 d following treatment and 24 heifers calved with an interval less than 10 d. This indicates the wide variation in gestation length or identification of accurate breeding dates that can occur and affect the risk for antibiotic residue contamination of milk.

The sixth milking or d 3 postpartum most closely represents the time when milk would be saleable after parturition as defined by the Pasteurized Milk Ordinance that specifies the milk regulations for the United States (US Department of Health and Human Services, 1989). Oliver *et al*. (1997) observed a lower rate of residues (3.1%) at d 3 postpartum compared to the present study where antibiotic residues were detected in 8.82% of heifers at the sixth milking postpartum. Hillerton *et al*. (1999) also reported antibiotics detected in milk at d 4 postpartum due to the previous cephalosporin dry cow IM antibiotic treatment, although the rate of positive samples was not reported. The sample size was greater in the present study compared to the study reported by Oliver *et al*. (1997) and included a wide range in geographical locations and presumably a wide range in management practices that represent typical risks for residue contamination of milk following prepartum antibiotic therapy. Although these are not great differences in residues detected between the two studies, the higher rate of residues in the present study suggest that testing milk for residues following prepartum antibiotic treatment will be necessary to reduce the risk for antibiotic contamination of commingled milk.

In this study, heifers with an interval longer than 20 d between IM treatment with cephapirin sodium and parturition had a cure rate of prepartum IM infections of 76% during the postpartum period versus 84.7% for heifers with an interval of less than 15 d. This suggests that the effectiveness of antibiotic treatment may be compromised as the interval increases. This is supported by other studies that have shown that IM treatment during early gestation was not as efficacious as treatment closer to parturition (Trinidad *et al*., 1990; Oliver *et al*., 1992). Therefore, in order to avoid antibiotic contamination of milk, effective testing of milk for antibiotics is needed if prepartum treatment is administered at 14 d prepartum.

Based on these results, it is recommended that the targeted treatment interval between prepartum IM antibiotic infusion with a cephapirin lactation preparation be greater than 14 d prior to anticipated parturition to reduce the risk of antibiotic residues in milk during the periparturient period. In addition, these results emphasize the importance of testing milk for antibiotic residues when antibiotics are administered prior to parturition.

Summary

Intramammary antibiotic treatment administered at 10 to 21 d prior to anticipated parturition resulted in 8.82% of heifers with detectable antibiotic residues in milk at the sixth milking postpartum and 3.68% residues at the tenth milking. The risk for residues decreased with increased milking number following parturition and increased interval (d) between prepartum antibiotic treatment and parturition. Treating heifers with an antibiotic at greater than 14 d prior to anticipated parturition and testing milk for antibiotic residues will reduce the risk of residues resulting from prepartum IM therapy of periparturient heifers.

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Mastitis control in emerging dairy countries

Role of selective dry cow therapy in prevention of mastitis in dairy herds with high disease prevalence

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Abstract

A field trial was conducted to assess the effect of selective dry cow therapy on quarter infection, milk cell count and occurrence of clinical mastitis at three organised dairy farms housing HF×Sahiwal cows. Animals approaching dry period were screened for mastitis and assigned two groups: Treatment group cows (n=59) each with at least one diseased quarter, were infused in all quarters irrespective of quarter health status, with "Cepravin dry cow" (cephalonium 250mg) at drying off. Control group cows (n=51) each having all quarters healthy received no treatment. Quarter foremilk samples were analysed for microbiology and cell count (California mastitis test, CMT) three times before drying, and three times after calving. Cows were followed throughout dry period and until disposed off or milked for at least 7 months post-calving. Therapy could eliminate 90.67% (68/75) existing subclinical infections and cured 14/19 (73.68%) clinical mastitis cases. Significantly less new infections established in treatment group (2.81%) than in control (14.42%) indicating a prevention of 80.51%. Occurrence of clinical mastitis during dry period reduced by 75.88%. Therapy resulted an appreciable fall in average CMT score of treated quarters i.e. 1.62 to 0.10, and at calving only 5.55% quarters were CMT positive among treated cows as compare to 53.85% in control. Follow up in subsequent lactation revealed significant reduction in clinical mastitis; occurrence in treated vs. control cows being nil vs. 6.03% (by 7d), 1.91 vs. 8.54% (by 30d), and 7.65 vs. 13.07% by 3 months post-calving. Thus, selective dry cow therapy appeared an effective tool for prevention of mastitis and lowering of milk cell count.

Keywords: dry therapy, infection, subclinical, clinical, CMT

Introduction

The importance of dry period in the dynamics of intramammary infections (IMI) has been studied extensively (Smith *et al.*, 1985, Eberhart 1986, Bradley and Green 2000, Green *et al.*, 2002). Some 48% of cows become infected, most in the first 3 weeks of the dry period, and half of these infections persist into the next lactation. While not all, some of these persistent infections develop into clinical mastitis in next lactation. This has recently been confirmed through the use of DNA finger printing (Bradley and Green 2001). Further, clinical mastitis associated with dry period infections was more likely to occur earlier in lactation than clinical mastitis not associated with dry period infections (Green *et al.*, 2002). Thus, the primary objective of udder health management during the dry period is to have as few infected quarters as possible at the next calving. The antibiotic dry cow therapy (DCT), initially developed in 1940's, seems to be the most effective means of achieving this objective even today. DCT primarily serves two purposes; eliminates the existing IMI and

prevents establishment of new IMI during dry period (Nickerson *et al*., 1999, Dingwell *et al*., 2003). In addition, it may regenerate damaged udder tissue during non-lactating period and result in increased milk production, reduced milk somatic cell count (SCC) and lower occurrence of clinical mastitis at freshening (Schott *et al*., 1994, Osteras and Sandvik 1996, Bradley and Green 2001). However, the role of DCT in deriving these benefits is very much affected by the initial level and type of bacterial infection, and bulk milk SCC of the herd under study (Schukken 2002). The present study was conducted to assess, under Indian dairy conditions, the effect of DCT on quarter infection, milk SCC and occurrence of clinical mastitis in three cattle herds with high disease prevalence.

Materials and methods

The study was conducted over a period 02 years beginning July 2002 at three organised dairy farms. The farms had semi-loose housing system and followed the practice of conventional feeding. The dairy farms had not dry-treated cows for at least 3 years prior to the start of trial, and followed the practice of post-milking teat dipping in a 0.5% iodine-based teat dip for all the lactating cows. A total of 110 HF $\times$ Sahiwal crossbred cows approaching dry period were assigned two groups; treatment group (n=59) and control group (n=51). The cows were enrolled to treatment group either because they had one or more quarters infected at drying off or because they suffered from clinical mastitis. Control group comprised of healthy cows that did not have any history of clinical mastitis during the last one month and harbored no intramammary infection at drying off. Immediately after last milking, each functional quarter of cows in treatment group was infused with 250 mg cephalonium (Cepravin dry cow, Schering-Plough Animal Health, UK). The cows in control group received no dry therapy. Cows were followed throughout the dry period and until disposed off or milked for at least 7 months post calving.

The cows were sampled for three consecutive days each time (i) before drying off (ii) within 3-5 days following calving and (iii) as and when cow suffered from clinical mastitis. The udder and teats of cow were thoroughly cleaned and subjected to California Mastitis Test (CMT, Schalm *et al*., 1971). About 5 ml of foremilk from each quarter was collected aseptically into separtae sterilized vial for microbial analysis (Brown *et al*., 1981). A quarter was considered infected if at least two of the three samples yielded the identical microorganism. The criterion for determining the elimination of infection was that the microorganism must not have been isolated from any samples taken after calving. By definition, it was possible for elimination (for one bacterial pathogen) and a new IMI (for a different bacterial pathogen) to occur in the same quarter. New IMI post-calving was identified when a microorganism was isolated from at least two post-calving samples with no microorganism prior to drying off or the microorganism post-calving differed from that before drying off. Clinical mastitis was identified on the basis of presence of abnormal secretion and/or udder tissue changes (e.g. hot, painful, swelling). A quarter experiencing relapse of clinical mastitis during the study period was classified as a new case. It got cured if the secretion became apparently normal and yielded no microorganism on culturing.

Significance of results was tested by Chi-square (χ^2) analysis (Gupta 1979).

Results

Of 59 cows initially enrolled in the treatment group, 05 lost to follow-up sample collection because of death or abortion in dry period or have been sold in-between. Three cows each in treatment and control group had one permanently blind quarter each. Thus, 213 quarters from 54 cows in treatment group and 201 quarters from 51 cows in control group were available for analysis of results.

The therapy could eliminate, in total, 68/75 (90.67%) of subclinical and 14/19 (73.68%) of clinical infections present at dry off in treated quarters. While 84.21% of *Staphylococcus aureus (S. aureus)*, 93.33% of coagulase negative staphylococci (CNS), 88.89% of *Str agalactiae* and 94.12% of non-agalactiae streptococci subclinical infections got cleared, therapy appeared less effective against clinical mastitis of *S. aureus* origin (Table 1).

The response of dry therapy with regard to occurrence of clinical mastitis and new IMI during the dry period is summarized in Table 2. Significantly less new subclinical IMI established in treated quarters than in un-treated (2.81% vs. 14.42%; $p < 0.01$) showing a reduction of 80.51%. The predominant organisms causing new IMI were CNS, *S. aureus an*d streptococci other than *Str agalactiae* (Table 3). Only one case of clinical mastitis occurred in treated cows compared to 4 in untreated.

The occurrence of clinical mastitis during the first three months of lactation showed a significantly lower incidence in treated quarters compared with those that did not receive any treatment at dry off (7.65 vs. 13.07%, $p < 0.1$). However, by 7 months lactation almost similar number of cases developed in two groups (Table 4).

Table 1. Elimination of intramammary infection from quarters receiving dry cow therapy.

Organism	Subclinical infections		Clinical infections	
	Present at dry off	Eliminated at calving	Present at dry off	Eliminated/ cured
S. aureus	19	16 (84.21)	08	05 (62.50)
CNS	30	28 (93.33)	02	02 (100.0)
Str agalactiae	09	08 (88.89)	05	04 (80.0)
Str (non-agalactiae)	17	16 (94.12)	02	02 (100.0)
No isolation	-	-	02	01 (50.0)
Total	75	68 (90.67)	19	14 (73.68)

(-) No data available in this context

Figures in parentheses indicate percentage

Table 2. Establishment of new intramammary infections during the dry period.

Treatment group	Clinical	Subclinical	Total number of quarters
Treated	01(0.48)[a]	06 (2.81)[b]	213
Control	04 (1.99)[a]	29 (14.42)[b]	201
Total	05	35	414

Figures in parentheses indicate percentage of total quarters

[a] $\chi^2 = 1.99$ ($p > 0.1$)

[b] $\chi^2 = 18.02$ ($p < 0.01$)

Table 3. Distribution of microorganisms causing new infections during the dry period.

Infection/ Group	*S. aureus*	CNS	*Str agalactiae*	Str (non- agalactiae)	Others*	Total quarters
Subclinical						
Treated	01[a]	02[b]	01[c]	01[d]	01[e]	213
Control	06[a]	10[b]	01[c]	07[d]	05[e]	201
Total	07	12	02	08	06	414
Clinical						
Treated	01	-	-	-	-	213
Control	-	01	-	01	02	201

(-) Indicates no isolation

*Includes corynebacteria and coliforms

[a]χ^2 =3.93 ($p < 0.05$); [b]χ^2 =5.97 ($p < 0.025$); [c]χ^2 =0.002 (ns); [d]χ^2 =4.97 ($p < 0.05$); [e]χ^2 =2.95 ($p < 0.1$)

Table 4. Occurrence of clinical mastitis in the post-calving period.

	Number (percent) cases in post-calving period					
Treatment group	7d	1m	2 m	3m	7m	Total quarters*
Treated	0 (0.00) [a]	4 (1.91)[b]	11 (5.26)[c]	16 (7.65)[d]	40 (19.14)[e]	209
Control	12 (6.03)[a]	17 (8.54)[b]	23 (11.56)[c]	26 (13.07)[d]	41 (20.60)[e]	199
Total	12	21	34	42	81	408

(Four quarters in treated and 2 in control cows got permanent blind during dry period

[a]χ^2 =13.01 ($p < 0.01$); [b]χ^2 =9.18 ($p < 0.01$); [c]χ^2 =5.30 ($p < 0.025$); [d]χ^2 =3.24 ($p < 0.1$); [e]χ^2 =0.03 (ns)

Table 5. Effect of dry cow therapy on CMT score at calving.

Parameter	Treated cows		Untreated cows	
	at dry off	at calving*	at dry off	at calving*
Total quarters	213	198	201	195
Quarters (≥ 1 CMT score)	173 (81.22)	11 (5.55)[a]	128 (63.68)	105 (53.85)[a]
Quarters (≥ 2 CMT score)	107 (50.23)	06 (3.03)[b]	50 (24.88)	46 (23.59)[b]
Average CMT score	1.62	0.10	1.07	0.94

*Some quarters could not be tested for CMT

Figures in parentheses indicate percentage

[a]χ^2 =110.12 ($p < 0.001$); [b]χ^2 =36.15 ($p < 0.001$)

The average CMT score of quarter samples at dry off was 1.62 in treatment group and 1.07 in healthy control. At post-calving it reduced to 0.10 and 0.94 in two groups, respectively, and only 5.55% of quarters had positive CMT in treated group as compare to 53.85% in control (Table 5).

Discussion

Cephalonium, a semi-synthetic cephalosporin antibiotic, when infused to cows as dry cow product, cured 14/19 (73.68%) of clinical mastitis and eliminated 90.67% of existing subclinical IMI present at dry off (Table 1). Since our study did not include an untreated infected control group for statistical, ethical and commercial reasons, this clearance of infection in the treated group reflects spontaneous as well as antibiotic mediated

elimination. Several reviews speak about this particular advantage of DCT; approximately 70 to 98% of IMI present at dry off got eliminated with DCT (Natzke 1981, Smith *et al*., 1985, Tuteja *et al*., 1994, Nickerson *et al*., 1999, Dingwell *et al*., 2003). The rate of elimination of infection in the herd may be affected by the intensity and type of bacterial infection (Dingwell *et al*., 2003), longevity of infection (Schultze 1983), age of cow (Browning *et al*., 1994) and type of dry cow product (Nickerson *et al*., 1999). We did not find much difference in rates of elimination for any pathogen due to treatment. This is in contrast to several studies that generally acknowledged DCT, compared with elimination of other infections, as being less successful in eliminating IMI caused by *S. aureus* (Browning *et al.,* 1990, Nickerson *et al.,* 1999).

DCT significantly prevented the establishment of new IMI during the dry period; new infection rate in untreated group was about 5 times higher than the rates in treated group (Table 2). This agrees with effects of DCT on new IMI reported by others (Eberhart 1986, Tuteja *et al*., 1994, Hillerton and Berry 2002). However, ineffectiveness of DCT in offering this protection to cows that were not infected at dry off has been documented (Kirk *et al.,* 1997). The majority of new infections were due to CNS, *S. aureus* and streptococci other than *Str agalactiae* (Table 3). Similar observations have been made (Browning *et al*., 1990, Dingwell *et al*., 2002).

As an additional benefit, treated cows had only one case of clinical mastitis compared to 04 in untreated cows in the dry period (Table 2). Also, significantly less number of clinical cases occurred in treated quarters than in untreated quarters during the first 3 months post calving (Table 4). The findings are supported by the work of Bradley and Green (2001) and Hillerton and Berry (2002) who found DCT as an effective tool for the prevention of clinical mastitis in early post-calving period. Beyond 3 months, difference in the occurrence of clinical mastitis in two groups became insignificant, bringing the incidence of disease almost similar by 7 months of lactation (19.14% for treated vs. 20.60% for control). Thus, it seems that DCT could offer prophylactic benefits up to a limited period of lactation. This is reasonable to believe because it is unlikely that DCT, which only persists until calving, influences occurrence of clinical disease during the whole lactation. Even the protection during early calving is not related to current prevention but to the elimination and shortening of IMI of dry period. This has been demonstrated that infection acquired during dry period result in long term colonization of mammary gland and increases the risk of clinical mastitis in the post-calving period (Green *et al*., 2002). The persistence of infection from dry period to lactation has been confirmed by DNA finger printing also (Bradley and Green 2001).

Our findings in regard to effect of DCT on CMT score (milk SCC) corroborate well with the findings of Schott *et al*. (1994) and Osteras and Sandvik (1996) who observed a significant reduction in SCC of the quarters receiving dry cow therapy.

Conclusions

Compared with untreated control, application of selective DCT resulted in significantly fewer new IMI and clinical cases of mastitis during the dry and early post-calving period. The udder health of treated cows was appreciably improved as evident form cure of existing infections, and lowering of milk SCC (CMT score) at subsequent lactation. However, our results indicated that in the absence of DCT risk of new IMI during dry period is significantly higher even for the cows not harboring any infection at dry off. Hence, probably

undertaking complete DCT would be the best answer in herds with high disease prevalence similar to our study, or alternatively role of teat seals may be evaluated in infection free quarters along with.

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Mastitis control in Uruguay: Strengths and weaknesses

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Abstract

This paper describes the mastitis prevalence and incidence in Uruguay, as well some of the key points (strengths and weaknesses) of implementing a mastitis control programme.

Keywords: Uruguay, control programme

Introduction

Uruguay is an emerging dairy country on the Atlantic Coast between Argentina and Brazil. It has a temperate climate with an average annual temperature of 17ºC and an annual rainfall of approx. 1200 mm. Rainfall is generally heavier in autumn and winter, but there is no rainy season (www.rau.edu.uy/uruguay/geografia/).

According to the 2003 statistics (www.mgap.gub.uy/Diea), there are over six thousand producers who milk about 440 thousand cows. Thirty eight percent of producers produce on less than 50 hectares, which is borderline for survival under Uruguayan circumstances. 3400 sell their milk to a dairy plant; the others either make cheese or sell raw milk.

In 2004, the dairy plants received 1229 million litres, of which over 50 % was exported, mainly as milk powder or cheese.

The production system is pasture based, which means that the cows are out all year round. The industry pays a bonus for winter milk, and many producers now start calving early in autumn, continue through the winter and calve the last cows on spring grass. Summer tends to be hot and usually dry, which has a marked influence on production.

There are no subsidies on milk production or exportation and there is no quota system.

Mastitis in Uruguay

Cell count

During many years, an annual California Mastitis Test (CMT) has been obligatory. However, there was no obligation to do anything with the results, and the milk was never rejected on the basis of a poor CMT.

In 1995, the government issued a decree that obliges the industry to test the individual supplies and to classify them. Many industries use the information for a payment system based on cell count, total bacterial count and kilos milk fat and protein, with penalties for inhibiting substances and extraneous water.

Milk quality has improved rapidly and keeps improving (table 1).

Table 1. Cell count and TBC from 1996 until 2004 (from: OPYPA, anuario 2004).

Year	Cell count (x thousand/ml)	TBC (x thousand/ml)
1996	546	558
1997	543	301
1998	491	157
1999	454	126
2000	422	88
2001	397	77
2002	396	66
2003	359	57
2004	341*	56[1]

[1]preliminary results

CONAPROLE is Uruguay's main dairy cooperative, receiving approximately 66% of all milk sold to the dairy industry. Its bonus for AAA quality milk (table 2) is high: approx. 14 % of the total milk price as compared to class B milk. C milk is penalized. With small variations, most other industries follow CONAPROLE's example.

Prevalence of subclinical mastitis

Early studies (del Baglivi *et al.,* 1976, Laborde *et al.,* 1981) found a predominance of Staphylococcus aureus and Str. agalactiae in the Southern half of the country where milk production is concentrated. Laborde *et al.* (1981) found a cow prevalence of 55 % and a quarter prevalence of 52 %. Overall, Str. agalactiae was the most commonly isolated pathogen (54 % of all isolates). Str. agalactiae was more common in hand milked cows, whereas S. aureus was much more common in machine milked cows. The authors postulated that the incidence of Str. agalactiae would decrease as a result of the wide spread use of intramammary penicillin.

The next study confirms that he was right: Gianneechini *et al.* (2002) took a representative sample in herds in the middle Northwest of the country (Paysandú and Rio Negro) in 1998. They found a quarter prevalence of 27 % and a cow prevalence of 52 %. S. aureus was by far the most common micro organism (63 % of all isolates, Str. agalactiae was second with 11 %).

When this study was extended to the more traditional milk-producing region in the South in 2002 (Gianneechini *et al.,* 2004), the quarter prevalence for subclinical mastitis was 26% and the cow prevalence 50%. Again, S. aureus was the most frequently isolated pathogen

Table 2. Typical payment scheme (CONAPROLE, Nuevo sistema de bonificación por calidad de la leche industria, 2004).

Class	Cell count[1]	TBC[1]	Bonus/penalty[2]
AAA	<= 400.000	< =50.000	18%
AA	400.001-700.000	51.001-100.000	5-17.9% (sliding scale)
A	400.001-700.000	100.001-120.000	2.5-7.5% (sliding scale)
B	700.001-800.000	100.001-200.000	No
C	800.001-1000.000	200.001->800.000	-10 to 30% (sliding scale)

[1]Geometric mean of min. 2-3 samples/month

[2]The scheme only applies to approx. 78 % of the milk

(48%), with an important presence of opportunistic pathogens (coagulase negative staphylococci 15%) and environmental streptococci (23%).

Incidence of clinical mastitis

Gianneechini's 2002 study includes the monitoring of clinical mastitis in 53 herds. He finds an incidence of 10.9 cases per 100 cows-years.

Mastitis control in Uruguay

There is no statistically reliable information available on mastitis control measures in Uruguay. When the first information on bulk tank cell count became available in 1996 and before a payment scheme was introduced, Bouman (unpublished) recorded the use of some basic mastitis control measures on 112 farms in the Southwest. On 63 % of farms, blanket dry cow therapy was used, 41 % used teat dip, and 52 % used relatively new liners. Routine milking machine testing was virtually inexistent, and all farmers routinely treated clinical mastitis with antibiotic therapy. On only 21 % of farms, the full Five Point Plan was implemented. The farms in the survey were subject to some sort of quality control from the dairy industry and it is likely that cheese makers and raw milk producers had not adopted these measures to the same extent.

In 2004, Bouman (unpublished) recorded the use of teat dip on 129 farms and found that 81 % of farms used teat dip all year round, whereas an additional 5 % used it in winter.

Information on mastitis control

A high percentage of farmers (66 %) who sell their milk to the industry, sell to the main cooperative CONAPROLE, which has an advisory service. This service has organized various campaigns over the years, focussing on correct milking technique, milking machine testing, dry cow therapy and other mastitis-related issues. However, without an indicator to monitor progress, the effect of these campaigns was difficult to measure. Possibly, the use of dry cow therapy and teat dip found in 1996 is related to the campaigns, but the pharmaceutical industry and the private advisers have played a role as well.

From 1995 onwards, most dairy industries organize courses and informative meetings for their producers on the subject of mastitis control. Some use the services of specialized advisers to visit problem farms.

In 1997 and 1999, two two-day conferences on mastitis are organized in an attempt to reach the on-farm advisers.

In 2002 the Unidad de Salud de Ubre, USU ("Udder Health Unit"), is formed. It aims to coordinate research, to offer training programmes for advisers and farmers, to liaise with sister organizations abroad and to be a central information point for those who wish to know more about mastitis.

Strengths of the present situation

1. A very high bonus for low cell count milk in most dairy plants.
2. A strong pressure on the industry to receive low cell count milk: over 50 % of the milk received goes for export and has to compete with "low cell count regions" like New Zealand, Australia or the European Union.

3. As compared to many surrounding countries, farmers and milkers are very well educated. The percentage of people who cannot read or write is less than 2 % in people up to 50 years of age (Instituto Nacional de Estadística, www.ine.gub.uy).
4. The cows are out all year: very few housing-related problems, as reflected in the low number of clinical mastitis and coliforms (Gianneechini *et al.*, 2004).
5. A small uniform country with good access to the farms and a reasonable infrastructure (roads, telephone, internet), as compared to surrounding countries.
6. Two individual cell count laboratories, and several laboratories for mastitis bacteriology. One of the laboratories specializes in tailor made on-farm advice. There are three cell count laboratories for bulk supplies and all are subject to an interlaboratory trial once a month organized by the Ministry of Agriculture. Two of these also participate in international interlaboratory tests (Hirigoyen *et al.,* 2004).
7. The bulk of the milk is sold to dairies that offer specialized advice.
8. A national database on bulk milk cell counts to monitor progress (OPYPA).
9. A national working party (the USU) on mastitis.
10. A new total quality related payment scheme ("Tambo Seguro") based on HACCP, run by CONAPROLE.

Challenges and weaknesses

1. The milk price is unpredictable, and many farmers find it hard to plan ahead, which reflects in many obsolete milking installations (Bouman 2003). When the milk price is low, many aspects of a full mastitis programme are abandoned and much long-term work is undone.
2. The sale of all types of antibiotics (also for human use) is unrestricted and there is little awareness of the limitations and dangers of blanket antibiotic use. Gianneechini *et al.* (2004) detected that 39.1 % of S. aureus strains isolated from subclinical cases of mastitis were resistant to penicillin, and 68.5 % to streptomycin. Streptomycin is never a drug of choice for mastitis treatment, but is part of the pen-strep combinations that are most frequently used for mastitis treatment.
3. On small family farms, the cows are milked by the family members. However, as soon as the farm passes the subsistence level, a hired milker takes over. These people often have very little status and salaries can be extremely low. Often, working and/or living conditions are precarious. No milker gets more than 4 days a month off (plus 20 days of holidays), and some self-employed milkers never get away from the farm. Many farms with mastitis problems suffer in fact from unmotivated staff. Research indicates that good management at cow level is essential for obtaining low cell count milk (Hutton *et al.,* 1990, Barkema *et al.,* 1998)
4. There is no training centre for milkers. There are several agricultural colleges that prepare young people for farm work (Universidad de Trabajo de Uruguay UTU), but graduates from these colleges tend to be overeducated for the milking parlour and end up in managing jobs. There is a need for a centre where common milkers can be instructed in the day-to-day business of milking and managing cows.
5. Unless a lot of work is done on farm roads, the grazing system creates an enormous mud pool when it rains, especially in autumn and winter. From 1994 until 1999, the volume of milk that was sent to the dairy plants increased 31 %. In most cases, this was due to an increase in herd size, because at the same time the number of milk

producers decreased considerably (DIEA). The worst problem is generated in the access to the milking parlour.

It is likely that the contamination of the mud is not the main risk factor, because the incidence of E. coli in intrammammary infections is negligible (Gianneechini *et al.*, 2004). However, the mud causes severe teat lesions (cuts, dryness) and the milkers also spend much of their time trying to remove it from the teats before milking, neglecting other jobs like removing clusters (automatic cluster removers are rare), detecting mastitis or teat dipping. Rough teat skin is a source of S. aureus and Str. dysgalactiae (Burmeister *et al.*, 1998).

Very few farmers dry teats with an individual cloth or paper towel, even though there is good evidence that this reduces total bacterial counts (Galton *et al.*1986, Taverna *et al.*1992). Occasionally a common udder cloth is used, something which is widely recognized as poor practice (Bramley 1992). Acuña *et al.* (2001) found that the use of washing without drying increased the number of S. aureus, streptococci and E. coli recovered from the teat in Argentina, in a similar grazing system to Uruguay.

6. Milking machines in Uruguay are rarely fit to milk cows (Bouman 2003). It is estimated that not even 10 % of all milking machines are checked annually. Of those that are checked, only 10-12 % doesn't show serious problems. There is no legislation regarding milking machine standards and there is no trade association. Many installers and testers have no formal training whatsoever and have never heard of standards, not even for new installations. Even though the milking machine is rarely considered to be of major importance in mastitis control, the severity of the problems found in Uruguayan milking machines make it likely that the estimated effect on overall new mastitis rate is closer to 20 than to 6 % (Mein 2004).
7. The cheese makers form a large group of producers (approx. 1800) that has little incentive to produce low cell count milk, even though high cell counts cause production losses in most types of cheese (Ballou *et al.*, 1995, Klei *et al.*, 1998, Ng-Kwai-Hang 1993). Only recently there is some control on these farms and cell count problems are not high on the list. The group is scattered and cheese makers often live in isolated places with a poor infrastructure
8. Not all dairies implement the payment system with all their clients. Large producers sometimes negotiate a milk price that depends to a lesser extent on cell count, or where the cell count limits are not so strict.

   Up until 2004, CONAPROLE implemented a strict cut-off system for high quality milk (AAA) in which almost 3 % of the bonus was lost when the milk had a cell count of over 400 thousand cells/ml. It has now introduced a sliding scale for milk that doesn't comply with this cut-off. Supplies that are a fraction over the limit lose a tiny percentage of their bonus, not 3 %. This is bound to increase overall cell counts, as there is little incentive to stay well below the 400 thousand cells/ml level.
9. By government decree 160/97, products used for teat disinfection and antibiotic therapy have to be registered at the Central Veterinary Laboratory (Chelle 1999), where information on safety, withdrawal time, etc. is required. However, controls on products for sale are not frequent and the results of these controls are not accessible to the general public. There is some anecdotal evidence that there have been batches of dry cow therapy that did not come up to standard. There is no independent laboratory to carry out relevant tests.

The National Mastitis Council in the US recommends efficacy tests for teat disinfection products. On the Uruguayan market there is only one (imported) product backed up by these tests.

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The impact of intervention on mastitis in an emerging dairy developing country-Uganda

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Abstract

An intervention study was undertaken for controlling mastitis in four different study groups: 1) selective dry cow therapy and improved hygiene, 2) improved hygiene and post milking teat dipping, 3) formation of 3 training groups of farmers in rural, peri-urban and urban herds in a modified Farmer Field School approach, and 4) a longitudinal study. Focus was made on effect of different control options on the occurrence of the disease, the economic benefits of the control options, and empowerment of the farmers. The purpose was to identify and recommend to farmers strategies to control mastitis, which are cost effective and easily adopted. The intervention study was carried out on 65 smallholder dairy farms with 153 lactating cows through qualitative, quantitative and microbiological methods for a period of 12 months. A participatory impact assessment (PIA) was carried out 12 months after intervention to determine the qualitative impact of the intervention project as perceived by the farmers. Overall the farmers ranked the benefits at household level as improved hygiene on the farms, reduced mastitis cases, increased cooperation with veterinarians, better access to mastitis control inputs, improved record keeping and increased milk production. The farmer's perception after having used the strategies for a period of time was absolutely necessary in estimating and recommending strategies to the goal that they must be 'easily adopted and used by the small holder dairy farmers sustainably'.

Keywords: selective dry cow therapy, improved hygiene, farmer field schools based training, participatory impact assessment

Introduction

Mastitis, especially subclinical mastitis, still remains a big challenge in dairy farming despite the amount of research that has been conducted over several decades. It is a complicated problem associated with almost every conceivable factor of management and environment and most dairymen often overlook it although it can be a serious problem even in herds where management and environment meet sensible standards. It is a disease that requires considerable diligence on the part of the milkers and veterinarians to control it. The disease is nearly always caused by microorganisms. The primary reservoirs of mastitis pathogens are infected cows' udders and teats and transmission from udder to udder occurs during the milking process via milker's hands, udder washcloths and milking machine teat cups (Blood and Raodostits, 1989). Transmission may also occur during the interval between

milkings from the environment as a result of contact of teats with contaminated beddings, flies and the tail switch.

The prevalence of the disease in Uganda has been ranging from 60-70% (Byarugaba *et al.*, 1998) and this has not changed over the years due to poor management and low hygiene (Byarugaba *et al.*, 2003). Mastitis control aims at reducing the losses the disease causes to the milk producer and processor (Philpot, 1975). It has a long lasting effect on milk yield, and cows with mastitis never reach their pre-mastitis milk yields during the remainder of the lactation after onset of the disease. Complete eradication is impossible due to the widespread environmental contamination with some of its causative agents such as coliforms. The level of infection, can however, be controlled to a minimum as is economically feasible. To be acceptable, the control system must cost less than the disease itself, be relatively simple to carry out, work under most management conditions and substantially reduce clinical mastitis soon after being adopted (Philpot, l975).

From the cross-sectional study, mastitis was confirmed as a problem with an over all prevalence of 61.3% of which sub-clinical mastitis was 60.7% (Byarugaba *et al.*, 2003). Individual farmer variations concerning farming systems, management factors, conditions and possibilities for solutions and control were noted. On this basis an intervention study was designed to identify and recommend to farmers the most appropriate strategies to control mastitis, which are cost effective, sustainable and easily adopted. Understanding farmers and working together with them is a very important process in designing interventions to address their constraints. This paper presents the results of a participatory evaluation exercise that was conducted to explore the farmers perceived impact of the project on mastitis control on their farms and changes in their households and communities as a result of the project.

Methodology

Study population

The farmers were selected from three areas in Jinja district representing urban (Jinja Municipality), peri-urban (Mafubira, Kakira, Mpumudde), and rural (Budondo and Butagaya) areas. The study involved cattle, which are intensively and extensively managed including the tethered, zero-grazed or paddocked animals.

Sampling design

A sampling frame of 7,000 adult smallholder dairy cows in the district was used to obtain the sample size. Farms were stratified by herd size (small 1-5, medium 6- 10, large 11 - 20) and by grazing system (zero-grazed and open grazing). At an estimated prevalence of 70% (other studies in Uganda) and at 95% confidence interval a samples size of 211 cows was used for the cross sectional study but the intervention study was carried out on 65 smallholder dairy farms with 153 lactating cows.

Intervention strategies

Three intervention strategies, teat dipping and improved hygiene and management, selective dry cow therapy and farmer-field based training were undertaken and a control longitudinal group without any intervention. Monthly socio-economic data (production, intervention costs, input-output-costs) was collected and milk examined quarterly for

mastitis causing agents together with associated risk factors and farm management factors. The formation of the four groups were based on the results of the cross-sectional study, experiences elsewhere and expert opinion.

Evaluation exercise

The evaluation exercise was conducted using qualitative methodologies with three different approaches, namely:- participatory impact assessment (PIA), in-depth individual interviews with the different treatment groups, and on-farm visits for physical assessment.

Participatory impact assessment (PIA)

Two PIA workshops were conducted following a field guide for conducting PIA (Oruko, 2002); one in Jinja municipality combining the urban and peri-urban farmers from the project, and the other one in Butagaya consisting of the rural farmers. In this project the P.I.A. process was guided by two trained facilitators.

In-depth interviews (IDIs)

Four IDIs with one farmer was selected from each of the four groups and an in-depth interview carried out on issues ranging from how the farmer learned of the project, benefits from the project individually and at community level as well as suggestions on how the project could be improved to benefit the farmers much more in controlling mastitis

On-farm visits

Three un-announced visits were done on selected farms to assess adherence to the treatments assigned to the groups, as well as note improvements on the farms that could be attributed to the project particularly relating to control of mastitis.

Results

Experiences of farmers from the project

Most farmers expressed that they had experienced a marked decrease in the number of mastitis cases, and an increased milk production. Improved knowledge had led to earlier detection of mastitis, and therefore cases were generally of a less severe nature. At the same time they received monthly visits from extension agents and researchers and so they could discuss some of the problems not only about mastitis but also other issues relating to dairy management. So in general the frequent contacts with the veterinary staff assisted them to improve general management particularly to do with control of mastitis. The different treatment groups had varied experiences such as the control group who received no particular control inputs while they heard that other had been supplied with control inputs. This group expressed disappointment although they also said they had gained from the frequent visits from the vets and the free treatment for all the clinical cases provided by the project. Table 1 lists some of the experiences related to mastitis control by the farmers.

Table 1. A list of the farmers' experiences during the mastitis project.

- Importance of hygiene in controlling mastitis
- Value of washing hands and udder before milking
- Proper milking procedures: squeezing the teat rather than pulling it
- Use of teat dips after milking
- Examination of cows for ill health
- Value of emptying the udder during milking
- Proper use of drugs
- The value of record keeping (production, treatments, sales, disease treatments)
- To detect mastitis in an earlier stage
- To develop a better relationship with the animal not to stress it

Benefits derived from the project at household and community level

The order of importance of the benefits from the project following intervention varied between the urban and rural farmers both at household and community level (Table 2) according to the farmers.

IDIs and on farm visits

The IDIs and farm visits revealed that some of the farmers tried to adhere to the recommended strategies, some did not take it seriously and many said that the procedures increased on the labour demands and the milkers did not follow them strictly whenever they were not being supervised.

Occurrence of mastitis

As a result of the intervention there was a decrease in prevalence of mastitis from 61.3% during the baseline cross-sectional study to 56.9% six months after intervention to 49.4

Table 2. Order of importance of household and community benefits.

Urban/peri-urban setting	Rural setting
Household level benefits	
1. Increased cooperation with vets	1. Improved hygiene on the farms
2. Increased knowledge and skills	2. Reduced mastitis cases
3. Improved hygiene on the farms	3. Increased cooperation with vets
4. Improved animals' health	4. Supply of mastitis control inputs
5. Decreased expenses on mastitis	5. Improved record keeping
6. Increased milk production	6. Increased milk production
Community level benefits	
1. More knowledge about mastitis	1. Training about mastitis
2. Improved animal health	2. Increased awareness about mastitis
3. Clean milk sold	3. Reduced expenses on mastitis
4. More milk produced	4. Improved quality of milk
5. Group work increased	5. Increased milk production
6. More jobs available	6. Better market for milk available

Table 3. Prevalence of Mastitis-causing pathogens after intervention.

	Prevalence (%)		
Mastitis pathogens	*Baseline*	*6 months*	*12 months*
Staphylococcus spp	69	32.7	11.5
Coliforms	14.4	6	1.5
Streptococci	12	1.4	0
Others	12	4.5	1.3

% after 12 months intervention. This was attributed to increased awareness about the disease and improved hygiene. A similar decrease in prevalence of mastitis pathogens was also observed (Table 3).

Milk production

According to the farmers in the intervention group, they experienced increase in milk production. This was verified by quarterly milk assessment and comparison of the milk production at different times during the intervention period as shown in Figure 1.

The teat dipping group included washing of udder before milking with cloth and disinfectant and application of an iodophor teat dip after milking. The Selective Rx group involved washing of udder with disinfectant before milking and selective treatment of cows that had mastitis causing organisms just before drying off. The FFS group were trained in mastitis control and adopted any of the control, strategies on their own

Discussion

Participation of farmers at all the stages of the research project cycles is a very important process for intervention strategies if they have to be sustainable and easily adopted. This

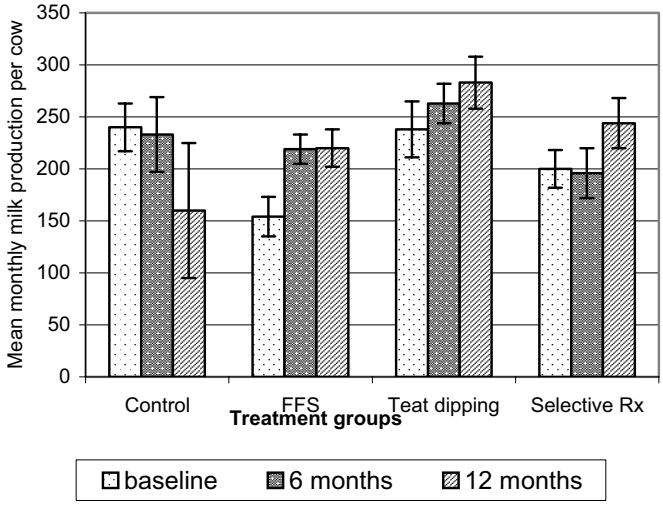

Figure 1. Milk production in the different intervention groups.

includes impact assessment, which involves systematically and continuously assessing progress and changes caused by implementation. The evaluation exercise of this project was therefore meant to further discuss with the farmers how the various strategies could be sustainably used to reduce mastitis occurrence. Several mastitis control strategies have been known for many years. Improved hygiene for example is a well-known method for reducing the level of new mastitis infections (Neave *et al.*, 1969). It has been shown that simple hygiene routines combined with antibiotic therapy for clinical mastitis and for all cows at drying off reduces the levels of infection of cows and of quarters as well as clinical mastitis. However treatment of all quarters at drying without demonstration of pathogens has no rationale and increases expenses and the risks associated with antibiotic use in animals. Benefits from mastitis control in the form of increased milk sales is the ultimate goal for mastitis control and was one of the expectations of the farmers. This combined with improvements attained with increased control effort can entice the farmers to be committed to designed control programmes and its sustainability. These methods were not being routinely followed and this could have been responsible for the high prevalence observed at the beginning of this study and other previous studies (Byarugaba *et al.*, 1998).

Participatory Impact Assessment (P.I.A.) is a widely used methodology of assessing impact among a group of participants in client- oriented research and dissemination projects. The impact of research project in general on households and communities is often not taken into consideration among the objectives of such projects. However by participating in projects, farmers often derive many benefits either both at household level and community level. From the workshops, the farmers' expected to reduce mastitis cases, and thus reduce on expenditures as well as improve the quality of milk. Generally the level of mastitis has decreased following increased awareness and improved hygiene on the farms. Each of the intervention groups including the control group apparently have benefited even just by monthly visits by the project team during data collection and interaction with their fellow farmers.

The evaluation exercises contributed directly to the fulfillment of the overall objective of identification of sustainable packages for mastitis control. The farmers' perception after having used the strategies for a period of time is absolutely necessary if estimating and recommending any strategy to the goal that they must be 'easily adopted and used by the small holder dairy farmers sustainably'. With regard to meeting the specific objectives, the evaluation gave valuable inputs to the establishment of the dairy farmers' knowledge, attitudes and practices towards mastitis and mastitis control and towards educating the farmers about mastitis and the benefits of control. These exercises were also valuable for farmers to hear the experiences of their fellow farmers as a result of the control strategies they were using.

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Environmental factors affecting somatic cells counts in milk from small dairies in Southern Brazil

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Abstract

Although somatic cell counts (SCC) in milk have been widely used as a subclinical mastitis monitoring tool, only a small number of dairy herds in developing countries have adopted this technology in a routinely basis. The objective of this research was to evaluate the impact of environmental effects on SCC in milk from small dairies in Southern Brazil. Milk recording data from 259 dairy herds associated to four cooperatives in Rio Grande do Sul state were collected from January 1998 to July 2003, yielding a total of 165,311 test day observations after editing. The average herd had 21.39 lactating cows, mostly Holsteins, under grazing with concentrate supplementation. The variability on somatic cells scores (SCS) was explained by a linear model containing the following effects: calving year (1997 to 2003), calving month, milk recording year (1998 to 2003), milk recording month, age at calving, cooperative (1 to 4), stage of lactation, years in milk recording (1, 2,..., 5 or greater), herd nested with cooperative and the interaction between herd (cooperative) and calving year. The average SCS was 3.57 and the standard deviation was 1.95. All effects were statistically significant, but years in milk recording. SCS increased progressively towards the end of lactation and as the cows got older. SCS were higher in April and May, and lower from August to December. Cows calving in January had the highest SCS average, while those calving in April had the lowest SCS, which seems to be related to better climatic and nutritional conditions.

Keywords: somatic cell scores, Holstein, dairy cooperatives

Introduction

Milk quality parameters have been used to detect failures in management practices and to serve as references in milk payment programs. The most commonly used characteristics by milk quality assurance programs are fat and protein contents, somatic cell counts (SCC), bacterial counts, water addition, presence of antibiotic residues, sensorial attributes and temperature of the raw milk (3). SCC in milk is a subclinical mastitis indicator worldwide accepted as a milk quality parameter. Most milk recording programs in North America, Europe and Oceania use somatic cell scores (SCS) to register mean SCC values of the controlled herds. Even though the level of infection of the mammary gland is the most important factor affecting SCC in milk (Harmon, 1994), the study of other factors on SCC variability in dairy herds is useful for management purposes.

Electronic cell counters in milk were introduced in Rio Grande do Sul (RS) state by Universidade de Passo Fundo (UPF) as a milk recording service only in 1997, and historical data is not available before that. The initiative to adopt SCC as an obligatory milk quality parameter has been mostly from the industry, and a new legislation has been implemented to ensure that in the whole country. A typical dairy herd in Southern Brazil is small scale (8 lactating cows) and low technology enterprises. However, data for this study were collected from herds that had a larger scale and received technical assistance from cooperatives in a regular basis.

The objective of this study was to evaluate environmental factors affecting SCS from milk recording data under Rio Grande do Sul conditions and establish a set of recommendations directed to the herds in Southern Brazil.

Materials and methods

Data was obtained from the Dairy Herds Analysis Service (SARLE) milk recording program, maintained by Universidade de Passo Fundo, in Rio Grande do Sul state, Brazil. Milk recording data from 259 dairy herds associated to four cooperatives in Rio Grande do Sul state (northwest region) were collected from January 1998 to July 2003, yielding a total of 165,311 test day observations, after editing. The average herd had 21.39 lactating cows, mostly Holsteins, under grazing with concentrate supplementation.

The variability on somatic cells scores (SCS) was explained by a linear model containing the following effects: calving year (1997 to 2003), calving month, milk recording year (1998 to 2003), milk recording month, age at calving, cooperative (1 to 4), stage of lactation, years in milk recording (1, 2,..., 5 or greater), herd nested with cooperative and the interaction between herd (cooperative) and calving year. Age at calving was organized in 6 classes, defined from the observed frequency distribution (class 1, cows calving from 20 to 32 months of age; class 2, from 33 to 45 months; class 3, from 46 to 58 months; class 4, from 59 to 71 months; class 5, from 72 to 84 months; and class 6, greater than 84 months).

SCC data were tranformed in SCS by a logarithmic transformation proposed by Shook (1982) before statistical analysis. Analysis of variance was undertaken using the General Linear Models procedure of SAS (SAS Institute, 1992), and the comparison of the treatment means was performed using Scheffé tests.

Results and discussion

The average SCS found in the study was 3.57 and the Standard deviation was 1.95. This corresponds to an inverval of 71,000 to 140,000 cells/mL, which can be interpreted as a low prevalence of subclinical mastitis in a general basis. Studies carried in other regions in Brazil found higher values of SCS, showing that regional differences in climate and management affect the health of mammary glands (Ostrensky *et al.*, 2000; Teixeira *et al.*, 2003).

The F test of the analysis of variance showed that all effects had a statistically significant effect on SCS at a 0.1% significance level, except years in milk recording. This is somehow surprising result, since one might expect that dairy producers recording SCC for a longer period of time would make a better use of this information, but the herds analyzed had a similar behavior, regardless the years in milk recording.

The interaction between herd nested with cooperative and calving year was significant, showing that management changed in these herds over the years of study and cows calving in different years in the same herd were exposed to different conditions related to the health of the mammary gland. Since the interaction was significant, the isolated effects will not be interpreted.

SCS was significantly affected by the cooperative in which the herd was associated to, and the average SCS ranged from 3.40 to 3.83. These differences are probably associated with the type of technical assistance offered by these cooperatives and also to the fact that the herd average profile (size, milk production, genetics, feeding strategy) differed among cooperatives.

SCS increased progressively from 1998 (3.08) to 2001 (3.68), decreasing in some extent in 2003 (3.68). This result disagrees with other studies (Schukken *et al.*, 1990) that described a descending trend in SCS over the sampling years. Apart from eventual differences in climate, which may increase the risk of mammary infections in given years, the goal of any dairy herd should always be to decrease the mean SCC over the years. The ascending trend observed in this study shows that producers are not fully aware of the damage caused in his budget by mastitis and how to prevent this disease and the dairy processors have not set a consistent program of penalties and prizes in which dairy producers can clearly perceive what kind of raw milk should be delivered to the industry.

Figure 1 shows that there was significant variation in SCS according to the month in which milk recording was performed. SCS were highest in April (3.65) and May (3.65), decreased to reach a bottom value in September (3,51), and remained low the rest of the year. For management purposes, the most important result is that the risk of mammary

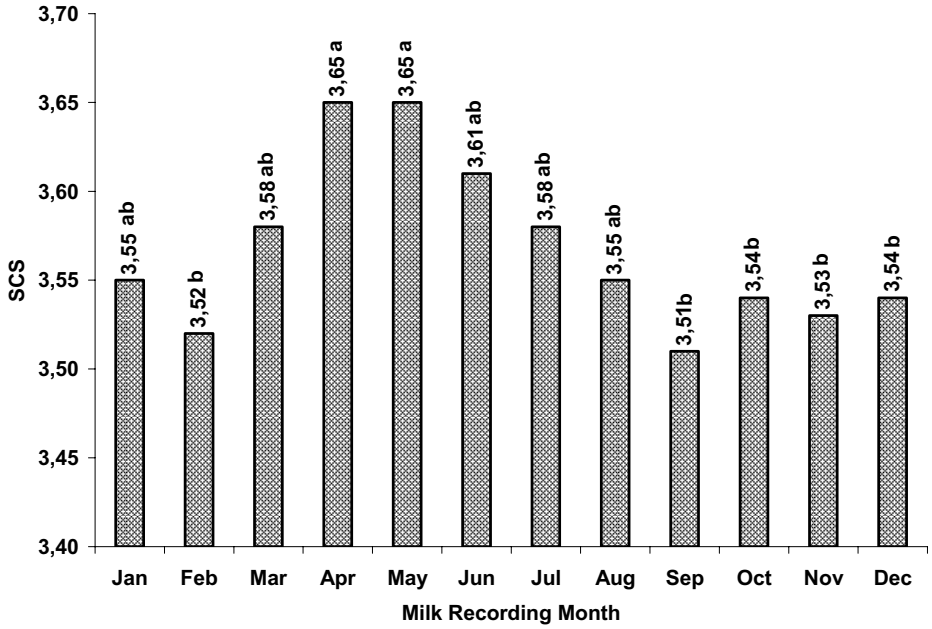

Figure 1. Effect of milk recording month on somatic cell scores (SCS). Means followed by same letter are not significantly different by the Scheffé test (p<0.05).

infections (indirectly assessed here by SCS) increases during the Fall and dairy producers should be more careful with milking procedures at this season. The reasons for that seem to be related to rainy conditions associated with nutritional management. At the Fall, estival pastures have finished their growth and winter pastures are not ready for grazing yet, therefore cows have to spend more time concentrated in small areas because most of their diets are based on conserved forrages that are offered in troughs. This result may be an indication that cows under grazing are less prone to mammay infections.

The effect of stage of lactation on SCS is shown in Figure 2. As the lactation advanced, SCS increased linearly from 2.96 in the beginnig of lactation to 4.08 at the end. This result is in agreement with most of the authors (Harmon, 1994), and indicates that the risk of mastitis increases towards the end of the lactation. Therefore, what makes the average SCC to rise from the begining to the end of the lactation is a larger number of cases of mastitis, and producers should not think that is normal to have cows with high SCC in any stage of lactation (Harmon, 1994).

The age at calving had a significant effect on SCS (Figure 3), increasing progresively from 2.79 in the first age class (20 to 32 months) to 4.17 in the fifth age class (72 to 84 months). The age classes were defined here to be an approximation of the lactation numbers, and the present results are similar to others findings in the literature (Cunha *et al.*, 2002). The observed trend might indicate a reduction in the immunocompetence, as cows get older. Likewise for the previous effect, producers should not consider normal high SCC in older cows.

Finally, Figure 4 shows that calving month had a significant effect on SCS, and the trend is practically inverse to the one observed for milk recording month (Figure 1). Cows calving in April (3.42), May (3.47) and June (3.48) had the lowest SCS during their lactations, while cows calving in December (3.72) and January (3.75) had the highest SCS. This effect is

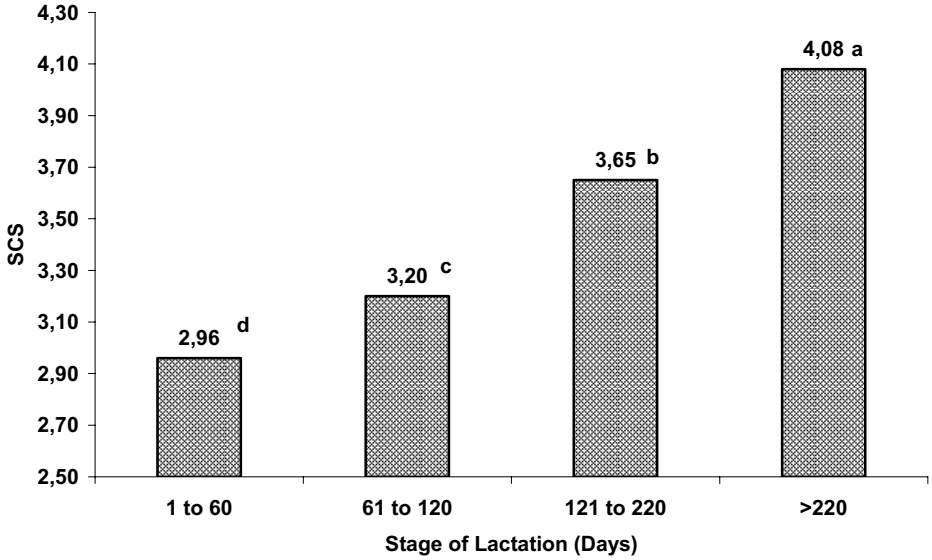

Figure 2. Effect of stage of lactatios on somatic cell scores (SCS). Means followed by same letter are not significantly different by the Scheffé test (p<0.05).

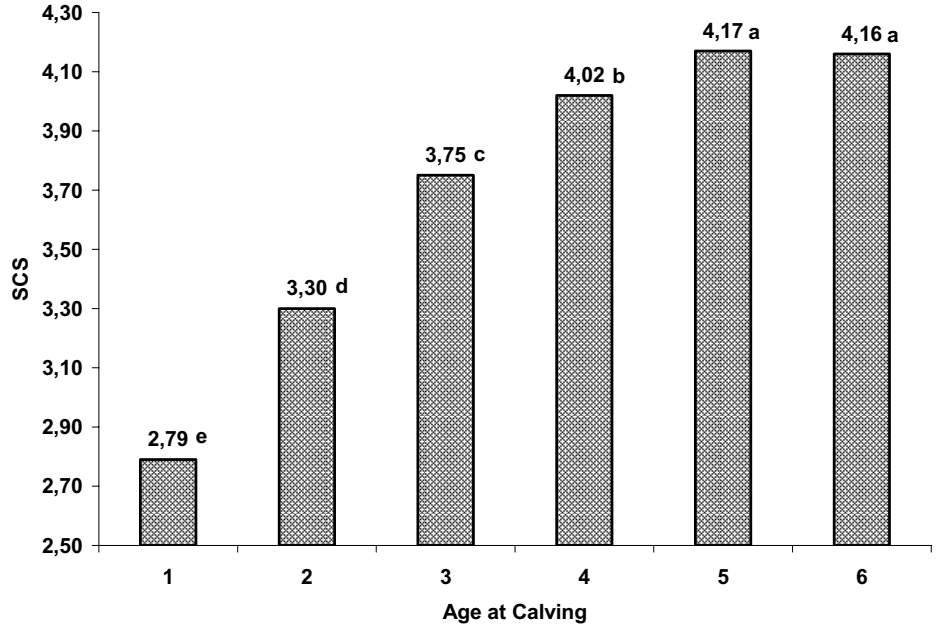

Figure 3. Effect of the class of age at calving on somatic cell scores (SCS). Means followed by same letter are not significantly different by the Scheffé test (p<0.05).

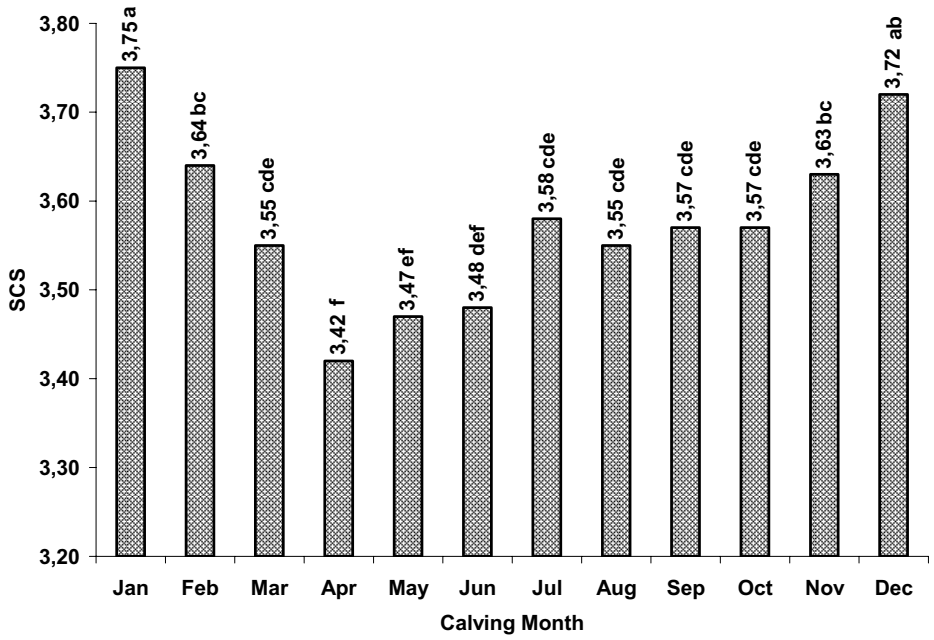

Figure 4. Effect of calving month on somatic cell scores (SCS). Means followed by same letter are not significantly different by the Scheffé test (p<0.05).

probably related to stage of lactation, meaning that cows calving at the Fall (highest SCS means, according to Figure 1) will counterbalance this factor by being at the most favorable stage of lactation (Figure 2). The higher SCS for cows calving in the Summer (December and January), might be related to a less favorable dry period prior to parturition, since most of the new infections in the mammary gland start between lactations (Radostitis *et al.*, 1994).

Conclusions

The average SCC in controlled dairy herds in Southern Brazil might be considered reasobly low, but an increasing trend was observed over the studied period. Different cooperatives in the same region congregate herds with different profiles. The Fall (April an May) is the season in which the highest mean SCC is verified, but cows calving in the Fall are at a lower risk of having mastitis during the lactation than cows calving in the Summer. SCC increases as stage of lactation advances and as the cows get older.

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Risk indicators associated with *Staphylococcus aureus* subclinical mastitis in smallholder dairy cows in Tanzania

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Abstract

A cross sectional study was carried out between June and September 2003 to establish the prevalence and related risks indicators for *Staphylococcus aureus* (*S. aureus*) subclinical mastitis on smallholder dairy herds in Dar es Salaam region, Tanzania. 191 lactating cows from 64 randomly selected herds were investigated. However, due to such reasons as aggressive cow, blind quarters and presence of open teat wound, the California mastitis test (CMT) was carried out on 726 (9 cows and 2 quarters were not sampled) quarter milk samples. Herd (at least one positive quarter per cow), cow and quarter level prevalence of subclinical mastitis defined by CMT ≥ + were 100 %, 91.2 % and 85.0 % respectively. *S. aureus* was isolated in 21.0 % of the 718 (11 cows and 2 quarters were not sampled) - bacteriologically examined quarter milk samples. The cow and quarter level prevalence of sub-clinical mastitis, defined by positive CMT (≥ +) score and S. *aureus* positive culture, was 97.4 % and 98.0 %, respectively. Water availability, residual suckling, single udder-towel, poor housing sanitation, teat-lesions and the failure to use dry-cow therapy were the most substantial ($\rho < 0.05$) risk indicators. It is concluded that intensive and continuous farmer education programmes particularly focusing on subclinical mastitis prevalence and ways to reduce or eliminate these infections, are necessary to improve udder health in Tanzania. These issues are discussed at the end of the paper.

Keywords*:* California mastitis test, smallholder dairy herds, *Staphylococcus aureus*, Tanzania

Introduction

Smallholder dairy producers are important contributors to the agricultural economy in Tanzania. More importantly, this sector employs many Tanzanians and provides a regular source of cash for farmers (Kivaria *et al.*, "in press"). Mastitis, a multifactorial disease with worldwide distribution, is usually associated with microbial infections (Quinn *et al.*, 2000, Radostits *et al.*, 2000), the isolation of which may be used as an aid to diagnosis and treatment of clinical mastitis. Epidemiologically, mastitis agents are categorized as either 'contagious' or 'environmental' (Radostits *et al.*, 2000). *Staphylococcus aureus* (*S. aureus*) a contagious pathogen, although some researchers consider it to be conditionally environmental (Zadoks *et al.*, 2000) is capable of establishing subclinical intramammary infections, which typically manifest as an elevation in the somatic cell count of milk from the affected quarter (Radostits *et al.*, 2000). *S. aureus* is propagated very easily among

cows (Schukken *et al.*, 1999), especially under poor milking hygiene. Various studies (Schukken *et al.*, 1991, 1999, Radostits *et al.*, 2000, Zadoks *et al.*, 2000) have shown that, besides cow-level factors, specific management and environmental factors influence the occurrence of subclinical mastitis.

Previous studies in Tanzania (Karimuribo *et al.*, 2003, Kivaria *et al.*, 2004), indicated that mastitis-especially subclinical mastitis-is also an important problem in smallholder dairy cows. However, smallholder farmers in Tanzania appear to be unaware of the subclinical mastitis situations in their cows. These infections may therefore hamper the control of mastitis because they often go unnoticed, resulting in a long duration and the possibility of spread. The objective of this study was therefore, to elucidate the prevalence, and the risk indicators for *S. aureus* subclinical mastitis in smallholder dairy cows.

Materials and methods

Herds and animals and data collection

Milk samples were collected between June and September 2003, from 191-lactating cows at different stages of lactation on smallholder dairy herds located within and around the Dar es Salaam region. Study population and herd characteristics have been previously described (Kivaria *et al.*, 2004, Kivaria *et al.*, "in press"). The investigation was planned as a cross-sectional observational study. During the study, farmers were questioned on relevant aspects of udder health. The questionnaires used in this study and the management practices studied have been described in an earlier paper (Kivaria *et al.*, 2004).

Screening for subclinical mastitis and microbiological examination of milk samples

CMT was applied as a cow-side test and evaluated as described by Hogan *et al.*, (1999). Based on the thickness of the gel formed by CMT reagent-milk mixture, test results were scored as negative / trace (0), weak positive (+), distinct positive (++), and strong positive(+++). In this study, milk samples with test results of negative / trace were assessed as having originated from cows free of subclinical mastitis while CMT results of $\geq$ + were classified as evidence of subclinical mastitis.

Milk samples for *S. aureus* culturing were taken from clinically "healthy" quarters of all lactating cows as described by IDF (1981) and Hogan *et al.*, (1999). Milk samples were stored and transported to the laboratory ice-cooled within six hours of the collection. *S. aureus* culturing and identification to the species level was performed according to standard procedures described by Hogan *et al.*, (1999).

Statistical analyses

The final data set available for statistical analyses at the herd, cow and quarter levels included 64 herds, 191 lactating cows and 764 quarters. Descriptive procedures for continuous data included calculations of frequencies; categorised data were summarised as contingency tables. Possible individual and environmental predictors of subclinical mastitis were identified using logistic models, as previously described (Kivaria *et al.*, 2004). In this analyses, *S. aureus* subclinical mastitic-sample was the target variable, defined as the one with a positive CMT score ($\geq$ +), and from which *S. aureus* was isolated. The statistical analyses were performed with SPSS 11.5 (SPSS Inc., 2002); the type 1 error was set at $\rho \leq 0.05$.

Results

Descriptive statistics

191 lactating cows from 64 herds were investigated, the average herd size being 12 ± 7 cattle. The mean parity was 2.8 ± 1.6_{Std}; the mean body condition score was 2.5 ± 0.4_{Std}; the average daily milk yield per cow was 8.0 ± 4 litres, the average lactation stage (days in milk) was 7.0 ± 5.0 months. All farmers applied hand milking, and had at least 5 years experience in dairying. In 11 herds 100 % of income was generated through dairying, the remaining 53 herds had additional non-farm income sources, like formal employment, and owing a kiosk, other small business activities. Distribution of some of the studied farm variables among 64 smallholder herds is summarised in Table 1.

Prevalence of subclinical mastitis

764 quarters from 191 lactating cows were examined. However, some cows had open-teat wounds, blind quarters or were aggressive, thus in some cows quarter milk samples could not be collected from all four quarters. 3.9 % of investigated quarters were blind, 31.4 %, had various forms of udder lesions and 7.5 % had increased udder fibrosis (palpable lumps in the gland). CMT was carried out on 726 quarters (9 cows and 2 quarters were not sampled) from 191 lactating cows. Herd-based prevalence of subclinical mastitis (at least one positive quarter per cow) was 100 %, the cow-level prevalence defined by CMT ≥ + was 91.2 %, Overall, 0, +, ++, and +++ CMT scores were observed in 7.0 %, 23.0 %, 24.0 % and 46.0 % of lactating cows, respectively. On quarter basis 85.0 % of all quarter samples tested were CMT positive (≥ +), CMT results of 0, +, ++, and +++ were observed in 15.0 %, 21.0 %, 21.0 %, and 43.0 % of quarter milk samples. *S. aureus* was isolated in 21 % of the 718 (11 cows and 2 quarters were not sampled) - milk samples that were microbiologically examined. The cow and quarter level subclinical mastitis prevalence defined by CMT ≥ + and *S. aureus* positive culture was 97.4 % and 98.0 %, respectively. The relationship between CMT scores and *S. aureus* isolates are displayed in Table 2. 100 (25 cows) quarter-samples had missing values for one or more variables presented for the logistic model. 664 (166 cows) samples were retained for the logistic model; the results of the final multivariable-logistic model are shown in Table 3.

Table 1. Descriptive statistics for the herd variables among 64 smallholder dairy herds in Dar es Salaam region, Tanzania

Variable studied	Number of herds	Percentage
Calf feeding (residual/bucket)	44/20	69/31
Housing sanitary (good/poor)	61/3	95/5
Water source (tap/bore-well)	42/22	66/34
Water availability (frequently/rare)	40/24	62/38
Dry cow therapy (Yes/No)	4/60	6/94
Screening for mastitis (Yes/No)	18/46	28/72
Udder cloth (single/individual)	59/5	92/8
Mastitic cow milked last (Yes/No)	60/4	94/6

Table 2. The relationship between CMT scores and Staphylococcus aureus isolated from 718-quarter milk samples of 191 lactating smallholder dairy cows in the Dar es Salaam region, Tanzania.

	Staphylococcus aureus - culture			
	Positive		Negative	
CMT - scores	Number of samples	Percent	Number of samples	Percent
0	3	2.0	105	18.5
+	9	6.0	143	25.1
++	26	17.5	126	22.1
+++	111	74.5	195	34.3

[a]Negative means that no other bacteria were found.

Table 3. Logistic regression model explaining the relationship between prevalence of Staphylococcus aureus subclinical mastitis and risk indicators for 166 lactating dairy cows on 64 smallholder dairy herds in Dar es Salaam region, Tanzania

Variable	β	SE (β)	Wald-Chi	ρ-value	OR	95% Confidence limits	
Intercept	1.781	0.817	4.752	0.029	-	-	-
Frequent water availability	1.087	0.111	95.899	0.000	2.965	2.385	3.686
Number of milkers (≥ 2)	0.812	0.475	2.922	0.087	2.252	0.888	5.713
Single udder towel	2.233	1.071	4.347	0.037	9.328	1.143	76.074
Residual suckling	-1.81	0.433	17.474	0.000	0.164	0.070	0.382
Poor housing - sanitation	2.293	1.080	4.508	0.034	9.905	1.193	82.215
Teat lesions	1.995	0.422	22.349	0.000	7.352	3.215	16.808
No dry cow therapy	1.633	0.211	59.897	0.000	5.119	3.385	7.740

Discussion

Our results indicate that *S. aureus* subclinical mastitis is prevalent in smallholder dairy cows in Tanzania. Omore *et al.*, (1996) and Workineh *et al.*, (2002) made similar observations among the smallholder and commercial dairy cows in Kenya and Ethiopia, respectively. Various interlinking factors are probably responsible for the observations made in this study.

The observed general deficiencies in milking hygiene in the smallholder dairy herds (Kivaria *et al.*, 2004), poor mastitis treatment protocols, absence of post milking teat dips and the failures by the farmers to use dry cow therapy, partially explain our observations. *Staphylococcus aureus* has adapted to survive in the udder; they frequently result in deep-seated abscesses in the udder (Radostits *et al.*, 2000) and are shed in the milk, which serves as a reservoir for further infection within the herd during the milking process. Moreover, *S. aureus* is transmitted very easily among cows (Schukken *et al.*, 1999), such that deficiencies in milking hygiene mean a rapid within herd transmission of *S. aureus* mastitis. *S. aureus* is a ubiquitous germ capable of contaminating different surfaces and easily isolated from the skin and mucosa of healthy animals, and humans. Therefore, milkers, contaminated water and udder towel, and even flies can spread it. Consequently, *S. aureus* intramammary infections are well established within the herd, and thus, the observed high prevalence of

S. aureus subclinical mastitis in smallholder dairy cows. The significant ($\rho = 0.000$) protective effect of residual suckling could be attributed to the fact that poor and irregular milking by the milker can be corrected by the calf. However, in herds with free-running calves, calves with infected mothers will transmit the infection to the rest of the lactating cows when trying to compensate for the milk that their mother cannot give them.

It worth mentioning that, the multifactorial causes of subclinical mastitis coupled with a failure to control a number of these factors in a cross-sectional study may, to some extent explain the observed wide, in particular, the 'single udder towel' and the 'poor housing sanitation' interval estimates in Table 3. The small number of cows studied in this survey may also have played a role in the observed wide interval estimates.

To reduce the high prevalence of mastitis in these herds, standard milking hygiene, with post-milking teat disinfection and improved herd nutrition, must be introduced. In addition, consecutive milking of infected and non-infected cows should be avoided. Early detection of mastitis, susceptibility testing of the mastitis pathogens before treatment, dry-cow therapy and culling of cows with persistent mastitis are also recommended for the control and prevention of mastitis in the study herds. To this end intensive and continuous farmer education programmes are necessary to improve udder health in Tanzania.

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Subclinical mastitis in buffaloes in different herd sizes and milking management systems

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Abstract

Influence of herd size and milking management on the udder health status of dairy buffaloes was studied. 749 quarter milk samples were taken from 190 animals distributed in two herd sizes and three milking management systems: calf suckling and hand milking (CH), manual pre-stimulation and hand milking (MH), and manual pre-stimulation and machine milking (MM). The samples were analysed for somatic cells (SCC), California Mastitis Test (CMT), proportion of neutrophils (NEU), and bacterial infection. Among the total quarters 97.5% had no clinical signs of mastitis. Milking management significantly influenced occurrence of mastitis while influence of herd size was weaker. Small CH-herds had lowest prevalence of mastitis while large MM-herds had highest. Among small herds, the MM- and MH-herds had similar prevalence of mastitis. Prevalence on *animal basis* was: CMT 37%, SCC 32%, increased NEU 21% and infection 33%, while the corresponding figures on quarter basis were: 15%, 14%, 8% and 12%. Among infected quarters environmental bacteria were predominant with large MM-herds having the highest prevalence. The results indicate that mastitis is mainly due to poor environment. Calf suckling had a positive effect on udder health. Machine milking *per se* did not appear to negatively influence udder health.

Keywords: CMT, SCC, neutrophil, bacteria, pre-milking routines

Introduction

Buffalo milk amounts for about 12% of the world's milk production and is widely consumed in the worlds fastest growing dairy markets. However there is very little information on udder health status of the animals utilised in its production. The buffaloes are reported to be less susceptible for mastitis with lower occurrences than cattle (Wanasinghe, 1985,. Bansal *et al.*, (1995). However studies on the occurrence of mastitis in buffaloes have been based on many different and sometimes unclear criteria for defining mastitis and seldom with data on clinical examination. The results are therefore difficult to interpret.

Factors such as teat injury, infectious load in environment and impaired immunological defence are predisposing for mastitis dependent on the management in particular the milking technique and routines (Carroll, 1977, Bramley and Dodd, 1984). The herd size and the degree of mechanization in the management system may also influence the occurrence of mastitis (Ekman 1998). The somatic cell count (SCC) which can be estimated by the California Mastitis Test (CMT) has also been used to diagnose mastitis in buffaloes (Singh *et al.*, 1982; Dhakal, 1994; Singh and Ludri, 2001). However, there is no established

threshold value for normal SCC in buffalo milk and the CMT reaction has not been properly evaluated when used in milk from this species.

The objective of this survey was to estimate occurrence of mastitis among buffaloes and to establish if and how herd size and milking management system affect udder health.

Material and methods

The study was performed during February and March 2002, in the Sangli district in the southwest of India. Buffalo herds studied were identified based on herd size and milking management system. The different milking management systems used were as follows: pre-stimulation by calf suckling and subsequent hand milking (CH); manual pre-stimulation and subsequent hand milking (MH); and manual pre-stimulation and subsequent machine milking (MM). Herds of 10-20 buffalo cows are referred to as small, and herds ≥ 30 are referred to as large. In total, 190 healthy buffalo cows of mainly Murrah breed was studied. From each herd eight animals were selected at random. In two small herds with CH however, samples were taken from seven animals only as the other animals were dry. Information on lactation number, lactation month, and milk yield at the last morning milking were collected based on the farmer's information. The udders and the milk were examined for clinical signs of mastitis such as general consistency, size and symmetry as well as visible abnormalities of the milk.

A total of 749-quarter milk samples were taken during the morning milking, of these eleven quarters were blind and could not be sampled. For each animal appearance of the milk was checked in a test cup. Samples of 10 ml and 2-5 ml were collected for CMT, SCC and bacteriological examination respectively. Samples for bacteriological examination were taken aseptically after milking in sterile disposal plastic test tubes with a screw capsule (National Mastitis Council, 1999). Samples were kept in a cooling box with ice packs and were stored at -20 °C within 6 h after sampling and later transported to the laboratory. Out of the total 749 samples, data for cell counts and bacteriology are missing in 1 and 46 samples, respectively.

Microscopic cell counting according to Prescott and Breed (1910) modified by Åström (1972) was used to make total and differential cell counts. SCC > 200×10^3/ml and a neutrophil count > 30% of the total count were regarded to be indicative of mastitis (Dhakal, 1994; Silva and Silva, 1994, Singh and Ludri, 2001). CMT was performed at the laboratory within 6 h after sample collection by mixing equal volumes milk and detergent (sodium lauryl sulphate with bromcresolpurple) in a 4-cup paddle. (Schalm *et al.*,1971). CMT scores ≥ 1 in quarter milk were considered as indicative of mastitis (Schalm *et al.*, 1971).

Bacteriological examination was performed at a routine bacteriological laboratory, the Poultry Diagnostic and Research Centre (PDRC), Pune, India (Venkateshwara Hatcheries Ltd.), according to the recommended tests and standard procedures (Honkanen-Buzalski and Seuna, 1995, National Mastitis Council, 1999). The result for each sample is given as either growth (bacteriological positive, bact+) or no growth (bacteriological negative, bact-) of udder pathogenic bacteria species.

The SAS software (SAS Institute Inc.,1997) was used for statistical analyses. Logarithmic transformation (*log* 10) of the SCC values and arcsine transformation for percentage neutrophil values were used to get a normal distribution. The SAS MIXED Model procedure was used for the analysis of variances where animal identity and farm identity were random

factors and fixed factors were system (CH, MH, and MM), herd size (small and large), milk yield in kg (< 4, 4-6 and > 6), lactation month (< 2.5, 2.5-6, and ≥ 6) and lactation number (≤ 3, 4, and > 4). Interactions between the factors were also analysed and later deleted from the model if not significant.

Results

Of 703 samples 85% had low CMT scores and cell counts, and 11% were bact+. The distribution of cell counts within each CMT score was wide. Of the 703 milk samples 15% had a CMT score ≥ 1, of which 34% were bact+ samples. Of the total number of bact+ samples in the study, 44% had a CMT score ≥ 1. Mean *log SCC* ±SEM per ml for *all quarters, bact-* and *bact+* quarters, respectively were *4.86* ± 0.01 (73×10^3, range 11×10^3 - $16\ 192 \times 10^3$); *4.80* ± 0.01 (63×10^3, range 11×10^3 - $11\ 561 \times 10^3$) and *5.25* ± 0.07 (178×10^3, range 32×10^3 - $15\ 849 \times 10^3$), respectively. The corresponding figures for *mean arcsine of proportion of neutrophils* were *0.07*± 0.01 (7.9%, range 0-96%), *0.05* ± 0.01 (6.0 %, range 0-86%) and *0.22* ± 0.03 (21.9%, range 0-95%), respectively. Four quarters (of 4 cows) were diagnosed as atrophic with decreased size and harder consistency than what is normal. Moderate clots were found in 13 milk samples while no swollen quarter was observed where as only 20 animals (25 quarters) had previously had mastitis.

In approximately half of the milk samples, structures with a characteristic appearance, looking like fragments of cells, were found (Table 1). They could be clearly identified as not being artefacts. These fragments were half-moon shaped with varying sizes of the sphere missing and a homogenous appearance without any nuclear structure. Imagining the size of the full sphere the size of a fragment was similar to that of a monocyte.

On an *animal basis,* the prevalence of mastitis based on increased value of the different inflammatory indicators was: CMT 38%, SCC 32%, and proportion of neutrophils 21%, respectively. On a *quarter basis*, the prevalence of mastitis was: CMT 15%, SCC 14%, and proportion of neutrophils 8%, respectively. The prevalence of mastitis based on *bact+* samples was 32% on an animal basis and 12% on a quarter basis. Of the bact+ samples, 32 % were diagnosed as udder-specific bacteria, *i.e. Staphylococcus aureus* 25%, *Streptococcus agalactiae* 5%, and *Streptococcus dysgalactiae* 2%. The remaining 68% of the bact+ samples were diagnosed as environmental bacteria, mainly coagulase-negative staphylococci (43%) and *Streptococcus uberis* (10%).

LS mean for *log SCC* in CH/small herds was significantly lower than in MH herds of both sizes and in MM/large herds, respectively (Table 2). The LS mean for the *proportion of neutrophils* was significantly lower in CH herds of both sizes and in MM/small herds, compared to the other groups of herds. Prevalence of mastitis in different management systems and herd sizes given as frequencies of quarters with increased content of inflammatory indicators in milk or with bact+ results are shown in Table 3. The frequency of suspected mastitic quarters was lowest in CH/small herds and highest in MM/large herds. The frequency of total bact+ positive samples was lowest in CH/small herds while it was highest in MM/large herds.

Discussion

The cells types found in the buffalo milk were similar to those found in bovine milk and in similar proportions with low and increased SCC, respectively although the variation between the samples was high (Hageltorn and Saad, 1986; Östensson 1993). The results also agree with what has been reported earlier in buffaloes by Dhakal, (1992), while Silva and Silva (1994) reported higher proportion of neutrophils in milk from healthy udder quarters. Fragments similar to those found in the present study have also been found in milk from other species. The presence of anucleated cell fragments has been reported in goat and camel milk (Wooding *et al.*1970; Abdurahaman *et al.*, 1992). In these species it was considered to be due to the apocrine milk secretory process. Since 97.5% of the quarters in this study did not show any clinical signs of mastitis the prevalence figures presented can be considered to reflect the occurrence of the subclinical form of mastitis.

The prevalence of subclinical mastitis based on bact+ results found in this study was similar to earlier reports (Chander and Baxi (1975), Bansal *et al.,* (1998). The prevalence of mastitis based on SCC was similar to the prevalence of bact+ mastitis, supporting SCC as the indicator that best reflects infectious mastitis. However, only 34% of the udder quarters with positive CMT reactions were found to be bact+, which is much lower than Singh *et al.*, (1982) found in buffaloes. In bovines the corresponding figure has been estimated to be between approximately 30% and 70% in milk with CMT scores from 1 to 3 (Schalm *et al.,* 1971).

The prevalence of subclinical mastitis based on CMT was higher than when based on the other parameters, which agrees with results presented by Singh *et al.*, (1982). CMT is the crudest method of those studied and actually only gives an estimation of the approximate cell count. The proportion of samples being incorrectly scored in the CMT is high when it is used both in buffalo and bovine milk and the intermediate reactions are not reliable (Smith and Schultze, 1966; Lund, 2003). Prevalence of mastitis was lowest with the proportion of neutrophils however there are no other reports on studies of prevalence based on this inflammatory parameter in buffalo milk. The amount of neutrophils is considered to most directly reflect inflammation and that could be relevant to use in practical work (for review see Harmon, 1994; Pillai *et al.*, 2001). In the present study the proportion of neutrophils was generally lower in milk with low SCC than in milk with high SCC, in accordance with previous studies of buffalo milk (Dhakal *et al.,*1992; Silva and Silva 1994).

The results show that udder health and occurrence of mastitis in buffaloes are highly influenced by the milking management system and to some extent also by the size of the herd. Udder health was found to be poorer in larger compared to smaller herds. The reasons behind the generally weaker udder health in the large herds are probably to be found in a lesser consistency in management routines. In this study, buffaloes in small herds are probably better managed than in large herds due to the fact that most small herds are family farms managed by the owners themselves and usually without hired labour. There is a general lack of important knowledge about proper milking routines, hygiene, milking machines and mastitis. The generally higher frequency of infections with environmental bacteria in large herds, except for MH herds, agrees with our general impression that in the large herds, environmental and management hygiene were often neglected and not as good as in the small herds. Among the milking management systems in this study, the udder health was best in CH herds. Calf suckling appeared to be a main factor contributing to good udder

health, which is in accordance with previous reports (for review see Krohn, 2001). It has also been shown that calf suckling is gentler to the teats resulting in less increase of the teat thickness compared to machine milking. (Hamann and Stanitzke, 1990) A good condition of the teat end is important for a proper closure of the teat canal, to prevent infections.

In the present study, large MM herds had the highest prevalence of mastitis both in terms of cell content in the milk and infections, mainly caused by environmental bacteria. It is however noteworthy that in the small MM herds the prevalence was markedly lower and based on cell counts even lower than in small HM herds. Hence, it appears that it is not the machine milking as such that is negatively influencing the udder health but rather reasons related to the management in the large herds. The reasons to the results may partly be deficiencies in environmental hygiene in the large MM herds giving a high infectious pressure. The milking machine and the milking animal are linked through the operator and a careless operator can cause teat injuries and the transfer of pathogens into the udder. As machine milking is introduced in buffalo herds, the need for proper knowledge and training among the milkers on correct milking routines and machine milking practises has to be met.

Table 1. SCC and percentage share of different cells, and the frequency of bacteriologically positive quarter samples within different cmt scores. Total n=703.

CMT score (n)	SCC × 10^3/ml Mean (min/max)	Monocyte/ macrophages (%) Mean (min/max)	Neutrophils (%) Mean (min/max)	Lymphocytes (%) Mean (min/max)	Epithelial cells (%) Mean (min/max)	Unidentified (%) Mean (min/max)	Bact.pos. total[1] (%)	EB[2] pos. (%)	USB[3] pos. (%)
- (162)	56 (11/506)	87 (6/99)	3 (0/56)	3 (0/36)	1 (0/10)	4 (0/56)	8	6	2
T (436)	112 (11/7 216)	88 (6/99)	3 (0/77)	3 (0/53)	1 (0/17)	4 (0/71)	7	4	3
1 (72)	460 (14/3 300)	69 (20/99)	21 (0/80)	4 (0/26)	2 (0/17)	3 (0/32)	30	22	8
2 (25)	2 370 (44/7 260)	43 (3/96)	48 (0/96)	3 (0/27)	3 (0/18)	2 (0/11)	36	24	12
3 (8)	6 946 (22/16 192)	24 (7/95)	71 (2/92)	1 (0/5)	3 (0/10)	0 (0/2)	62	50	12

[1] Total bacteriologically positive samples

[2] Samples positive for environmental bacteria

[3] Samples positive for udder specific bacteria

Table 2. Antilog somatic cell count and percentage share of neutrophils (lsm) in quarter milk samples from different herd sizes and management systems.

Parameter	Herd size 10-20			Herd size ≥ 30		
	CH	MH	MM	CH	MH	MM
Antilog SCC × 10^3 /ml	41.7[a1]	89.1[b]	72.4[ab]	67.6[ab]	95.5[b]	81.2[b]
Neutrophils (%)	1.7 [a]	13.2 [b]	5.6 [a]	5.4 [a]	9.1 [b2]	10.3 [b2]

CH: Calf suckling and hand milking, MH: Manual pre-stimulation and hand milking,

MM: Manual pre-stimulation and machine milking.

[1]ab: Figures with one letter in common within row are not significantly different (P <0.05).

[2]Figures with this superscript showed a tendency to differ to from each other (P=0.06)

Table 3. Frequency of mastitic udder quarters, based on inflammatory indicators and bacteriological results respectively, in different herd sizes and milking management systems.

Indicator of inflammation/infection	Herd size 10-20			Herd size ≥ 30		
	CH (%)	MH (%)	MM (%)	CH (%)	MH (%)	MM (%)
	n = 120	n = 126	n = 125	n = 125	n = 126	n = 127
CMT ≥ 1	5.8	15.1	15.2	9.6	19.1	25.1
	n = 120	n = 126	n = 125	n = 125	n = 125	n = 127
SCC > 200 × 103/ml	3.3	14.2	8	14.4	19.2	25.2
Neutrophils > 30%	3.3	12.1	4.8	5.6	7.2	15.1
	n = 116	120	n = 117	n = 123	n = 111	n = 116
Total bact. positive quarters	0.8	16.6	16.2	8.1	7.2	19.8
Environmental bact. positive quarters	0	8.3	11.1	5.6	4.5	17.2
Udder specific bact. positive quarters	0.8	8.3	5.1	2.4	2.7	2.5

CH: Calf suckling and hand milking, MH: Manual pre-stimulation and hand milking

MM: Manual pre-stimulation and machine milking.

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