Coagulase-Negative Staphylococci in Bovine Sub-Clinical Mastitis

Britt-Marie Thorberg

Division of Bacteriology and Food Hygiene
Department of Biomedical Sciences and Veterinary Public Health
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences

Licentiate Thesis

Department of Biomedical Sciences and Veterinary Public Health
Swedish University of Agricultural Sciences

Report no. 2

Uppsala 2008

Report / Department of Biomedical Sciences and Veterinary Public Health
Swedish University of Agricultural Sciences
Report no 2
ISSN 1653-8315
ISBN 978-91-85911-58-5

Print: SLU Service/Repro, Uppsala 2006

Coagulase-negative staphylococci in bovine sub-clinical mastitis.

Abstract

Mastitis is a common disease in dairy cows. The majority of the cases are sub-clinical, and many of those are due to infection with coagulase-negative staphylococci (CNS). CNS is a heterogeneous group of bacteria consisting of a large number of different species, but limited information is available on the epidemiology of mastitis due to these species.

The overall aim of the thesis was to improve the knowledge on prevalence and significance of different CNS species in connection with sub-clinical mastitis in dairy cows. In the first study, the epidemiology of CNS species, i.e. ability to induce persistent intra-mammary infections (IMI), and association with milk production, SCC, parity and month of lactation, was studied in dairy herds with problems due to sub-clinical CNS mastitis. The most commonly isolated CNS species were *S. epidermidis*, *S. simulans*, *S. chomogenes*, *S. xylosus* and *S. haemolyticus*. Persistent IMI were common in quarters infected with *S. chromogenes, S. epidermidis* and *S. simulans*. The results did not indicate differences between these CNS species in their association with daily milk production, cow SCC, and month of lactation. *S. epidermidis* was mainly found in multiparous cows, and *S. chromogenes* in primiparous cows.

The second study concentrated on *S. epidermidis* by investigating possible transmission of *S. epidermidis* from milkers to cows, the discriminatory capacity of the sub-typing methods used and the clonal diversity within unrelated bovine *S. epidermidis* strains. Pulsed-field gel electrophoresis had high discriminatory power and showed that many different *S. epidermidis* types exist in bovine milk samples. Identical isolates were found in samples from the milker's skin and in milk samples. As dairy cows are not a natural host for *S. epidermidis* the results suggest a human source of these udder infections.

Keywords: sub-clinical mastitis, bovine, coagulase-negative staphylococci, CNS species, epidemiology, *Staphylococcus epidermidis*, human skin, phenotyping, genotyping

Author's address: Britt-Marie Thorberg, Department of Biomedical Sciences and Veterinary Public Health, SLU,
Box 7036, 750 07 Uppsala, Sweden
E-mail: Britt-Marie.Thorberg@bvf.slu.se

Dedication

To myself, for finally being ready…

Förlossare av all strålglans och vibration. Mildra motstånd i mitt väsen och skulptera ut ett rum inom mig att fylla med närvaro i frihet.

Fyll mig med kreativitet så jag får kraft att bära frukten av mina föresatser.

Lös upp de snåriga ödestrådar som håller mig borta från att vila närvarande, låt mig även få befria andra från snarorna av gångna misstag.

Låt mig inte förföras av sådant som får mig att vika från min sanna natur, utan klargör de möjligheter som finns i allt som sker nu.

För i grunden är jag del i den fruktbringande visionen, födelsen, kraften och förverkligandet, då allt är samlat och helt än en gång.

Fri tolkning av äldre version av Fader vår

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I B.-M. Thorberg, M.-L. Danielsson-Tham, U. Emanuelson, and K. Persson Waller. Study on bovine sub-clinical mastitis caused by different types of coagulase-negative staphylococci (manuscript).

II B.-M. Thorberg, I. Kühn, F.M. Aarestrup, B. Brändström, P. Jonsson, and M.-L. Danielsson-Tham (2006). Pheno- and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. *Veterinary Microbiology* 115, 163-172.

Paper II is reproduced with the permission of the publisher.

Abbreviations

CMT	California mastitis test
CNS	Coagulase-negative staphylococci
CSCC	Cow somatic cell count
IMI	Intra-mammary infections
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MLST	Multilocus sequence typing
NOSEC	Novobiocin-sensitive CNS
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RFLP	Restriction fragment length polymorphism
SCC	Somatic cell count
SH	Swedish Holstein
SOMRS	Swedish official milk recording system
SR	Swedish Red

1 Introduction

1.1 Milk Production in Sweden

In 2007, the Swedish dairy cow population consisted of around 370 000 cows in 7 100 dairy herds, and the average herd size was 52 cows/herd (SCB, 2008). The majority of the dairy cows are of the two breeds Swedish Holstein (SH) and Swedish Red (SR). On average, 37% of the cows in a herd are first-parity cows, and the cows are culled after 2.7 lactations. The dairy herds are to a large extent run as family businesses, and most herds still house their cows in tie stalls. Most cows are fed a diet based on grass-silage, grains, and industrially processed concentrates.

Around 80% of the herds are enrolled in the Swedish official milk recording system (SOMRS; Olsson *et al.*, 2001), which includes herd and cow data for example regarding milk production, and milk somatic cell count (SCC). In Sweden all antimicrobial treatments of dairy cows must be initiated by a veterinarian, and all veterinary diagnoses and treatments should be reported to the Swedish animal disease recording system (Emanuelson *et al.*, 1988; Olsson *et al.*, 2001), which is linked to the SOMRS. All herd and cow data are reported back to the farm on a monthly basis, and each year, the Swedish Dairy Association publishes a report summarising national data on milk production and disease incidence. The most common veterinary-treated disease affecting Swedish cows is mastitis, and the yearly incidence of veterinary-treated clinical mastitis is at least 16% (Swedish Dairy Association, 2008). Typically, a difference in udder health and milk production is observed between the two dairy breeds. SR-cows have lower incidence of veterinary-treated mastitis, lower cow SCC, and lower milk production than SH-cows.

1.2 General Introduction to Bovine Sub-Clinical Mastitis

Mastitis, defined as an inflammation of the udder, is the most costly disease in dairy farming, and is mostly caused by bacterial intra-mammary infections (IMI). Mastitis is characterized as clinical or sub-clinical mastitis depending on if clinical signs are present or not. Clinical mastitis can be further classified as mild, moderate or severe mastitis, and does often require antimicrobial therapy.

This thesis focuses on sub-clinical mastitis, which is defined as mastitis that can not be diagnosed by clinical examination. Instead analysis of inflammatory products in the milk is needed. In most cases, direct or indirect measurement of the milk SCC is used to diagnose sub-clinical mastitis. This is often combined with bacteriological examination of the milk to get an aetiological diagnosis. The costs caused by sub-clinical mastitis are mainly due to loss of milk production, reduced payment caused by lower milk quality, and costs for culling and replacement of cows (Sandgren *et al.*, 2008). To reduce the incidence of sub-clinical mastitis new infections must be prevented. Control measures to reach this goal may differ depending on the character of the udder pathogens prevalent in the herd. Sub-clinical mastitis is often caused by bacteria, which can be transmitted between and within udders. In these cases, ways to prevent such transmission, e.g. at milking, must be considered. Udder pathogens can, however, also derive from the environment of the cow. If so, ways to improve environmental hygiene and general cow health must be considered.

In many programs on control of sub-clinical mastitis, antimicrobial dry-cow therapy of all or most cows in a herd has been considered a corner stone. The Scandinavian view is, however, to use dry-cow therapy only on a selection of cows. Cows with sub-clinical mastitis are selected for antimicrobial therapy based on an overall evaluation of success of treatment, which includes economical benefits. The policy is to only use antimicrobials with a documented effect, to base the choice of antimicrobials on bacteriological examinations, and to avoid broad-spectrum antimicrobials (Ekman *et al.*, 1995). If antimicrobial therapy of sub-clinical mastitis is considered, it is recommended to treat at dry-off rather than during the lactation period. Based on the current low prevalence of antimicrobial resistance among udder pathogens in Sweden, the policy has been successful (SVARM, 2007).

As already mentioned, most cases of sub-clinical mastitis have bacterial aetiology. The prevalence of different bacteria differs from country to country, but contagious udder pathogens pre-dominate (Djabri *et al.*, 2002). For decades, the genus *Staphylococcus* has been the dominating finding in

sub-clinical mastitis in Sweden. In 2007, *Staphylococcus aureus* was found in 46% of bacteriologically positive cases of sub-clinical mastitis, while coagulase-negative staphylococci (CNS) were found in 25% of such cases (Swedish Dairy Association, 2008). *S. aureus* mastitis has been described in detail elsewhere in numerous reports (Kerro Dego *et al.*, 2002; Sears and McCarthy, 2003; Barkema *et al.,* 2006). CNS mastitis has, however, not been so well elucidated. This is partly explained by the heterogeneity of this group of bacteria consisting of a large number of different CNS species.

1.3 The Genus *Staphylococcus*

Rosenbach introduced the generic name *Staphylococcus* in 1884 for the round organisms in pus that several others had described earlier, and classified species of staphylococci on the basis of colony colour. He called the orange-yellow staphylococci *S. aureus* and the white staphylococci *S. albus*. *S. albus,* later named *S. epidermidis,* was presumed to be a commensal, but already in 1882 Ogston was aware of the presence of staphylococci on the skin of the human body and questioned their innocence (Kloos, 1980). Using colony colour for species classification later proved to be unsatisfactory.

The progress in taxonomy and discovery of phenotypic properties useful for classification of the genus *Staphylococcus* was slow. It was not until the 1930s that the correlation between coagulase reaction, first introduced by Daranyi (1925), and pathogenicity became apparent. Two new groups were formed, coagulase positive pathogenic staphylococci and coagulase-negative non-pathogenic staphylococci. During the 1960s Baird-Parker conducted some of the most comprehensive taxonomic studies of the genus *Staphylococcus* and proposed a sub-division into sub-groups I-VI (Baird-Parker, 1963). Sub-group I contained the species *S. aureus*, and sub-groups II-VI were distinguishable types of CNS. Later, the sub-groups II-VI were included under the group name *S. epidermidis* and sub-groups were changed into biotypes. Today Sub-group II, later named *S. epidermidis* biotype I, has essentially become the species *S. epidermidis* sensu stricto.

The organisation in biotypes and a new series of studies focusing on different host-adapted populations of staphylococci in this complex genus lead to the introduction of new species and sub-species. Most of this work was performed by Kloos and Schleifer and published in the 70s and 80s (Kloos and Schleifer, 1975a; Schleifer and Kloos, 1975; Kloos *et al.*, 1976; Devriese *et al.*, 1978; Kloos *et al.*, 1983; Schleifer *et al.*, 1984; Devriese *et al.,* 1983). The many changes made in the taxonomy of staphylococci can still cause confusion as the old classification of *S. epidermidis* is sometimes

thought to be today's species *S. epidermidis* sensu strico, and vice versa. Conclusions concerning the aetiology of CNS infections reported prior to the 1980s should be made with some caution due to the early use of the name *S. epidermidis* as a group name and later as a species name. *S. hyicus* could also have been interpreted to occuvery frequently in early studies due to the fact that *S. chromogenes* was a sub-species of *S. hyicus* until 1987 (Hajek *et al.*, 1986).

1.3.1 Species identification in clinical bacteriology

To identify bacterial species medical bacteriologist have learned to rely on phenotypic properties that are relatively easy to recognize such as shape, size, colour, staining, motility, capsule, colony morphology, formation and characteristic of fermentation products, ability to metabolise various substrates, antimicrobial sensitivity and habitat (including the host range and pattern of disease for pathogens). New simplified classification systems based on biochemical tests were proposed for human (Kloos and Schleifer, 1975b) and bovine (Devriese, 1979) staphylococci. The latter scheme was later extended to other farm animals (Devriese *et al.*, 1985). However, such conventional biochemical tests are time- and labour consuming, and are mostly unsuitable for routine microbiological diagnostics. Commercial kits for fast identification of staphylococci are available, but without evaluation of performance of host specific CNS strains they are not suitable for research (Thorberg and Brändström, 2000). There might also be local differences in CNS typeability. Some commercial kits have a very limited value in routine diagnostics of bovine CNS as they are mainly designed for identification of human strains. Poor performance compared to molecular methods has been reported both for bovine and human strains (Couto *et al.*, 2001; Taponen, 2008).

New methods for species identification are being developed, based on genotypic characteristics. Comparisons with conventional biochemical typing methods have, however, been lacking until recently when Santos *et al.* (2008) used restriction fragment length polymorphism (RFLP)-PCR of the *groEL* gene for species identification of bovine mastitis strains and was able to confirm the results with conventional biochemical species typing. In addition to identification of species-specific genes, genotyping is also useful for identification of genes involved in virulence and antimicrobial resistance.

1.3.2 Sub-typing of CNS

Sub-typing is the identification of certain types or groups of bacteria, which can be identified below the species or sub-species level. Sub-typing is

essential in bacterial epidemiology when monitoring the distribution of infections, and in determining the etiologic agent or reservoir for infections. The repeated isolation of a particular sub-type or strain is more clinically significant than the repeated isolation of a specific species. The work of finding clinically relevant strains is complicated by the fact that CNS is a part of the normal microflora on the skin and mucous membranes. To identify a host habitat for CNS is also complex as both true colonisation (resident microflora) and environmental contamination (transient microflora) can be found on the skin and mucous membranes. Bacteria isolated from areas protected from environmental contamination are more liable to reflect true colonisation.

Several different sub-typing methods of staphylococci have been tested and can be divided into molecular and conventional methods. Today, the molecular methods are dominated by different genetic fingerprinting techniques. Generally accepted and widely used conventional sub-typing methods of CNS have not yet been established. Phage typing has been tested, but was shown to be inefficient for CNS (Holmberg, 1973; de Saxe *et al.*, 1981; Aarestrup, 1995). Biotyping has been working for some species, but was thought to be too slow and some isolates change phenotype from time to time (Christensen, 1987; Pfaller and Herwaldt, 1988). A combination of several methods has also been proposed (Hebert *et al.*, 1988).

Antibiograms have also been used and are of value when searching for special strains linked with a multiple resistance pattern (Widerström *et al.* 2006). In human medicine, dissemination of methicillin-resistant *S. aureus* (MRSA) is an important and costly problem. This problem is also emerging in veterinary medicine. The consensus in clinical diagnostics in the latest decade is that pulsed-field gel electrophoresis (PFGE) of genomic DNA macrorestriction fragments is the method of choice when typing MRSA (Mulvey *et al.*, 2001; Murchan *et al.*, 2003) and *Sma*I has been the restriction enzyme of choice. Other enzymes have been tested, but it seems like methodology and interpretation using *Sma*I suits most laboratories. Sub-typing of methicillin-resistant *S. epidermidis* (MRSE) isolates by PFGE has been done with similar methodology as described for MRSA, but has not resulted in any standard guidelines. Guidelines for optimal epidemiological markers to use for the definition of a *S. epidermidis* clone have not been defined (Miragaia *et al.*, 2008), as information regarding evolutionary development of *S. epidermidis* clones has been missing.

Other DNA based CNS sub-typing methods that have been tested in smaller scale are for example single gene typing systems like ribotyping (Aarestrup, 1995; Andollina *et al.*, 2004), and multilocus sequence typing

(MLST) based on sequencing of conserved housekeeping genes (Miragaia *et al.,* 2007).

1.3.3 Pathogenic significance of CNS

Prior to the 1970s, clinicians and microbiologists generally regarded CNS as contaminants in clinical specimens and *S. aureus* as the only pathogenic *Staphylococcus* species. In humans it became clear, however, that CNS could cause sepsis and mortality in extremes of life, like in geriatric patients and neonates (Wade *et al.*, 1982). Such CNS infections, most frequently caused by *S. epidermidis*, are mainly associated with the use of intravascular catheters and other indwelling or prosthetic material used in intensive medicine and surgical therapy (Kloos and Bannerman, 1994). *S. saprophyticus* has also been frequently found in urinary tract infections in young women (Latham *et al.*, 1983).

Today 41 different staphylococcal species have been described plus a number of sub-species (Euzeby, 2008). In veterinary medicine bovine mastitis is the dominating infection in association with CNS. Studies on identification of different sub-types of CNS, and different CNS species in association with mastitis have been performed since in the 1970s (Holmberg, 1973; Devriese, 1979).

1.4 Bovine Sub-Clinical CNS Mastitis

CNS have often been considered as minor udder pathogens, causing relatively small udder health problems. However, CNS infections may cause substantial herd problems due to high prevalence of sub-clinical and/or clinical mastitis (Wilson *et al.*, 2007). Another concern is the higher prevalence of ß-lactamase production in CNS compared to *S. aureus*. In Sweden, ß-lactamase production was found in 32% and 15% of sub-clinical CNS and *S. aureus* isolates, respectively (Swedish Dairy Association, 2008). The occurrence of antimicrobial resistance like ß-lactamase production or oxacillin resistance can be a reason to identify and reduce certain CNS IMI.

The prevalence of CNS mastitis varies markedly between studies as reviewed by Taponen (2008). The variation may at least partly be due to differences in sampling and diagnostic techniques, making direct comparisons between studies difficult. Other factors of importance are parity and stage of lactation. According to several studies, the highest prevalence of CNS IMI occurs in heifers around parturition (Oliver and Sordillo, 1988; White *et al.,* 1989; Fox *et al.*, 1995). Housing system and production level can also be of importance. For example, heifers on pasture have a low

prevalence of CNS IMI (Compton *et al.*, 2007), and cows with a high milk production have been associated with more frequent CNS IMI (Gröhn *et al.*, 2004).

The udder quarter prevalence of CNS IMI may vary between 4% (Aarestrup, 1995) and 50% (Trinidad *et al.*, 1990a). In two recent studies from Switzerland and Estonia, each including a large number of herds, the prevalence of CNS IMI was 8.3% on cow level (approximately 3.5% of udder quarters) (Roesch *et al.*, 2007) and 4.5% on quarter level (Haltia *et al.*, 2006), respectively. The most recent Scandinavian surveys indicate that the overall prevalence is quite different in Finland and Norway despite substantial similarities in dairy production between the two countries. The udder quarter prevalence of sub-clinical CNS mastitis was 16.6% in Finland (Pitkälä *et al.*, 2004) and 3.3% in Norway (Østerås *et al.*, 2006). The discrepancy may be explained by differences in the number of CFU/ml needed for a sample to be considered as CNS positive as the limit used was 10 times higher in the Norwegian study (Whist, A.-C., personal communication). A similar study has not yet been performed in Sweden. In a Danish study conducted in 20 herds in 1993, the udder quarter prevalence of CNS IMI was 4.1% (Aarestrup, 1995). In many studies, the prevalence of udder pathogens is presented on udder quarter level, which may be less complex to present than cow level data. However, for studies on associations between IMI and milk production, or other cow-related outcomes, the cow level prevalence is needed.

Conflicting results have been presented concerning the association between CNS IMI and milk production. Early studies described no (Eberhart *et al.*, 1982) or negative (Timms and Schultz, 1987) correlation between such infections and milk production. Later, Wilson *et al.* (1997) found a higher milk production in cows with CNS IMI than in healthy cows in a large US survey. In a study on associations between milk production and clinical mastitis, multiparous cows affected by clinical CNS mastitis had a higher milk production before the onset of mastitis than healthy cows (Gröhn *et al.*, 2004).

The SCC associated with CNS IMI has been reported to vary markedly within and between studies. In a meta-analysis of Djabri *et al.* (2002), the geometric mean udder quarter SCC in CNS IMI was 138 000 cells/ml (95% CI 20 000 – 210 000 cells/ml). Taponen *et al.* (2008) found the geometric mean and median in CNS infected udder quarters to be 647 600 cells/ml (95% CI 223 400 – 1 091 800 cells/ml) and 355 400 cells/ml, respectively, which was a 10-fold rise compared with healthy udder quarters.

In some studies, no effect of stage of lactation on prevalence of CNS infections was found (Harmon and Langlois, 1989), while other studies reported that CNS IMI mainly occur in later stages of lactation for older cows (Davidson *et al.*, 1992) and in early lactation for first parity cows (Gröhn *et al.,* 2004; Taponen *et al.*, 2007). The latter study found higher prevalence of CNS IMI in first parity cows, which is consistent with other studies (Matthews *et al.*, 1992; Rajala-Shultz *et al.*, 2004). Overall, the prevalence of CNS IMI seems to be 2- or 3-fold higher in first parity cows than in older cows (Harmon and Langlois, 1989).

1.4.1 Different CNS species associated with bovine sub-clinical mastitis

With some variation, the most frequently isolated CNS species from bovine IMI are *S. chromogenes*, *S. epidermidis*, *S. haemolyticus, S. hyicus, S. simulans*, and *S. xylosus* (Jarp, 1991; Birgersson *et al.*, 1992; Matthews *et al.*, 1992; Aarestrup, 1995; Taponen *et al.*, 2006), but a number of other species have also been reported.

Some CNS species have been associated with the bovine skin microflora or the close environment of the cow. *S. chromogenes* seems to be closely adapted to dairy cows and can be isolated from the udder skin in heifers as well as from teat canals and mammary secretions in newly calved heifers and lactating cows (Trinidad *et al.*, 1990a; Matthews *et al.*, 1992; Nickerson *et al.*, 1995; Aarestrup and Jensen, 1997; Taponen *et al.*, 2008). *S. chromogenes* has also been isolated from other body sites like nares, hair coat and vagina (White *et al.*, 1989). *S. epidermidis* is well adapted to the human host and may prevail in large numbers in the skin microflora (Kloos, 1980), while it is absent or rare in the bovine microflora (Devriese and de Keyser, 1980; White *et al.,* 1989). *S. haemolyticus* has been isolated from udder skin of cows (Devriese and de Keyser, 1980; Baba *et al.* 1980). *S. hyicus* has most commonly been found in clinical mastitis (Honkanen-Buzalski *et al.*, 1994; Waage *et al.*, 1999). In this species two ecovars have been defined, one from pigs and one from cows (Chesneau *et al.*, 2000). *S. simulans* has been found frequently both in clinical and subclinical mastitis (Jarp, 1991; Birgersson *et al.*, 1992; Waage *et al.,* 1999), but has been found in low frequency in extra-mammary sites (Devriese and de Keyser, 1980, Taponen *et al.*, 2008). *S. xylosus* is ubiquitous (Kloos, 1980), and can be found in various ecological niches. It can be present in raw meat and milk, and is often used as a starter culture for their fermentation (Dordet-Frisoni *et al.*, 2007). *S. xylosus* and *S. sciuri* are often found to be a part of the skin flora of cattle as well as of other mammals and of birds (Devriese and de Keyser, 1980; Kloos, 1980; White *et al.* 1989; Nagase *et al.*, 2002; Taponen *et al*, 2008). These species have,

however, also been isolated from bedding material in cow stables (Matos *et al.*, 1991).

Among CNS species found less frequently in samples from mastitis is *S. sciuri*, a species that has gained recent attention as it carries a *mecA* homologue from which the *mecA* gene of methicillin-resistant staphylococci may have developed (Couto *et al.*, 1996; Kloos *et al.*, 1997; Fuda *et al.*, 2007). Antimicrobial resistance like oxacillin resistance is common in human infection caused by *S. epidermidis* and *S. haemolyticus* (Nunes *et al,* 2005; Miragaia et al 2007; Schuenck *et al.*, 2008) and it might be that exchange of resistance genes between non-pathogenic, pathogenic strains and also different staphylococcal species might occur more frequently than earlier anticipated.

It can be concluded that the knowledge on the epidemiology of different CNS species IMI in dairy cows is very limited. Some studies report that first parity cows have a higher prevalence of *S. chromogenes* IMI than older cows (Trinidad *et al.*, 1990a; Matthews *et al.*, 1992; Rajala-Shultz *et al.*, 2004; Taponen *et al.*, 2006). For other CNS species there is no information on parity distribution. As mentioned earlier, conflicting results have been reported regarding stage of lactation and CNS species IMI, and the same goes for the relationship between milk production and CNS IMI. As milk production is crucial for the dairy industry it would be important to explore if different CNS species have different impact on milk production by studying commercial herds. The same is also valid for the relationship between CNS species and milk SCC.

Over-estimation of some CNS species when diagnosing IMI could be one reason for varying results between studies. For example, *S. xylosus* IMI has been reported rather frequently, but has also been regarded as environmental and non-pathogenic bacteria. Knowledge concerning the origin of bacteria, and significance of different strains in CNS IMI is also very limited. Taponen *et al.* (2008) found the same strain of *S. chromogenes* in milk isolates from IMI and in skin samples from cows, but other transmission routes have to our knowledge not been studied in detail.

2 Aims

The overall aim of the thesis was to improve the knowledge on prevalence and significance of different CNS species in connection with sub-clinical mastitis in dairy cows.

The specific aims were to:

- Investigate the epidemiology of different CNS species in dairy herds with problems due to sub-clinical CNS mastitis with emphasis on persistence of infection, and associations with milk production, SCC, parity and month of lactation.
- To investigate the possible transmission of *S. epidermidis* from milkers to the cows.
- To investigate the discriminatory capacity of some sub-typing methods and the clonal diversity within unrelated bovine *S. epidermidis* strains.

3 Material and Methods

Detailed information on material and methods are given in Papers I-II. Here, a general overview and some comments are given.

3.1 Study Populations (Papers I and II)

Field veterinarians working with udder-health on herd basis were asked to report herds with prevalent problems associated with CNS-induced clinical or sub-clinical mastitis, where no other obvious disease or management problems prevailed. Thus, all commercial herds in Sweden could take part in the study if they could present monthly records on milk yield, cow somatic cell count (CSCC), parity, and time of calving, and if the herd size exceeded 20 cows. The final eleven study herds included in Paper I were scattered throughout the southern and middle parts of Sweden.

In Paper II, 341 isolates of *S. epidermidis* were included. Of those, 105 milk isolates and 17 human isolates emanated from two herds also included in Paper I. In addition, 212 milk isolates and 7 human isolates epidemiologically unrelated to the other material were also included. This addition was done as a larger number of isolates was needed to evaluate the methods and results in Paper II, and for survey of oxacillin resistant isolates.

3.2 Samplings (Papers I and II)

From all cows included in Paper I, milk samples for bacteriological examination and California Mastitis Test (CMT) were taken twice, one month apart, in connection with the monthly milk recordings. During the study the importance of taking milk samples strictly after milking became clear. If samples were taken before milking, a large number contained mixed flora, and bacterial findings and the inflammatory reaction were poorly

correlated. Skin samples were also taken from the milkers, and the isolates found were included in Paper II. Milk and skin samples were transported chilled to the laboratory. Milk and skin samples were cultured on blood agar, and milk samples were CMT-scored within 24 hours after sampling. Cow data on daily milk production, CSCC, parity and month of lactation were collected from the monthly milk recordings.

3.3 Biochemical Identification of CNS (Papers I and II)

Identification of CNS isolates was based on colony morphology, Gram-staining, catalase reaction and coagulase test.

Based on findings in an earlier study (Thorberg and Brändström, 2000) a conventional biochemical typing system was chosen for CNS species identification. Commercial identification kits, e.g. ID Staph, had been found to have a poor capacity to identify bovine CNS species by not meeting the standards desired in epidemiological studies. In Paper I, CNS isolates from the first sampling were identified by conventional identification schemes, while a simplified system for novobiocin-sensitive CNS (NOSEC) was used for CNS isolates from the second sampling.

In the conventional identification scheme, CNS isolates were tested for susceptibility to novobiocin (5 μg) and furazolidone (100 μg). Novobiocin-sensitive isolates were tested for aerobic acid production from trehalose, mannitol, maltose, sucrose, mannose, ribose and lactose. All sugars were added to phenyl red broth, except mannose where a bromcresol purple agar plate was used. Tests for DNase, calcium caseinate, urease, alkaline phosphatase, Tween-80, -galactosidase and -glucuronidase were also performed. Novobiocin-resistant isolates were identified using the carbohydrates cellobiose, xylose, arabinose, raffinose, mellibiose and sucrose in phenyl red broth. For mannose, a bromcresol purple agar plate was used. Production of oxidase, calcium caseinate, urease and alkaline phosphatase was also tested

The aim of the NOSEC method (Table 1) is to identify the most significant and prevalent CNS isolated in bovine mastitis. Only ß-toxin-negative, novobiocin-sensitive CNS strains showing a negative reaction in the oxidase test should be tested. Results of the novobiocin sensitivity test, using the disc diffusion method, were compared with the results from tests on Muller-Hinton agar plates containing novobiocin. Up to nine strains per agar plate could be tested with the latter method. Substrates selected for the NOSEC identification were DNase agar, calcium caseinate agar, phosphatase agar, and bromcresol agar containing 1% trehalose and 1% mannitol. ß-

Galactosidase activity was tested on Luria-Bertani agar plates, and an agar acetoin test was performed on tryptose-yeast extract-glucose agar. All tests were performed on agar plates where nine test strains on each agar plate were inoculated as a short streak or a spot. For identification of *S. hyicus* its ability to cause a unique haemolysis in the ß-toxin zone of *S. intermedius* was tested using unwashed bovine blood in agar plates. Moreover, a rapid additional test, the PYR test (L-pyrrolidonyl-beta-naphthylamide), was used to differentiate *S. haemolyticus* from *S. warneri*.

Table 1. *Simplified identification scheme for novobiocin-sensitive CNS (NOSEC) isolated from bovine mastitis (Thorberg and Brändström, 2000)*

Species	ß-CAMP, synergistic haemolysis[1]	CCA	Trehalose-Mannitol	VP	Alk P	X-gal
S. chromogenes	-	+	+	-	+	-
S. epidermidis	-/+	-/w	-	+	+	-
S. felis[2]	+	+-	+	-	+	-
S. haemolyticus	++	-	+	+	-	-
S. hyicus	+++	+	+/-	-	+	-
S. simulans	+/d	-/w	+	-	-	+
S. warneri	+/d	-	+	+	-	-

CCA, calcium calseinate; VP, Vogues-Proskauer; Alk P, alkaline phosphatase; X-gal, ß-galactosidase.

[1] +, clear synergistic haemolysis (narrow); ++, clear synergistic haemolysis three times the size of bacteria streak; +++, opaque synergistic haemolysis shaped like an arrow or an arch; d, different reactions; w, weak reaction.

[2] Isolates from cats.

3.4 Strain Typing (Paper II)

Isolates of *S. epidermidis* were genetically fingerprinted by PFGE and ribotyping. In these methods restriction enzyme is used for cleavage of bacterial DNA. The DNA fragments are separated by electrophoresis in gel and the restricted DNA fragments are visualized as banding patterns. The banding patterns generated can be used to compare different isolates. For the study, the most important method was PFGE using the restriction enzyme *Sma*I, as this method has become a routine method in epidemiological studies on *S. aureus* and later also on *S. epidermidis*. The advantage with this method is that results can be compared between laboratories, provided that a standardized protocol is used as in line with Murchat *et al.* (2003)

3.5 Determination of Antimicrobial Resistance (Paper II)

A commercial microdilution system, SVA VetMIC®, providing minimum inhibitory concentration (MIC) values for a set of 17 antimicrobials was used. The most common substance responsible for penicillin resistance in staphylococci, -lactamase, can have a delayed production and not be detected in this system. Therefore, -lactamase production was evaluated using the clover-leaf method. Detection of methicillin/oxacillin resistance is important in staphylococci since this resistance makes the bacterial strain resistant against all -lactam antimicrobials. Methicillin resistance is also associated with multi-resistance against antimicrobials. CNS strains can express hetero-resistance against methicillin, which means that only a fraction of cells growing from one original bacterial cell will express resistance and the resistance can thereby be undetected in ordinary test panels. The oxacillin-salt agar screening test was performed on all *S. epidermidis* isolates as this species had a higher degree of resistance against -lactamase than other CNS species frequently found in mastitis. All oxacillin resistant isolates were examined for presence of the *mecA* gene by PCR.

4 Results and Discussion

4.1 Occurrence of CNS Species in Dairy Herds with Prevalent CNS IMI (Paper I)

The most commonly isolated CNS species in the study herds, regardless of inflammatory reaction in the udder quarter, were *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus*. These species consisted of 87% and 92% of the CNS isolates at the first and second sampling, respectively. Infrequent isolations of six other CNS species were also made. In most herds, one or two of the five CNS species mentioned above dominated. The findings are in line with a Danish study of early lactation heifers in multiple farms (Aarestrup and Jensen, 1997) using similar bacteriological diagnostics. In a Canadian study in herds with high prevalence of CNS, *S. hominis, S. sciuri* and *S. xylosus* were the most prevalent species followed by *S. epidermidis* and *S. warneri* (Davidson *et al.*, 1992). In that study, however, bacteriological diagnostics were performed using a commercial kit.

4.2 Differences in Persistence Between CNS Species (Paper I)

Persistence of CNS IMI, i.e. finding the same CNS species in the same udder quarter at two samplings one month apart, was common (58-76% of the infections) for *S. chromogenes, S. epidermidis*, and *S. simulans*, but infrequent (11-22%) for *S. xylosus* and *S. haemolyticus*. Most (95%) quarters persistently infected with these CNS species had an inflammatory reaction in the udder quarter as measured by the CMT reaction.

S. chromogenes has been found to cause persistent infections also in previous studies (Todhunter *et al.*, 1993; Taponen *et al.*, 2007). Aarestrup

and Jensen (1997) found, however, that the prevalence of *S. chromogenes* declined rapidly in heifers after calving, but the study material used was different from that of the present study as herds representative for the whole dairy cow population was used. Few studies have been published on persistence of *S. simulans* IMI, but in line with our findings a high proportion of persistent *S. simulans* IMI was reported by Aarestrup *et al.* (1999) and Taponen *et al.* (2006). To our knowledge, conclusive information on persistence of *S. epidermidis* IMI has not been reported. In our study, almost 60% of such infections were persistent, but the prevalence varied markedly between herds (data not shown). In some herds most *S. epidermidis* IMI were transient, but in one herd, where *S. epidermidis* was the most dominating udder pathogen, 100% of these IMI were persistent. This indicates a large variation in virulence among *S. epidermidis* strains, and that this CNS species may cause long-lasting udder health problems in some herds.

The reasons why some CNS species are more prone than other species to cause persistent IMI are not known, but may indicate differences in virulence and/or adaptation to the environment of the udder. For example, *S. chromogenes* seems well adapted to the udder as it has been isolated from the teat canal as well as the skin of the udder (Boddie *et al.*, 1987; Harmon and Langlois, 1989; Trinidad *et al.*, 1990a; Matthews *et al.*, 1992; Taponen *et al.*, 2008). In contrast, *S. simulans* is not frequently found in the teat canal or on the udder skin in cattle (Taponen *et al.*, 2008). The origin of this IMI is uncertain. Moreover, in contrast to several other CNS species involved in bovine mastitis, *S. epidermidis* is not commonly found in the normal bacterial flora of bovine skin or mucous membranes (Devriese and de Keyser, 1980; White *et al.*, 1989, Taponen *et al.*, 2008). *S. epidermidis* is, however, one of the most prevalent staphylococcal species found on human skin, and Kloos (1980) regarded this species as exclusively human. It has therefore been suggested that udder infections caused by *S. epidermidis* may be of human origin (Watts and Owens, 1989). A human origin of bovine *S. epidermidis* IMI was supported by findings in Paper II. In that study the same genotypic patterns were found in *S. epidermidis* isolates from bovine milk and the milkers' skin in two herds with a high prevalence of *S. epidermidis* IMI.

S. haemolyticus and *S. xylosus* were found rather frequently in the study herds, but less than 20% of these IMI were persistent despite the fact that these species often have been isolated from the skin of cattle (Devriese and de Keyser, 1980; Baba *et al.*, 1980; Kloos, 1980). *S. xylosus* has, however, also been isolated from the environment of the cow such as in the bedding (Matos *et al.*, 1991), and considerable diversity has been found among *S.*

xylosus strains (Dordet-Frisoni *et al.*, 2007). *S. xylosus* has been considered to be non-pathogenic, but has frequently been reported to be involved in mastitis (Harmon and Langlois, 1989; Jarp, 1991; Birgersson *et al.*, 1992; Todhunter *et al.*, 1993; Aarestrup *et al.*, 1995). Findings of pure growth of *S. xylosus* in milk samples in connection with sub-clinical mastitis may be an indication of teat canal colonisation rather than of IMI as observed in this study and other unpublished observations done in connection to this study.

4.3 CNS Species and Cow Characteristics (Paper I)

For analysis of associations between CNS species, and milk production, SCC, parity and month of lactation 380 of the cows in Paper I were classified as sub-clinical cases infected with *S. chromogenes, S. epidermidis* or *S. simulans,* or as healthy cows. The cows in the three CNS classes were further classified as having persistent or non-persistent IMI.

The cow SCC did not differ significantly between cows with non-persistent and persistent IMI, which is in line with Taponen *et al.* (2007) who found that the SCC varied considerably both in quarters with persistent and non-persistent CNS IMI. Moreover, the cow SCC did not differ between cows with sub-clinical *S. chromogenes, S. epidermidis* or *S. simulans* IMI*,* but these groups had higher SCC than healthy cows. An inflammatory reaction, as measured by CMT or SCC, to *S. chromogenes*, *S. epidermidis* and *S. simulans* IMI has been reported also in previous studies (Birgersson *et al.*, 1992; Todhunter *et al.*, 1993; Taponen, 2008).

Among cows with sub-clinical mastitis due to *S. chromogenes, S. epidermidis* or *S. simulans,* cows with non-persistent IMI had significantly lower daily milk production than cows with persistent IMI and healthy cows. Significant differences in production between CNS species were, however, not observed within cows with persistent or non-persistent IMI. The lowest milk production was found in cows with non-persistent *S. simulans* IMI. The reasons behind the observed differences are not clear. One possible explanation is that high-yielding cows are more prone to persistent IMI, which is supported by the findings by Gröhn *et al.* (2004) that cows with clinical CNS mastitis were higher producers than healthy control cows before the onset of mastitis. Another explanation could be that cows categorized as non-persistently infected had been infected for some time before the first sampling resulting in a reduction of milk production. Thus, the negative bacteriological sample at the second sampling could indicate that the infection had been cleared from the udder. If this was the case, the lower production could have been a result of a more intense

inflammatory reaction in the udder, which was not detected by the sampling strategy used herein.

The distribution between parities differed between CNS species. *S. epidermidis* IMI was more common in older cows than in first parity cows, while *S. chromogenes* IMI was most common in first parity cows. *S. simulans* IMI was equally distributed between first parity and older cows. Matthews *et al.* (1992) also found that the prevalence of *S. chromogenes* was approximately twice as high in first parity cows compared with older cows post partum, and similar results, although using different cow materials and sampling strategies, were also found in other studies (Harmon and Langlois, 1989; Todhunter *et al.*, 1993; Aarestrup and Jensen, 1997; Taponen *et al.*, 2006). Studies on *S. epidermidis* and parity are, however, not available. It may be hypothesized that at least some *S. epidermidis* strains are more contagious than others, i.e. the longer the time in the herd the higher the risk for IMI, which would explain the higher prevalence in older cows. Differences in the immune system have also been found between first lactation and older cows (Mehrzad *et al.*, 2002), which could be of importance for their susceptibility and response to IMI. Preliminary results from one of the herds indicate that the inflammatory response to *S. epidermidis* IMI may differ from that of other CNS infections as udder tissue infiltration of eosinophilic granulocytes rather than neutrophilic granulocytes was observed in connection with *S. epidermidis* IMI (unpublished results). Trinidad *et al.* (1990b) also observed tissue infiltration of eosinophils in connection with some CNS IMI. In that study, CNS were not identified to the species level.

4.4 Sub-Typing of *S. epidermidis* (Paper II)

PFGE typing of milk isolates of *S. epidermidis* collected from a routine diagnostic laboratory showed a large diversity of patterns, as 59 different PFGE patterns were found among 71 isolates. Seven isolates from human patients were also investigated using PFGE. When comparing these strains with the milk strains they were more closely related to the milk strains than to each other. No similar studies on PFGE analysis of bovine *S. epidermidis* isolates have been found. All studies so far are mainly on human strains isolated within hospital units. The high level of diversity obtained by PFGE when analysing the unrelated *S. epidermidis* isolates used in this study is in line with Miragaia *et al.* (2008) who found a Simpsons diversity index of 96.4% when analysing 419 human *S. epidermidis* isolates originating in different countries. Despite the high diversity found within this population, PFGE was considered useful when tracking spread of specific *S. epidermidis*

stains between patients, wards and hospitals (Miragaia *et al.*, 2008). The high diversity found among unrelated milk isolates in the present study indicates that the method used was a good tool for selective epidemiological typing, and that the risk to identify non-related strains as identical was small.

From the two study herds 8 different PFGE patterns were found among milk isolates. One or two identical patterns were also found in human samples taken at the herd. In the second herd two patterns were found in human samples, but not in milk samples. Ribotyping yielded a total of four patterns in the two herds. The patterns differed only by one or two bands between isolates. One single ribotype was found in herd 1 and this ribotype was identical to the most dominant ribotype in herd 2. These findings indicate a lower discriminatory capacity of ribotyping than PFGE, and ribotyping may have limited use in molecular epidemiological tracing. Three ribotypes were found in milk isolates from the second herd, which was in close agreement with the PFGE typing and typing by antibiograms. Thus, in this herd all three methods had equal capacity. Several antibiogram types with close agreement with PFGE types were found in the milk isolates in the herds.

Ribotyping gave patterns with small differences in this study. This method has not been widely used in molecular epidemiology of staphylococci, but has been described as a useful method in species identification and definition of different ecovars within the different staphylococcal species (Chesneau *et al.*, 2000; Aarestrup, 2001; Carretto *et al.*, 2005). High discriminatory power did not seem to be in conflict with typeability properties for PFGE as epidemiologically related strains could be identified in the herds. Based on these results it was concluded that PFGE was the most valuable method for the study.

A large proportion (75%) of the *S. epidermidis* antibiograms from the isolates collected at the routine laboratory showed either sensitive results for all antimicrobials tested or only penicillin resistance measured as ß-lactamase production. As many different PFGE patterns were found among these isolates there was a low agreement between PFGE and antibiograms. One exception was in the case of one cluster of 5 oxacillin resistant strains with identical PFGE patterns. Four of those strains were milk isolates and one isolate originated in a case of human infection. All those isolates had the same antibiogram, and were resistant to penicillin, oxacillin, erythromycin, fucidic acid and oxytetracycline. Thirteen isolates were detected in the oxacillin-salt agar screening test and all these isolates carried the *mecA* gene, which is the highest prevalence reported among farm animal staphylococcal isolates in Sweden (SVARM, 2007).

4.5 Transmission of *S. epidermidis* from Milkers to Cows in Two Herds (Paper II)

In the two study herds having high prevalences of *S. epidermidis* IMI 8 different PFGE patterns were found among the milk isolates. The patterns differed between the herds, but one PFGE pattern dominated in each herd. The dominating PFGE patterns were found in consecutive samplings and in cows with an inflammatory reaction in the udder. Thus, there seemed to be a selection pressure for strains more adapted to cause IMI. In both herds the same one or two PFGE patterns were also found in *S. epidermidis* isolates from the skin flora of the milkers. In one herd, the dominating *S. epidermidis* strain in milk was the same as was found on the milker's hands and the bends of the elbows. In the other herd, the two dominating sub-types were also found on the milker's hands and bends of the elbows. The *S. epidermidis* type found on the milker's spouse, who was not in contact with the udders or teats, were not recovered in the milk samples. Almost identical ribotyping patterns in the two herds and milkers also suggest that this is a human ecovar of *S. epidermidis*. These findings support the hypothesis of Watts and Owens (1989) that the *S. epidermidis* strains that cause mastitis emanate from humans. Studies performed on the microflora of cattle indicate that CNS species other than CNS commonly isolated from mastitis can also be found in the microflora of the mucous membranes or the skin in cattle (Devriese and de Keyser, 1980; White *et al.*, 1989). However, *S. epidermidis* is absent or very rare in the bovine microflora of the mucous membranes or skin (Devriese and de Keyser, 1980; White *et al.*, 1989).

If *S. epidermidis* is to become established in herds in a more permanent way, the bacteria must either find susceptible host tissue or be continuously supplied. Significant contributions to IMI might have come from the farmers who transferred these bacteria during daily contact with the udders. Impaired local resistance of the teats to environmental infections due to poor milking technique or milking equipment may also favour the establishment of *S. epidermidis*. A follow up study in one of the herds showed faulty pulsation in the milking unit resulting in no teat massage during milking. Other disturbances in the herds affecting the immune system of the cows could also be of importance, but such effects would be difficult to analyse. The observed findings could also be an indication that adaptation of human strains to bovine udders has happened mainly by genetic alterations in *S. epidermidis* clones. By adapting methods for evaluation of evolutionary events, as done in a recent study (Miragaia *et al.*, 2008), it has been suggested that *S. epidermidis* has a population with an epidemic structure. One of the clones of *S. epidermidis,* mostly found in clinical isolates, showed

considerable speed in diversity of DNA information. The origin of the changes initiating diversification of *S. epidermidis* clones has mainly been found to be due to recombination of DNA and less to point mutation. In the model, accession of the staphylococcal chromosomal cassette carrying the *mecA* gene was estimated to have taken place 56 times. This exemplifies the capacity of the species *S. epidermidis* to quickly induce genetic changes and adapt to new environments. The observed findings could also be an indication that adaptation of human strains to bovine udders has occurred mainly by genetic alterations in *S. epidermidis* clones. Some interesting information concerning epidemiology of *S. epidermidis* could perhaps be gained in the future by conducting studies in automatic milking systems where limited contact occurs between human hands and udders.

5 Conclusions

- Persistent CNS IMI was most common in quarters infected with *S. chromogenes*, *S. epidermidis* and *S. simulans* indicating that those CNS species are of more relevance for bovine sub-clinical mastitis than other species.
- *S. epidermidis* IMI were mainly found in multiparous cows and *S. chromogenes* IMI in primiparous cows.
- The results did not indicate that cows with *S. chromogenes*, *S. epidermidis* and *S. simulans* IMI differed in milk production, CSCC, and month of lactation.
- Cows with non-persistent *S. chromogenes*, *S. epidermidis* and *S. simulans* IMI had lower milk production than cows with persistent IMI and healthy cows.
- The PFGE method had high discriminatory power and showed that many different *S. epidermidis* types exist in milk samples.
- The same *S. epidermidis* PFGE patterns were found in isolates from bovine milk and from the skin of the milkers indicating that bovine *S. epidermidis* mastitis may be a zoonosis emanating from man.

6 References

Aarestrup, F.M. (1995). *Characterization of Staphylococcus aureus from bovine mastitis*. Diss. The Royal Veterinary and Agricultural University Copenhagen, Denmark.

Aarestrup, F.M., Wegener, H.C., Rosdahl, V.T. & Jensen, N.E. (1995). Staphylococcal and other bacterial species associated with intramammary infections in Danish dairy herds. *Acta Veterinaria Scandinavica* 36, 475-487.

Aarestrup, F.M. & Jensen, N.E. (1997). Prevalence and duration of intramammary infection in Danish heifers during the peripartum period. *Journal of Dairy Science* 80, 307-312.

Aarestrup, F.M., Larsen, H.D. & Jensen, N.E. (1999). Characterization of Staphylococcus simulans strains isolated from cases of bovine mastitis. *Veterinary Microbioliology* 66, 165-170.

Aarestrup, F.M. (2001). Comparative ribotyping of *Staphylococcus intermedius* isolated from members of the Canoidea gives possible evedence for host-specifity and co-evalustion of bacteria hosts. *Inernal Journsl of Systematic and evolutionary microbiology* 51, 1343-1347.

Andollina, A., De Cesare, A., Bertoni, G., Modelli, L. & Manfreda, G. (2004). Identification and genetic characterisation of orthopaedic Staphylococcus isolates collected in Italy by automated EcoRI ribotyping. *FEMS Microbiolgy Letters* 234, 275-280.

Baba, E., Fukata, T. & Matsumoto, H. (1980). Ecological studies on coagulase-negative staphylococci in and around bovine udder. *Bulletin of University of Osaka Prefecture, B* 32, 69-75.

Baird-Parker, A.C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *Journal of General Microbiology* 30(3), 409-427.

Barkema, H.W., Schukken, Y.H. & Zadoks, R.N. (2006). Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. *Journal of Dairy Science* 89, 1877-1895.

Birgersson, A., Jonsson, P. & Holmberg, O. (1992). Species identification and some characteristics of coagulase-negative staphylococci isolated from bovine udders. *Veterinary Microbiology* 31, 181-189.

Boddie, R.L., Nickerson, S.C., Owens, W.E. & Watts, J.L. (1987). Udder microflora in nonlactating heifers. *Agri-Practice* 8, 22-25

Carretto, E., Barbarini, D., Couto, I., De Vitis, D., Marone, P., Verhoef, J., De Lencastre, H. & Brisse, S. (2005). Identification of coagulase-negative staphylococci other than Staphylococcus epidermidis by automated ribotyping. *Clinical Microbiology and Infection* 11, 177-184.

Chesneau, O., Morvan, A., Aubert, S. & el Solh, N. (2000). The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus Staphylococcus. *Internal Journal of Systematics and Evoltionary Microbiology* 50, 689-697.

Christensen, G.D. (1987). The confusing and tenacious coagulase-negative staphylococci. *Advances in Internal Medicine* 32, 177-192.

Compton, C.W., Heuer, C., Parker, K. & McDougall, S. (2007). Epidemiology of mastitis in pasture-grazed peripartum dairy heifers and its effects on productivity. *Journal of Dairy Science* 90, 4157-4170.

Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J.A., Hubner, R.J., Sanches, I.S. & Tomasz, A. (1996). Ubiquitous presence of a mecA homologue in natural isolates of Staphylococcus sciuri. *Microbial Drug Resistance*, 377-391.

Couto, I., Pereira, S., Miragaia, M., Sanches, I.S., de Lencastre, H. (2001). Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. *Journal of Clinical Microbiology*, 39, 3099-3103.

Daranyi von, J. (1925). *Arch. Hyg.* 96, 182-184.

Davidson, T.J., Dohoo, I.R., Donald, A.W., Hariharan, H. & Collins, K. (1992). A cohort study of coagulase negative staphylococcal mastitis in selected dairy herds in Prince Edward Island. *Canadian Journal of Veterinary Research* 56, 275-280.

de Saxe, M.J., Crees-Morris, J.A., Marples, R.R. & Richardson, J.F. (Eds.) (1981). *Evaluation of current phage-typing systems for coagulase-negative staphylococci.* In (Staphylococci and staphylococcal infections). Stuttgart.: Gustav Fischer Verlag.

Devriese, L.A. (1979). Identification of clumping-factor-negative staphylococci isolated from cows udders. *Research in Veterinary Science* 27, 313-320.

Devriese, L.A. & De Keyser, H. (1980). Prevalence of different species of coagulase-negative staphylococci on teats and in milk samples from dairy cows. *Journal of Dairy Research* 47, 155-158.

Devriese, L.A., Hajek, V., Oeding, P., Meyer, S.A. & Schleifer, K.H. (1978). Staphylococcus-hyicus (Sompolinsky 1953) comb nov and Staphylococcus-hyicus subsp chromogenes subsp nov. *International Journal of Systematic Bacteriology* 28, 482-490.

Devriese, L.A., Poutrel, B., Kilpperbalz, R. & Schleifer, K.H. (1983). *Staphylococcus-gallinarum* and *Staphylococcus-caprae*, 2 new species from animals. *International Journal of Systematic Bacteriology* 33, 480-486.

Devriese, L.A., Schleifer, K.H. & Adegoke, G.O. (1985). Identification of coagulase-negative staphylococci from farm animals. *Journal of Applied Bacteriology* 58, 45-55.

Devriese, L.A., 1986. Coagulase-negative staphylococci in animals. In: P.-A. Mårdh and K.H. Schleifer (Editors), *Coagulase-negative Staphylococci.* Almqvist & Wiksell International, Stockholm, Sweden, pp. 51-57.

Djabri, B., Bareille, N., Beaudeau, F. & Seegers, H. (2002). Quarter milk somatic cell count in infected dairy cows: a meta-analysis. *Veterinary Research* 33, 335-357.

Dordet-Frisoni, E., Dorchies, G., De Araujo, C., Talon, R. & Leroy, S. (2007). Genomic diversity in Staphylococcus xylosus. *Applied Environmental Microbiology* 73, 7199-7209.

Dordet-Frisoni, E., Talon, R. & Leroy, S. (2007b). Physical and genetic map of the Staphylococcus xylosus C2a chromosome. *FEMS Microbiology Letters* 266, 184-193.

Eberhart, R.J., Hutchinson, L.J., & Spencer, S.,B. (1982). Relationships of bulk tank somatic cell counts to prevalence of intramammary infections and to indices of herd production. *Journal of Food Protection* 45, 1125-1128.

Ekman, T., Franklin, A, Hallen-Sandgren, C. & Jonsson, P, (1995). Ny policy vid antibiotikabehandling av mastit hos ko. *Svensk Veteriniärtidning* 47, 59-61.

Emanuelson, U., Olsson, T., Mattila, T., Astrom, G. & Holmberg, O. (1988). Effects of parity and stage of lactation on adenosine triphosphate, somatic cell count and antitrypsin content in cows' milk. *Journal of Dairy Research* 55, 49-55.

Euzeby, J., P. *List of procaryotic namnes with standing in nomenclature* [online]. Available from: http://www.bacterio.net [Accessed 13 Augusti 2008].

Fox, L.K., Chester, S.T., Hallberg, J.W., Nickerson, S.C., Pankey, J.W. & Weaver, L.D. (1995). Survey of intramammary infections in dairy heifers at breeding age and first parturition. *Journal of Dairy Science* 78, 1619-1628.

Fuda, C., Suvorov, M., Shi, Q., Hesek, D., Lee, M. & Mobashery, S. (2007). Shared functional attributes between the mecA gene product of *Staphylococcus sciuri* and penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *Biochemistry* 46, 8050-8057.

Grohn, Y.T., Wilson, D.J., Gonzalez, R.N., Hertl, J.A., Schulte, H., Bennett, G. & Schukken, Y.H. (2004). Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *Journal of Dairy Science* 87, 3358-3374.

Hajek, V., Devriese, L.A., Mordarski, M., Goodfellow, M., Pulverer, G. & Varaldo, P.E. (1986). Elevation of *Staphylococcus-hyicus* subsp *chromogenes* (Devriese et-al, 1978) to species status - *Staphylococcus-chromogenes* (Devriese et-al, 1978) comb-nov. *Systematic and Applied Microbiology* 8, 169-173.

Haltia, L., Honkanen-Buzalski, T., Spiridonova, I., Olkonen, A. & Myllys, V. (2006). A study of bovine mastitis, milking procedures and management practices on 25 Estonian dairy herds. *Acta Veterinaria Scandinavica* 48, 22-27.

Harmon, R.J. & Langlois, B.E. (1989). Mastitis due to coagulase-negative *Staphylococcus* species. *Agri-Practice* 10, 29-34.

Hebert, G.A., Cooksey, R.C., Clark, N.C., Hill, B.C., Jarvis, W.R. & Thornsberry, C. (1988). Biotypingcoagulase-negative staphylococci. *Journal of Clinical Microbiology* 26, 1950-1956.

Holmberg, O. (1973). Staphylococcus epidermidis isolated from bovine milk. Biochemical properties, phage sensitivity and pathogenicity for the udder. In: *Acta Veterinaria Scandinavica Supplementum*. p. 1-144.

Honkanen-Buzalski, T., Myllys, V. & Pyorala, S. (1994). Bovine clinical mastitis due to coagulase-negative staphylococci and their susceptibility to antimicrobials. *Zentralblatt fur Veterinarmedizin B* 41, 344-350.

Jarp, J. (1991). Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinidal mastitis. *Veterinary Microbiology* 27, 151-158.

Kerro Dego, O., van Dijk, J.E. & Nederbragt, H. (2002). Factors involved in the early pathogenesis of bovine Staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. *Veterinary Quarterly,* 24, 181-198.

Kloos, W.E. (1980). Natural populations of the genus *Staphylococcus*. *Annual Review of Microbiology* 34, 559-592.

Kloos, W.E., Ballard, D.N., Webster, J.A., Hubner, R.J., Tomasz, A., Couto, I., Sloan, G.L., Dehart, H.P., Fiedler, F., Schubert, K., de Lencastre, H., Sanches, I.S., Heath, H.E., Leblanc, P.A. & Ljungh, A. (1997). Ribotype delineation and description of *Staphylococcus sciuri* subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. *Internal Journal of Systematics and Bacteriology* 47, 313-323.

Kloos, W.E. & Schleifer, K.H. (1975a). Isolation and characterization of staphylococci from human skin .2. Descriptions of 4 new species - *Staphylococcus-warneri*, *Staphylococcus-capitis*, *Staphylococcus-hominis*, and *Staphylococcus-simulans*. *International Journal of Systematic Bacteriology* 25, 62-79.

Kloos, W.E. & Schleifer, K.H. (1975b). Simplified scheme for routine identification of human *Staphylococcus* species. *Journal of Clinical Microbiology* 1, 82-88.

Kloos, W.E. & Schleifer, K.H. (1983). *Staphylococcus-auricularis* sp-nov - an inhabitant of the human external ear. *International Journal of Systematic Bacteriology* 33, 9-14.

Kloos, W.E., Schleifer, K.H. & Smith, R.F. (1976). Characterization of *Staphylococcus-sciuri* Sp-Nov and its subspecies. *International Journal of Systematic Bacteriology* 26, 22-37.

Kloos, W.E. & Bannerman, T.L. (1994). Update on clinical significance of coagulase-negative staphylococci. *Clinical Microbiology Reviews* 7(1), 117-140.

Latham, R.H., Running, K. & Stamm, W.E. (1983). Urinary tract infections in young adult women caused by *Staphylococcus saprophyticus*. *JAMA* 250, 3063-3066.

Matos, J.S., White, D.G., Harmon, R.J. & Langlois, B.E. (1991). Isolation of *Staphylococcus aureus* from sites other than the lactating mammary gland. *Journal of Dairy Science* 74, 1544-1549.

Matthews, K.R., Harmon, R.J. & Langlois, B.E. (1992). Prevalence of Staphylococcus species during the periparturient period in primiparous and multiparous cows. *Journal of Dairy Science* 75, 1835-1839.

Mehrzad, J., Duchateau, L., Pyorala, S. & Burvenich, C. (2002). Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *Journal of Dairy Science* 85, 3268-3276.

Miragaia, M., Carrico, J.A., Thomas, J.C., Couto, I., Enright, M.C. & de Lencastre, H. (2008). Comparison of molecular typing methods for characterization of Staphylococcus epidermidis: Proposal for clone definition. *Journal of Clinical Microbiology* 46, 118-129.

Miragaia, M., Thomas, I.C., Couto, I., Enright, M.C. & de Lencastre, H. (2007). Inferring a population structure for *Staphylococcus epidermidis* from multilocus sequence typing data. *Journal of Bacteriology* 189, 2540-2552.

Mulvey, M.R., Chui, L., Ismail, J., Louie, L., Murphy, C., Chang, N. & Alfa, M. (2001). Development of a Canadian standardized protocol for subtyping methicillin-resistant *Staphylococcus aureus* using pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 39, 3481-3485.

Murchan, S., Kaufmann, M.E., Deplano, A., de Ryck, R., Struelens, M., Zinn, C.E., Fussing, V., Salmenlinna, S., Vuopio-Varkila, J., El Solh, N., Cuny, C., Witte, W., Tassios, P.T., Legakis, N., van Leeuwen, W., van Belkum, A., Vindel, A., Laconcha, I., Garaizar, J., Haeggman, S., Olsson-Liljequist, B., Ransjo, U., Coombes, G. & Cookson, B. (2003). Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *Journal of Clinical Microbiology* 41, 1574-1585.

Nagase, N., Sasaki, A., Yamashita, K., Shimizu, A., Wakita, Y., Kitai, S. & Kawano, J. (2002). Isolation and species distribution of staphylococci from animal and human skin. *Journal of Veterinary Medicine* Series A 64, 245-250.

Nickerson, S.C., Owens, W.E. & Boddie, R.L. (1995). Mastitis in dairy heifers: initial studies on prevalence and control. *Journal of Dairy Science* 78, 1607-1618.

Nunes, A.P., T.L., Bastos, C.C., Silva, M.G., Ferreira, R.B., Fonseca, L.S. & Santos, K.R. (2005). Genomic characterization of oxacillin-resistant *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* isolated from Brazilian medical centres. *Journal of Hospital Infections* 59, 19-26.

Oliver, S.P. & Sordillo, L.M. (1988). Udder health in the periparturient period. *Journal of Dairy Science* 71, 2584-2606.

Olsson, S.O., Baekbo, P., Hansson, S.O., Rautala, H. & Osteras, O. (2001). Disease recording systems and herd health schemes for production diseases. *Acta Veterinaria Scandinavica Supplementum* 94, 51-60.

Østerås, O., Sölveröd, L. & Reksen, O. (2006). Milk culture results in a large Norwegian survey-effects of season, parity, days in milk, resistance, and clustering. *Journal of Dairy Science* 89, 1010-1023.

Pfaller, M.A. & Herwaldt, L.A. (1988). Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. *Clinical Microbiology Reviews* 1, 281-299.

Pitkälä, A., Haveri, M., Pyorälä, S., Myllys, V. & Honkanen-Buzalski, T. (2004). Bovine mastitis in Finland 2001--prevalence, distribution of bacteria, and antimicrobial resistance. *J Dairy Sci* 87, 2433-2441.

Rajala-Schultz, P.J., Smith, K.L., Hogan, J.S. & Love, B.C. (2004). Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. *Veterinary Microbiology* 102, 33-42.

Roesch, M., Doherr M.D., Scharen, ,W., Schallibaum, M. & Blum, J.W. (2007). Subclinical mastitis in dairy cows in Swiss organic and conventional production systems. *Journal of Dairy Research* 74, 86-92.

Sandgren, C.H., Waller, K.P. & Emanuelson, U. (2008). Therapeutic effects of systemic or intramammary antimicrobial treatment of bovine subclinical mastitis during lactation. *Veterinary Journal* 175, 108-117.

Santos, O.C., Barros, E.M., Brito, M.A., Bastos Mdo, C., Dos Santos, K.R. & Giambiagi-Demarval, M. (2008). Identification of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the groEL gene. *Veterinary Microbiology* 130, 134-140.

SCB (Statistiska Centalbyrån) (2008). *Antal husdjur av olika slag 1980-2007 - Statistik från SCB* [online]. (Jordbruksstatistisk Årsbok (JÅ)). Available from: http://www.scb.se/templates/tableOrChart37589.asp

Schleifer, K.H. & Kloos, W.E. (1975). Isolation and Characterization of Staphylococci From Human Skin .1. Amended Descriptions of *Staphylococcus-epidermidis* and *Staphylococcus-saprophyticus* and Descriptions of 3 New Species - *Staphylococcus-cohnii, Staphylococcus-haemolyticus,* and *Staphylococcus-xylosus*. *International Journal of Systematic Bacteriology* 25, 50-61.

Scleifer, K.H., Kiopperbalz. R. Devriese, L.A.(1984) *Staphylococcus arlette* sp-nov, *Staphylococcus equorum* and *Staphylococcus kloosii* sp-nov - 3 new coagulase-negative, novobiocin-resistant species from animals. *Systematic and Applied Microbiology*, 5, 501-509.

Schuenck, R.P., Pereira, E.M., Iorio, N.L., Dos Santos, K.R. (2008). Multiplex PCR assay to identify methicillin-resistanst *Staphylococcus haemolyticus*. *FEMS Immunology and Medical Microbiology,* 52, 431-435.

Sears, P.M. & McCarthy, K.K. (2003). Management and treatment of staphylococcal mastitis. *Veterinary Clinics North American Food Animal Practitioners* 19, 171-185.

SVARM. (2007). Swedish veterinary antimicrobial resistance monitoring 2007. National Veterinary Institute (SVA) (Ed.) Wikströms tryckeri. Uppsala. Sweden.

Swedish Dairy Association. (2008). Annual report from the animal health section. *Animal health 2006/2007.*

Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D. & Pyorala, S. (2006). Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary Microbiology* 115, 199-207.

Taponen, S., Koort, J., Björkroth, J., Saloniemi, H. & Pyorälä, S. (2007). Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. *Journal of Dairy Science i* 90, 3301-3307.

Taponen, S. (2008). *Bovine mastitis caused by coagulase-negative staphylococci*. Diss. University of Helsinki.

Taponen, S., Björkroth, J. & Pyorälä, S. (2008). Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd. *Journal of Dairy Research,* 1-8.

Thorberg, B.M. & Brändström, B. (2000). Evaluation of two commercial systems and a new identification scheme based on solid substrates for identifying coagulase-negative staphylococci from bovine mastitis. *Jounal of Veterinary Medicin series B Infectious Diseases and Veterinary Public Health* 47, 683-91.

Timms, L.L. & Schultz, L.H. (1987). Dynamics and significance of coagulase-negative staphylococcal intramammary infections. *Journal of Dairy Science* 70, 2648-2657.

Todhunter, D.A., Cantwell, L.L., Smith, K.L., Hoblet, K.H. & Hogan, J.S. (1993). Characteristics of coagulase-negative Staphylococci isolated from bovine intramammary infections. *Veterinary Microbiology* 34, 373-380.

Trinidad, P., Nickerson, S.C. & Alley, T.K. (1990a). Prevalence of intramammary infection and teat canal colonization in unbred and primigravid dairy heifers. *Journal of Dairy Science* 73, 107-114.

Trinidad, P., Nickerson, S.C. & Adkinson, R.W. (1990b). Histopathology of staphylococcal mastitis in unbred dairy heifers. *Journal of Dairy Science* 73, 639-647.

Waage, S., Mörk, T., Röros, A., Aasland, D., Hunshamar, A. & Ødegaard, S.A. (1999). Bacteria associated with clinical mastitis in dairy heifers. *Journal of Dairy Science* 82, 712-719.

Wade, J.C., Schimpff, S.C., Newman, K.A. & Wiernik, P.H. (1982). Staphylococcus epidermidis: an increasing cause of infection in patients with granulocytopenia. *Annals of Internal Medicine* 97, 503-508.

Watts, J.L. & Owens, W.E. (1989). Prevalence of staphylococcal species in four dairy herds. *Research In Veterinary Science* 46, 1-4.

White, D.G., Harmon, R.J., Matos, J.E. & Langlois, B.E. (1989). Isolation and identification of coagulase-negative Staphylococcus species from bovine body sites and streak canals of nulliparous heifers. *Journal of Dairy Science* 72, 1886-1892.

Widerström, M., Monsen, T., Karlsson, C. & Wiström, J. (2006). Molecular epidemiology of meticillin-resistant coagulase-negative staphylococci in a Swedish county hospital: evidence of intra- and interhospital clonal spread. *Journal of Hospital Infections* 64, 177-183.

Wilson, D.J., Ruben, N., Gonzalez, Helena, H. Das (1997). Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. *Journal of Dairy Science,* 80, 2592-2598.

7 Acknowledgments

This project was carried out at the Department of Biomedical Sciences and Veterinary Public Health (BVF), Division of Food Hygiene and Bacteriology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The studies were supported by the Swedish Farmer's Foundation for Agricultural Research.

Over the years many people have contributed to this project and to my need for reliable friendship and I like to thank you all, I like to send special thanks to:

My first supervisor Per Jonsson for introducing me to the endless field of mastitis and the true concept "if you know for sure what you search for you don't need to do research".

Marie-Louise Danielsson-Tham for generously taking me under her wings as Head of the former Department of Food Hygiene and also for becoming my second supervisor.

Karin Persson Waller, my third supervisor, for coming in late in the project but indeed prioritising my project and helping me very much with all her skills. I am very grateful, finally I was ready.

Eva "Puck" Olsson Engvall, my co-supervisor in the end of the project, well known for her cheerful attitude and competence in bacteriology.

Boel Harbom (born Brändström) for excellent assistance in the laboratory and never fading engagement, covering up all my shortcomings. We have done so many things.

Lolita Nilsson former Head of the Mastitis laboratory at the National Veterinary Institute (SVA), for being a never ending friend in the field of mastitis and elsewhere, always supportive and generous.

Ulf Emanuelson, for exclusive combination of statistical skills and being really nice.

Christina Greko, my mother of bacteriology who have all answers, in secret we are Professor's material since we on average produce material above mean quality, not so frequently then.

Susanna Sternberg-Lewerin for standing up for what is right and for making me feel sound an in sane when I didn't, and I wont forget Boel Fransson and Ragnvi Hagman for joyful friendship at the old Section of Clinical Microbiology (KLM).

All service and help provided by nice persons at BVF, Mastitis Laboratory and Department of Antibiotics.

Lars-Erik Edqvist, not knowing his contribution, but we were offered coffee *and* buns as students in his laboratory, after been out a year on the field with no coffee brakes in sight. It made me reconsider laboratory work, which I never would have done otherwise.

My long lasting friends through the years Catrin and Per Carlson, Andrea Ingvarsson and Johan Alvner.

All my friends with four legs, small and large that mean so much to me.

My whole family Thorberg, special thanks to my brother Göran Thorberg for help with computer assistance and my father and mother Vera and Gösta Thorberg who assisted in practical ways.

My daughter Petra for introducing me to ever lasting love and for making me aware of the importance to be a human *being*.