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Bovine mastitis caused by coagulase-negative staphylococci

by

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ACADEMIC DISSERTATION

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ABSTRACT

Coagulase-negative staphylococci (CNS) are a frequent cause of bovine intramammary infections (mastitis) in modern dairy herds. They have become the most common bacteria isolated from milk samples in many countries. Mastitis caused by CNS in most cases remains subclinical, or the clinical signs are mild. For some reason, heifers and primiparous cows are most susceptible to CNS mastitis. CNS mastitis increases milk somatic cell count (SCC) in the infected udder quarter. The increase in milk SCC is usually moderate compared with mastitis caused by many other common pathogens, including *Staphylococcus (S.) aureus* and streptococci. However, high prevalence of CNS mastitis in a herd can affect the herd bulk milk SCC.

CNS comprises almost 40 different species of staphylococci. In mastitis diagnostics they are differentiated from the coagulase-positive mastitis pathogen *S. aureus* using a test to gauge the ability of the bacteria to coagulate plasma. CNS are not identified further by species but are treated as a uniform group. Many different CNS species have been isolated from bovine milk. Many CNS species can also be isolated from cows' hair coat, udder skin and teat canals, and are therefore often considered to be opportunistic skin organisms rather than real mastitis pathogens. CNS mastitis is generally expected to be eliminated spontaneously and is commonly left without any antimicrobial treatment.

This thesis focused on identification of the most relevant CNS species causing bovine mastitis, and the possible differences in clinical characteristics between mastitis caused by different CNS species. CNS species isolated from milk and from skin and other extramammary sites were compared. The response of CNS mastitis to antimicrobial therapy was studied, as well as the ability of CNS infections to persist in the mammary gland. Two identification methods based on genotyping of bacteria, amplified fragment length polymorphism (AFLP) analysis, and the 16S and 23S rRNA gene restriction fragment length polymorphism (RFLP) method, commonly termed ribotyping, were evaluated and compared with results from using a commercial test kit, API Staph ID 32, which is based on various phenotypic biochemical reactions of the bacteria. AFLP and ribotyping appeared to be more accurate than the API test. The agreement of the identification results between genotypic and phenotypic tests ranged from 47% to 75%. The agreement between the results was better for isolates originating from the milk than for those originating from the skin samples.

The CNS species causing mastitis were identified in studies I, III, and IV. In study I, milk samples were collected during daily practice from cows suffering from mastitis in commercial dairy herds in the practice area of the Ambulatory Clinic of Faculty of Veterinary Medicine, University of Helsinki. In studies III and IV, the milk samples originated from the Viikki research dairy herd of the University of Helsinki. The predominant CNS species isolated from milk samples in all studies were *S. chromogenes* and *S. simulans*. *S. chromogenes* was predominant in heifers around calving and at the first lactation, whereas *S. simulans* was predominant in cows in subsequent lactations.

The CNS species on bovine skin and other extramamary sites were investigated in study IV. Swab samples were collected from the perineum, udder skin, teat apex and teat canals of cows, hands of milkers, and teat cup liners in the Viikki research dairy herd. The CNS species isolated from the samples were identified and compared with the CNS species

isolated from milk samples from cows with mastitis in the same herd. The CNS isolated were further typed by strain using pulsed field gel electrophoresis, and the strains in milk and extramammary samples were compared. The predominant CNS species in the extramammary samples were mainly different from the predominant species in milk samples. *S. succinus* and *S. xylosus* predominated in samples from cows' skin. *S. chromogenes* and *S. saprophyticus* were also isolated from several extramammary sampling sites. The same strains of *S. chromogenes* were found both in the milk samples and from cows' skin. *S. simulans* was found from the samples originating from cows' skin only three times, indicating that *S. simulans* may be solely a mastitis pathogen that has adapted to the conditions of the bovine mammary gland. In contrast, *S. chromogenes* is able to live on bovine skin, but can also infect the udder and cause mastitis.

In studies I and II, the response of CNS mastitis to antimicrobial treatment was evaluated under field conditions in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, University of Helsinki (I, II), and in the practice areas of four municipal veterinarians in southern Finland (II). CNS mastitis caused by β-lactamase negative CNS isolates was treated with penicillin, and mastitis caused by β-lactamase positive CNS isolates with cloxacillin. In study II, 41% quarters with mastitis were left without antimicrobial treatment. The bacterial cure rates for 69 (study I) and 28 (study II) quarters with mastitis caused by β-lactamase negative CNS isolates and treated with penicillin G were 88% and 79%, respectively. In study I, six of nine quarters (67%) infected by β-lactamase positive CNS isolates and treated with cloxacillin were cured. The spontaneous bacterial cure rate of 55 quarters left without antimicrobial treatment was 46%.

In study III, all quarters of all cows (30 primiparous and 52 multiparous) of the Viikki research dairy herd were sampled once a month throughout one entire lactation. At parturition, 37.5% of quarters of primiparous cows and 5.8% of quarters of multiparous cows were infected with CNS. Forty-nine percent of these infections present at calving were also detected later during the subsequent lactation. During lactation, a total of 63 CNS intramammary infections were detected. Half of the infections persisted from one sampling to the next, and often to the end of lactation. The persistence of the same bacterial clonal lineage was confirmed by strain typing with AFLP. The geometric mean of milk somatic cell count (SCC) was 658 000 cells/ml in the quarters infected persistently with CNS. This is about a tenfold increase compared with the geometric mean of milk SCC in the quarters without bacterial growth, which was 65 000 cells/ml. The conclusion from this study was that about half of the intramammary infections due to CNS during lactation persist for long periods, if not treated with antimicrobials, and increase milk SCC moderately.

About half of the cows with CNS mastitis showed some clinical signs, but in most cases the signs were mild. Often only changes in milk appearance, such as clots and flakes, were detected, but sometimes also slight swelling of the affected quarters. Not more than 7% of cows showed systemic signs. No statistically significant differences between the main mastitis causing species *S. chromogenes* and *S. simulans* in the clinical characteristics of mastitis or persistence of infections were recorded, although *S. simulans* tended to cause clinical signs of mastitis slightly more often than *S. chromogenes*.

ORIGINAL ARTICLES

This thesis is based on the following original articles, referred to in the text by their Roman numerals:

I   Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D., Pyörälä, 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.

II   Taponen, S., Dredge, K., Henriksson, B., Pyyhtiä, A.-M., Suojala, L., Junni, R., Heinonen, K., Pyörälä, S. 2003. Efficacy of intramammary treatment with procaine penicillin G vs. procaine penicillin G plus neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria – a double blind field study.
Journal of Veterinary Pharmacology and Therapeutics 26:193-198.

III   Taponen, S., Koort, J., Björkroth, J., Saloniemi, H., Pyörälä, 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 90:3301-3307.

IV   Taponen, S., Björkroth, J., Pyörälä, S. 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd.
Journal of Dairy Research. Submitted 2008.

ABREVIATIONS

AFLP	amplified fragment length polymorphism
ATCC	American Type Culture Collection
CCUG	Culture Collection, University of Göteborg
CMT	California mastitis test
CNS	coagulase-negative staphylococci
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
IMI	intramammary infection
MLST	multilocus sequence typing
NAGase	N-acetyl-β-glucosaminidase
PFGE	pulsed field gel electrophoresis
RFLP	restriction fragment length polymorphism
SAA	serum amyloid A
SCC	somatic cell count

INTRODUCTION

Coagulase-negative staphylococci (CNS) have traditionally been considered to be minor pathogens. Their importance has increased and they have become the predominant pathogens isolated from subclinical mastitis in several countries (Tenhagen et al., 2006; Koivula et al., 2007; Lim et al., 2007). In a nationwide survey in Finland, 50% of bacterial isolates from randomly sampled quarters were CNS (Pitkälä et al., 2004). In clinical mastitis the proportion of CNS is generally lower. However, in Finland they are the most common isolates in milk samples from cows with clinical mastitis, especially mastitis with mild clinical signs (Nevala et al., 2004; Koivula et al., 2007). It seems that CNS mastitis is a particular problem in well managed, high producing farms, which have successfully controlled udder infections caused by major mastitis pathogens (Myllys and Rautala, 1995). For reasons not yet known, CNS mastitis is especially common in heifers and in cows during first lactation (Myllys, 1995).

CNS are generally associated with subclinical mastitis and occasional bouts of clinical mastitis. Usually the clinical signs remain mild. The increase of somatic cell count (SCC) in the affected quarter is only moderate, which has resulted in underestimation of the occurrence of CNS mastitis. Quality requirements for raw milk in the EU are high and the price of bulk tank milk is linked with the SCC of the milk. In Finland, the requirement for best bulk milk price is a SCC <250 000 cells/ml and a bacterial count <50 000/ml. CNS can affect the quality of bulk tank milk as they are a frequent cause of mastitis, although they only slightly increase the SCC.

CNS are generally considered to be opportunistic pathogens. Controlling CNS mastitis is difficult because the epidemiology is not clear, and the CNS group consists of about 40 different *Staphylococcus* species. These different CNS species are not necessarily a uniform group: some CNS may be more contagious in nature than others and some may be more virulent than others. The reservoirs of mastitis causing CNS remain unclear, although a number of CNS species have been isolated from different bovine body and other extramammary sites (White et al., 1989; Matos et al., 1991).

A variety of CNS species has been isolated from mastitis. *S. chromogenes, S. simulans* and *S. hyicus* are reported most often, but also many other species are frequently mentioned. Identification of CNS species in different studies usually rests on use of commercial identification kits based on biochemical profiles. Recently, diverse identification methods based on genotype have been developed and compared with identification methods based on phenotype. Agreement between genotypic and phenotypic identification methods has varied from satisfactory to poor (Ruegg, 2007). Differences in clinical characteristics between mastitis caused by different CNS species can exist, but for studying these hypothetical differences accurate identification methods are needed. Effective control of CNS mastitis presumes knowledge about characteristics and epidemiology of different CNS species.

This dissertation focuses on characterization of bovine CNS mastitis, i.e. clinical characteristics, tendency for persistence, response to treatment, epidemiological aspects of CNS mastitis, possible differences in these traits among CNS species, and accurate identification of the relevant CNS species causing bovine mastitis. Such knowledge is essential for effective control and prevention of CNS mastitis.

REVIEW OF LITERATURE

1. Significance of coagulase-negative staphylococci in bovine intramammary infections

1.1. Classification of staphylococci in mastitis diagnostics

In mastitis diagnostics, staphylococci are divided into coagulase-positive and coagulase-negative staphylococci on the basis of the ability to coagulate rabbit plasma. The major pathogen, *S. aureus*, is regarded as being coagulase-positive, although some strains have been suggested in some studies to be coagulase-negative (Fox et al., 1996). Some other *Staphylococcus* species may also be coagulase-positive (Hajek, 1976; Devriese et al., 1978; Devriese et al., 2005), but in mastitis diagnostics all coagulase-positive isolates are usually classified as *S. aureus* and all other isolates as coagulase-negative staphylococci, CNS. In diagnostics of bovine mastitis, this classification has been considered adequate because the CNS usually cause subclinical or only mild clinical mastitis and are therefore traditionally regarded as minor pathogens of minor importance.

1.2. Proportion of CNS causing mastitis

CNS are a frequent cause of bovine mastitis in modern dairy herds in many countries. In many countries they have even become the predominant pathogen isolated from milk samples from cows with mastitis. CNS are most frequently isolated from quarters with subclinical or clinical mastitis with only mild clinical signs. In national survey studies, where prevalence of mastitis is investigated based on sampling from all quarters of all cows in randomly selected herds, those quarters with bacteriological growth represent mainly subclinical mastitis. The clinical status of the quarters is usually not recorded but the prevalence of clinical cases is probably very low in these surveys. In a nationwide survey in Finland, CNS were isolated from 17% of the quarters and from 50% of the quarters positive for bacterial growth (Pitkälä et al., 2004). In a similar survey in Norway, CNS were isolated from 3% of all sampled quarters and from 14% of the quarters positive for bacterial growth (Østerås et al., 2006). The detection limit for a positive diagnosis in the Norwegian study was almost ten times higher than in Finland, which certainly led to underestimation of the number intramammary infections in the Norwegian survey. In a recent survey-type study from Germany, CNS were isolated from 9% of the quarter milk samples in a total of 80 dairy herds, and they comprised 35% of samples positive for bacterial growth (Tenhagen et al., 2006). In the Netherlands, CNS have not been so dominant as mastitis causing agents: they were isolated from 3% of quarters and 6% of cows with milk SCC above 250 000 cells/ml, and the proportion of CNS among bacteria isolated from quarters with bacterial growth was 16% (Poelarends et al., 2001). The most frequent bacterial isolate found in this study was the major pathogen *S. aureus* (49% of quarters positive for bacterial growth).

Intramammary infections caused by CNS have also become common in the USA and Canada. In two dairy research herds in Ontario, Canada, CNS were the most common bacteria (51%) causing intramammary infection (IMI) at drying off (Lim et al., 2007). In a study carried out a decade ago in New York and Pennsylvania in the USA, prevalence of CNS infections among all sampled cows was 11%, and 23% of the bacteria isolated from the milk samples were CNS (Wilson et al., 1997). In a more recent study from Wisconsin

in the USA, CNS were isolated from 13% of all milk samples submitted for microbiological examination and were the most commonly isolated bacteria; 24% of all samples positive for bacterial growth (Makovec and Ruegg, 2003). In another study in Wisconsin on 20 conventional and 20 organic dairy farms, the prevalence of CNS IMIs was 14% on conventional farms and 17% on organic farms, and CNS were recovered from 38% and 30% of milk samples with bacterial growth on conventional and organic farms, respectively (Pol and Ruegg, 2007). In Tennessee in the USA, the average proportion of CNS IMIs in high SCC herds was 28% (Roberson et al., 2006), and herd prevalence varied from 12 to 41%. In a study carried out in the US and Canada, 15% of new IMIs detected post partum were caused by CNS (Dingwell et al., 2004). The results from different countries are not easily comparable because the number of cfu per ml considered as positive for CNS growth varies. In the Finnish survey with the high prevalence, growth of ≥ 5 cfu per 0.01 ml was considered positive for all bacteria, whereas in the Norwegian survey, the respective cut-off value for CNS was 40 cfu. In the study by Dingwell et al. (2004), the limit was 50 cfu.

CNS are generally associated with subclinical mastitis. The proportion of CNS among bacteria isolated from milk samples from clinical mastitis is very low in many countries. In a recent study from Canada, CNS did not appear on the list of pathogens causing clinical mastitis (Barkema and Olde Riekerink, 2006). Only few studies report separately the proportion of CNS as a cause of clinical mastitis. In Sweden, CNS comprised only 6% of bacteria isolated from clinical mastitis (Ekman and Østerås, 2004). In Switzerland the respective figure was 17% (Schällibaum, 2001) and in Israel 9% (Shpigel et al., 1998). Among 77 051 routine samples from mastitis submitted to laboratories in Finland during 2004-2006, CNS were the most frequently isolated bacteria in clinical (18%) and subclinical mastitis (24%) (Koivula et al., 2007). In the practice area of the Faculty of Veterinary Medicine, University of Helsinki, Finland, 21% of the bacteria isolated from milk samples from clinical mastitis were CNS (Nevala et al., 2004). In a Norwegian study by Waage et al. (1999), CNS caused 13% of mastitis with clinical signs in heifers prior to or within 14 days after parturition. In that study, *S. aureus* was most frequently associated with clinical mastitis in heifers (44% of the quarters), and in mastitis with systemic signs the proportion of *S. aureus* was >50%. In New Zealand, 8% of isolates from clinical mastitis were CNS (McDougall et al., 2007). Classification of clinical mastitis can differ between studies; in the Finnish studies, mastitis with any clinical sign, such as clots in milk, was classified as clinical. All Nordic countries have national databases for disease recording of dairy cattle, but codes used to classify type of mastitis somewhat differ (Valde et al., 2004).

1.3. Precalving prevalence of CNS intramammary infection

Precalving heifers were earlier suggested to be free from mastitis, and studies were not targeted at heifer populations. Oliver and Mitchell (1983), Oliver (1987), and numerous other authors thereafter showed that a marked proportion of heifers had IMI already before, at, or soon after parturition. The majority of these infections were found to be caused by CNS. The precalving prevalence of CNS IMI varies greatly among herds. In many studies conducted in the USA, the precalving prevalence of CNS IMI is rather high. In the study of Oliver and Mitchell (1983) on 32 heifers in Massachusetts in the USA, 69% of heifers and 27% of the quarters were infected at parturition, and CNS were isolated from 20% of quarters. In Oliver's (1987) study on 75 heifers, where heifers were

sampled from two weeks before parturition to two weeks after, the highest frequency of isolation of mastitis pathogens was at parturition (28% of samples). CNS accounted for 56% of the organisms isolated in total. The proportion of CNS markedly decreased in the samples taken after calving, and only 38% of the CNS infections persisted into early lactation. In a study conducted on a research herd in Louisiana in the USA, 10 heifers were sampled from about 11 months of age throughout freshening (Boddie et al., 1987). That study reported that bacteria were isolated from 86% of the secretion samples, and 72% of the bacteria isolated were CNS. Prepartum IMI was detected as early as at 11 months of age and infection could persist into lactation. Another study, conducted on 116 heifers on the same research herd and on three commercial herds, reported that 75% of 370 sampled quarters were infected, and CNS were isolated from 75% of the quarters (Trinidad et al., 1990a). Fifteen percent of the quarters showed clinical signs, and CNS were isolated from 52% of the quarters with clinical signs. After parturition the CNS infections decreased markedly, and in early lactation only 8% of quarters were infected with CNS, as also reported in the study of Oliver (1987). In Vermont in the USA, 46% of heifers and 19% of quarters had IMI at parturition (Pankey et al., 1991). CNS were the most common cause of IMI, affecting 23% of the heifers and 11% of the quarters. Matthews et al. (1992) also found CNS IMI to predominate during the periparturient period both in primiparous and in multiparous cows. The quarter prevalences of CNS IMIs prepartum and at parturition in primiparous cows were 39% and 28%, respectively, and in multiparous cows 50% and 12%, respectively. One to five weeks postpartum the quarter prevalence of CNS ranged from 13% to 15% in primiparous cows, but the prevalences were lower, from 6% to 11%, in multiparous cows. Fox et al. (1995) did a survey of heifer IMI on 1583 heifers in 28 herds in four different locations in the USA. The quarter prevalence of CNS IMI in pregnant heifers averaged 27% and varied by location and by season from 19% to 36%. At calving, the quarter prevalence of CNS IMI was 22%. It seems that in the USA the management of heifers may particularly favor development of IMIs caused by CNS.

Heifer mastitis is a common problem also in other countries. In Finland, Myllys (1995) studied mastitis in heifers before and after calving. He found CNS infection before calving in 29% of a total of 236 healthy mammary quarters of heifers; 50% of 74 quarters with clinical signs of mastitis were infected. In the same study, 19% of the 527 healthy quarters and 34% of the 275 quarters with clinical signs were infected after calving. In a Danish study conducted on 180 heifers in 20 herds, the most common CNS species in heifers, *S. chromogenes*, was isolated from 15% of quarters before calving (Aarestrup and Jensen, 1997). The overall quarter prevalence of CNS IMI pre-calving was about 17%. In a Japanese study in a dairy herd with a high prevalence of *S. aureus* infection, CNS were isolated from 54% of the quarters of 15 heifers at four to five weeks before parturition (Nagahata et al., 2006); *S. aureus* was not isolated from those heifers. A lower precalving prevalence of CNS infection was reported by Parker et al. (2007). In the pasture-fed heifers in New Zealand, the total quarter prevalence of IMI pre-calving was 16% and the quarter prevalence of CNS IMI 12%. Within four days after calving the quarter CNS prevalence was only 5%. The conditions under which heifers are raised in New Zealand on pasture differ from those where animals are kept in closed barns or yards and where probably spread of CNS IMIs in the udder of heifers is not that efficient.

1.4.Clinical signs of CNS mastitis

CNS have been regarded as minor pathogen that mostly infect heifers around calving, do not cause clinical signs, cause only a slight increase in the somatic cell count, and disappear soon after parturition. It is generally held that in CNS mastitis only mild local signs are usually seen, such as slight swelling and changes in the milk appearance, but studies that have thoroughly investigated clinical characteristics of mastitis caused by CNS are very few. Jarp (1991) reported that clinical signs of CNS mastitis most often were subclinical or mild clinical, although severe clinical signs occasionally were recorded. One recent study (Bleul et al., 2006) reported on three cases of toxic mastitis caused by staphylococci other than *S. aureus*. Unfortunately, status of coagulase production of the isolates was not reported. In a pilot study, in which five lactating cows were experimentally infected with *S. choromogenes*, the concentrations of different inflammation parameters in milk were 10 to 100 times lower than in an experimentally induced *Escherichia coli* (*E. coli*) mastitis, and the clinical signs were very mild (Simojoki et al., 2007).

Histopathologic changes caused by staphylococcal infection in bovine mammary glands were investigated in three studies. According to results from the studies, CNS infection causes a similar type of but possibly less serious damage in the mammary gland than *S. aureus* infection. Boddie et al. (1987) observed a strong leukocyte response to CNS colonization in the teat canal and mammary tissues of two heifers. Trinidad et al. (1990b) studied histopathologic changes in 7 mammary glands of unbred heifers experimentally infected with *S. aureus* Newbould 305 (ATCC 27940), one quarter naturally infected with *S. aureus*, and three quarters naturally infected with CNS. The quarters infected with *S. aureus* and CNS showed less alveolar, epithelial and luminal areas, more interalveolar stroma and greater leucocyte infiltration compared with the uninfected quarters. In quarters infected with CNS, histopathologic changes were not as marked as in quarters infected with *S. aureus*. Benites et al. (2002) studied histopathology of lactating dairy cows culled due to mastitis. The histopathologic changes of 99 quarters infected with CNS and 14 quarters infected with *S. aureus* mainly showed a chronic inflammatory response or a chronic inflammatory response with repair, and no differences in the histopathologic changes were observed between *S. aureus* and CNS infected quarters.

1.5. Effects of CNS mastitis on milk quality

Compared with infections caused by other common Gram-positive mastitis pathogens, such as *S. aureus* and streptococci, the SCC in quarters infected with CNS is rather low. It is, however, about 10-fold higher than the SCC of healthy quarters, which typically remains under 50 000 cells/ml (Boddie et al., 1987; Laevens et al., 1997b; Barkema et al., 1999). Staphylococcal infections typically increase milk SCC for a long time (de Haas et al., 2004), but even a transient CNS infection causes a temporary increase in SCC (Laevens et al., 1997b). In the meta-analysis of Djabri et al. (2002), the geometric mean SCC in the CNS infected quarters was 138 000 cells/ml and in the *S. aureus* infected quarters 357 000 cells/ml. Although the mean SCC in CNS infected quarters is rather low, CNS infection can occasionally raise SCC markedly. Simojoki et al. (2007) reported that the mean peak SCC after an experimental challenge with *S. chromogenes* was 2 000 000 cells/ml. In the study of Rainard et al. (1990), SCC was >500 000 cells/ml in 38% of the quarters infected with CNS, in 42% of the quarters SCC was from 200 000 to 500 000

cells/ml, and in 20% of them <200 000 cells/ml. In most studies, the reported milk SCC in CNS infected quarters varied from about 200 000 to 600 000 cells/ml (Boddie et al., 1987; Pyörälä and Syväjärvi, 1987; Matthews et al., 1990; Nickerson and Boddie, 1994; Chaffer et al., 1999).

The direct economic impact of high SCC depends on the violation limits for poor quality milk or possible quality premiums paid for high quality milk, which differ considerably among countries. In Finland, the requirement for best bulk milk price is SCC <250 000 cells/ml and bacterial count <50 000/ml. The dairy producers pay much attention to keeping the SCC low, and any bacteria causing udder infections and increasing the SCC are in this respect harmful.

Intramammary infection caused by CNS causes inflammatory reaction in the infected gland, which can be detected using various indicators for inflammation in the milk. Some studies are available where parameters other than milk SCC were studied in CNS mastitis. Elevated concentrations of milk N-acetyl-β-glucosaminidase (NAGase) activity, antitrypsin, and serum amyloid A (SAA) were reported during IMI caused by CNS (Pyörälä and Syväjärvi, 1987; Myllys, 1995; Pyörälä and Pyörälä, 1998; Simojoki et al., 2007).

1.6. The effect of CNS mastitis on milk yield

It has been speculated that IMIs caused by minor pathogens may also have a negative impact on the milk yield. The normal variation in milk production of different cows is high, and therefore large datasets are needed to verify significant differences in the effects on the milk production caused by IMIs with different mastitis pathogens. Kirk et al. (1996) did not detect a significant effect of minor pathogens on the milk production or SCC in first-lactation heifers in one large herd. In contrast, Timms and Schultz (1987) established a large difference, 821 kg, in milk production between uninfected cows and cows infected with CNS in two herds with high prevalence of CNS IMI.

Large databases on milk production records of dairy cows that would include information on bacteriological diagnoses of mastitis are usually not available. In contrast, milk SCC data are usually available from production recording systems. Comparing milk production of first-lactating cows with high or low milk SCCs has been used to estimate the impact of mastitis on milk production. CNS are the main cause of mastitis in cows in their first lactation, so it is likely that CNS are largely responsible for possible production losses. In the study of Coffey et al. (1986), the mean milk production of first-lactating cows with milk SCC in early lactation <100 000 cells/ml was 400 kg more than the mean production of heifers with milk SCC between 100 000 and 400 000 cells/ml, and 750 kg more than the mean milk production of heifers with SCC >400 000 cells/ml. De Vliegher et al. (2005) showed that elevated SCC was associated with reduced milk production during the first lactation. For example, a heifer with an SCC value of 500 000 cells/ml about two weeks after parturition produced 119 kg less milk during the first lactation than a heifer with a SCC value of 50 000 cells/ml. Hortet et al. (1999) found also that an increase in milk SCC was associated with a reduction in milk yield both in primiparous and multiparous cows. According to the recent results of Schukken et al. (2007), obtained from a very large database combining SCC data and milk bacterial culture results from 352 614

quarters, approximately two percent of herds in Ithaca, USA, would potentially have a high bulk milk SCC (>400 000 cells/ml) due to CNS.

Myllys and Rautala (1995) reported that heifer mastitis was most common in herds with a high mean milk production and a low bulk tank milk SCC. Heifers with mastitis had slightly higher genetic potential for milk production, but a slightly lower actual milk production than the healthy control heifers. The actual milk production was 70 to 80 kg lower than expected. Similar results indicating that CNS mastitis particularly affects highly productive cows were shown by Gröhn et al. (2004). They found that multiparous cows that developed clinical CNS mastitis produced from 2.3 to 2.7 kg/d more milk before the onset of mastitis than the control cows without CNS mastitis. After the diagnosis of clinical CNS mastitis, no difference in the milk production between cows with and without CNS mastitis was recorded. CNS mastitis seems to be concentrated in the higher producers, and the comparison of milk production of cows with and without CNS mastitis may thus have led to an underestimation of the production losses caused by CNS mastitis.

1.7. Protective effect of CNS infection against infections caused by major pathogens

Some authors have suggested that infections with minor pathogens like *S. chromogenes* or other CNS and *Corynebacterium* sp. could be beneficial as they might protect the quarter from mastitis caused by major pathogens such as *S. aureus* (Schukken et al., 1989; Matthews et al., 1990). Possible mechanisms for this effect could be the increased SCC in the milk of the infected quarters or bacteriocins produced by the bacteria (Matthews et al., 1990; De Vliegher et al., 2004). Several i*n vitro* studies indicated that a selection of minor pathogens and normal skin microbiota (*Corynebacteria, Staphylococcus, Bacillus* and *Acinetobacter*) can inhibit growth of mastitis pathogens *in vitro* (Woodward et al., 1987; De Vliegher et al., 2004). The inhibitory ability varies among bacteria and seems not to be associated with certain genera or species. A strong inhibitory capacity seems not to be very common among staphylococci, and only some strains possess considerable inhibitory capacity. Strains that have been able to show inhibitory activity have inhibited growth of staphylococci and streptococci, but not of *E. coli* or other Gram-negative pathogens (Woodward et al., 1987; De Vliegher et al., 2004). De Vliegher et al. (2003) studied the protective *in vivo* effect of teat skin colonization with *S. chromogenes* on milk SCC three to five days after parturition. In the study, with limited material, teat apex colonization with *S. chromogenes* was found to be slightly protective against milk SCC ≥200 000 cells/ml in quarters during the early lactation period.

The protective effect of CNS IMI against IMI with major pathogens has not been confirmed either in challenge studies or under field conditions, as the results from the studies have been conflicting. In a *S. aureus* challenge study by Matthews et al. (1990) on 35 udder quarters, experimental infections with *S. aureus* were established in all 18 out of 18 previously uninfected quarters, whereas only 8 out of 17 quarters with pre-existing *S. chromogenes* infection became infected. Nickerson and Boddie (1994) used data from five challenge trials on one research herd and recorded a total of 346 new IMIs caused by *S. aureus* or *Streptococcus agalactiae*. In those data a new IMI caused by *S. aureus* was observed in 13% of previously uninfected quarters and in 4% of quarters infected with CNS, indicating that CNS IMI could protect against *S. aureus* IMI. In contrast, a new IMI caused by *Str. agalactiae* developed in 5% of the uninfected and in 8% of the CNS

infected quarters, indicating that CNS IMI would not be a protective but rather a predisposing factor for infection caused by *Str. agalactiae*.

Observations under field conditions do not completely support the hypothesis of the protective effect of CNS infection on IMI with major pathogens. Schukken et al. (1989) compared ten herds with a low bulk milk SCC and a low incidence of clinical mastitis caused by *E. coli, Str. ubris, S. aureus* and CNS with ten herds with a low bulk milk SCC and a high incidence of clinical mastitis caused by the same four pathogens. The prevalence of CNS, *C. bovis* and *Micrococcus* species was higher in the herds with a low incidence of clinical mastitis, indicating that infections with minor pathogens tended to protect cows against clinical mastitis. Matthews et al. (1991) found 13% of new IMIs in previously uninfected quarters compared with 7% of new IMIs in previously CNS infected quarters. For the major pathogens, however, the difference in the incidence of new IMIs was not statistically significant. In the study of Myllys (1995), the majority of the infections pre-calving were eliminated at around parturition, but the quarters infected before parturition were more susceptible to new infections by other pathogens after parturition than the uninfected quarters. Lam et al. (1997a) reported that the rate of infection with major pathogens was lower in quarters infected with *C. bovis* but higher in quarters infected with coagulase-negative micrococci, compared with uninfected quarters. Parker et al. (2007) found that precalving IMI with CNS increased the risk of post-calving IMI with CNS, *Str. uberis*, and *S. aureus*. Isolation of CNS in 54% of quarters of heifers, but not *S. aureus* in a dairy herd with a high prevalence of *S. aureus* infection, may also indicate that CNS infection did not protect cows against subsequent *S. aureus* infections (Nagahata et al., 2006).

Even if CNS infection would have some protective effect against clinical IMI caused by major pathogens, the negative effects cancel out the hypothetic positive effect. CNS infection induces an inflammation reaction in the udder, increases the concentrations of various inflammation markers, and causes damage to the mammary tissue, leading to some decrease in milk production. The possible pain or discomfort caused by subclinical or mild clinical mastitis is also an unstudied subject (Milne et al., 2004). Teat skin or teat canal colonization with CNS might have some protective effect against mastitis pathogens without having the negative effects of an intramammary infection (De Vliegher et al., 2003). The problem is how to limit colonization of the teat skin and the teat canal, from where the CNS can invade the udder and develop into an intramammary infection. There is no information about the possible protective effects of some non-pathogenic CNS against major mastitis pathogens, nor is there information about CNS species or strains that are non-virulent and non-pathogenic in the udder.

2. Treatment of CNS mastitis

2.1. Spontaneous cure and persistence of CNS mastitis

Spontaneous elimination rate of CNS mastitis is generally regarded as high. Some studies have demonstrated spontaneous elimination rates of as high as 60-70% for IMIs caused by CNS (McDougall, 1998; Wilson et al., 1999). On the other hand, markedly lower rates, 15%-44%, have also been reported (Rainard and Poutrel, 1982; Timms and Schultz, 1987, Deluyker et al., 2005). There is also evidence that CNS infections may persist for long periods in the mammary gland (Rainard et al., 1990; Aarestrup and Jensen; 1997; Laevens

et al., 1997a; Chaffer et al., 1999). In the study of Rainard et al. (1990), 76% of the CNS infections persisted until the end of the lactation period and the mean duration of infections was 236 days. Timms and Schultz (1987) found that only 15.5% of CNS infections were eliminated spontaneously, and many CNS infections persisted for long times. In a recent Norwegian study (Mørk et al., personal communication), where udder quarters of cows on four farms were followed-up monthly, a high proportion of CNS infections were found to persist.

Differences in the persistence between infections caused by different CNS species may exist: Aarestrup and Jensen (1997) followed quarters of heifers from 4 weeks prepartum until 4 weeks after calving. They showed that infections caused by *S. chromogenes* disappeared shortly after parturition, and infections caused by *S. epidermidis* were transient. In contrast, infections caused by *S. simulans* persisted for longer. The persistence of the same *S. simulans* clone was later confirmed by ribotyping (Aarestrup et al., 1999). However, the number of infected quarters in that study was limited.

2.2. Antimicrobial treatment during lactation

The strategies for treatment of mastitis vary among different countries. In some countries sublinical mastitis is treated during lactation, but in others subclinical and mild clinical mastitis such as CNS mastitis is left untreated or treated using conservative means such as frequent milking-out. In Finland and in the other Nordic countries, the national policy is to avoid unnecessary use of antimicrobials in animal husbandry (Anon., 2003). Subclinical and mild clinical mastitis caused by CNS are often left untreated, the rationale being that CNS will be eliminated spontaneously.

Not many treatment studies that separately report results for quarters infected by CNS have been published. Based on those available, it seems that mastitis caused by CNS responds well to antimicrobial treatment. The reported bacteriological cure rates have been 70%-90% for treatment with β-lactam antimicrobials (Rainard et al., 1990; McDougall, 1998; McDougall et al. 2007; Pyörälä and Pyörälä, 1998; Waage et al., 2000). Somewhat lower cure rates, 53% and 78%, after pirlimycin treatment for eight and two days, respectively, were reported by Deluyker et al. (2005). Elimination rates for mastitis caused by penicillin-resistant CNS seem to be somewhat lower (Pyörälä and Pyörälä, 1998). The phenomenon that cure rates for mastitis caused by penicillin-resistant isolates, even if the isolate is *in vitro* sensitive to the antimicrobial used, is known from *S. aureus* mastitis (Sol et al., 2000; Taponen et al., 2003). Cows with higher parity have significantly lower tendency for cure (Pyörälä and Pyörälä, 1998; Deluyker et al., 2005). The usual treatment length in CNS mastitis in Finland is 3 to 5 days. Optimum duration of treatment for CNS mastitis is not known, but according to the study of Deluyker et al. (2005), extending treatment to 8 days did not improve cure rates of CNS mastitis, as compared with a 2-d treatment. In that study, the cure rate of untreated CNS mastitis was 44%, which did not differ significantly from that for the treated groups.

2.3. Prevention of CNS mastitis

Very little information is available about prevention of CNS mastitis, reflecting the fact that mastitis control measures are usually targeted against major mastitis pathogens. It has been suggested that control measures against contagious mastitis pathogens, such as post-

milking teat disinfection, reduce CNS infections in the herd. Discontinuation of teat dipping was shown to increase the prevalence of udder infections with *Corynebacterium bovis* and CNS significantly (Lam et al., 1997b). Most IMIs caused by CNS occur around parturition, which is probably related to initiation of milk production and increased susceptibility of the mammary gland to mastitis during that period (Oliver and Sordillo, 1988). Measures to improve heifer immunity around parturition include maintaining optimal environment, feeding and management for the heifers. Welfare and comfort may also be significant factors for good udder health of heifers.

Dry cow therapy is generally considered to be an effective tool for mastitis control, but as regards CNS mastitis, very little published information is available. Somewhat surprisingly, preliminary results of the studies by Rajala-Schultz et al. (2007) showed some evidence that CNS infections persist over the dry period despite dry cow therapy. In a recent meta-analysis of the general efficacy of dry cow treatment (Robert et al., 2006), no significant benefit from dry cow therapy was found for the prevention of CNS infections.

Prepartum intramammary antibiotic therapy for heifers was suggested to reduce CNS mastitis during the first lactation (Oliver et al., 2003; Oliver et al., 2004; Middleton et al., 2005). Oliver et al. (2003) showed that the number of clinical IMIs was lower and the milk production higher in heifers that were treated prepartum with antimicrobials when compared with untreated heifers. In other studies, differences in milk production between treated and untreated heifers have not been demonstrated (Middleton et al., 2005; Borm et al., 2006). Antimicrobial treatment of heifers before parturition did not protect them from new infections during lactation (Oliver et al., 2004; Middleton et al., 2005; Borm et al., 2006), and new IMIs during early lactation were as common in antibiotic-treated as in untreated quarters (Oliver et al., 2004).

3. Identification of CNS species and their importance in bovine mastitis

In mastitis diagnostics, CNS are normally not identified at species level but are treated as a uniform group. Some CNS species may be more virulent or have different clinical characteristics, but evidence is still lacking. Moreover, species identification is costly and guidance for tailoring treatments to different CNS species does not exist.

3.1. Identification methods

To date 40 *Staphylococcus* species have been characterized, ten of which have subspecies (in total 52 species and subspecies). The majority of the CNS species were characterized in the 1970s and 1980s (and a few as late as in the 2000s) based on various phenotypic characteristics (http://www.bacterio.cict.fr/s/staphylococcus.html). Changes to the nomenclature occur frequently as new species are identified or previously identified species are found to represent the same species. Most of the species are determined based on various phenotypic characteristics, such as colony morphology and haemolysis patterns, and various biochemical reactions. Identification based on these conventional tests is time-consuming and costly, and therefore test series like API Staph (bioMérieux, France) and Staph-Zym (Rosco, Denmark), for rapid identification of staphylococcal species, are commonly used. However, these tests do not identify all *Staphylococcus* species, especially those from veterinary samples (Bes, 2000), and false identification

results have been reported also from human medicine (Couto et al., 2001; Heikens et al., 2005; Skow et al., 2005). The methods based on molecular genetics are developing rapidly and this seems to have created a new problem in that the bacterial phenotypes and genotypes do not necessarily match (Heikens et al., 2005).

In human medicine, staphylococci mainly cause nosocomial infections, posing a serious health risk to immuno-compromised patients or those with implanted medical devices (Grosserode et al., 1991; Hussain et al., 2000). The majority of nosocomial *Staphylococcus* infections are caused by few staphylococcal species: 95% of bloodstream infections are caused by *S. aureus, S. epidermidis, S. haemolyticus* and *S. hominis* (Marshall et al., 1998; Martin et al., 1989). Other coagulase-negative staphylococci frequently mentioned are *S. warneri, S. saprophyticus, S. schleiferi* and *S. lugdunensis* (Kloos and Bannerman, 1994). In human medicine there is a need for a simple and rapid method, which reliably and at low cost identifies the bacterial species from clinical isolates. Various new methods, mainly targeting at house-keeping genes (*tuff,* 16S rRNA, *sodA, rpoB, hsp60*) have been introduced (Martineau et al., 2001; Poyart et al., 2001; Drancourt and Raoult, 2002; Kwok and Chow, 2003; Heikens et al., 2005; Skow et al., 2005). Of these methods, sequencing of the 16S rRNA gene seems to be the most reliable (Boerlin et al., 2003), although its discriminative capacity may be insufficient for closely related species (Patel, 2001; Heikens et al., 2005). A multiplex PCR assay simultaneously targeting the gene on which identification is based and some clinically relevant genes for antibiotic resistance could offer a possibility for rapid diagnosis directly from certain types of clinical sample (Martineau et al., 2001).

In bovine practice, the need for a CNS identification method is somewhat similar as for human medicine. An ideal system would identify the CNS species relevant in mastitis rapidly and accurately directly from milk, and perhaps simultaneously detect the *blaZ*– gene, indicating resistance to penicillin G. It would be important to know about penicillin resistance of the isolate because it affects the choice of antimicrobial treatment and also prognosis for cure. Some other antimicrobial resistance genes, if such resistance genes are considered important in relation to mastitis treatment, could also be determined. CNS species responsible for the majority of bovine CNS mastitis cases are probably different from those in human medicine (Jarp, 1991; Waage et al, 1999). There is no knowledge about how many of the isolates identified as a particular CNS species do genetically really belong to that species. Most studies concerning CNS species isolated from bovine mastitis were conducted a long time ago, using methods based on phenotypic characteristics of the bacterial isolates. Before any identification method can be adopted into mastitis diagnostics, more knowledge about CNS species involved in bovine mastitis is needed. Further studies in the field of identification of CNS species from cows are necessary.

3.2. CNS species associated with bovine intramammary infections

The CNS species most commonly isolated from bovine intramammary infections is *S. chromogenes* (Trinidad et al., 1990a; Matthews et al., 1992; Nickerson et al., 1995; Aarestrup and Jensen, 1997; De Vliegher et al., 2003; Rajala-Schulz et al., 2004). *S. chromogenes* has been isolated not only from lactating cows, but also from secretion samples of heifers before parturition (Boddie et al., 1987; Trinidad et al., 1990a). Another species frequently isolated, especially during lactation, is *S. simulans* (Jarp, 1991; Myllys, 1995; Waage et al., 1999). *S. hyicus* was reported to be one of the predominant CNS

species in mastitis (Jarp, 1991; Honkanen-Buzalski et al., 1994; Myllys, 1995; Waage et et al., 1999; Rajala-Schultz et al., 2004). *S. epidermidis* has also been isolated frequently from CNS mastitis (Aarestrup and Jensen, 1997; Thorberg et al., 2006). In addition to these CNS species, a large variety of different CNS species have been isolated from bovine intramammary infections (Devriese and De Keyser, 1980; Rather et al., 1986; Jarp, 1991; Davidson et al., 1992; Aarestrup et al., 1995; Myllys, 1995; Chaffer et al., 1999; Waage et al., 1999; Lüthje and Schwarz, 2006).

4. Epidemiology of CNS mastitis

CNS have traditionally been considered to be normal skin microbiota, which as opportunistic bacteria can cause mastitis (Devriese and De Keyser, 1980). The epidemiology of CNS mastitis still is unclear, although a number of studies have been conducted to identify the reservoirs of CNS. CNS were isolated from different body sites of cows, heifers and calves, from udder secretions and milk, and from the cows' environment (Devriese and Keyser, 1980; Boddie et al., 1987; White et al., 1989; Trinidad et al., 1990a; Matos et al., 1991; Matthews et al., 1992). A wide range of CNS species have been isolated, identified most often using methods based on the phenotype. *S. chromogenes, S. epidermidis, S. intermedius, S. warneri, S. haemolyticus, S. sciuri* and *S. xylosus* were isolated in several studies from milk samples, teat canals, teat skin or skin on other body sites (Devriese and Keyser, 1980; Boddie et al., 1987; Trinidad et al., 1990a; Matthews et al., 1992; Aarestrup et al., 1995; Chaffer et al., 1999). *S. xylosus* and *S. sciuri* were shown to be part of the normal bovine skin microbiota and have been isolated from bedding and the cows' environment (Matos et al., 1991). *S. cohnii* and *S. saprophyticus* were also common in the cows' environment. In contrast to CNS typically isolated from milk, which are novobiocin sensitive, CNS mainly originating from the cows' environment are novobiocin resistant (Devriese, 1979).

S. chromogenes, which in several studies was shown to be the predominant CNS species isolated from bovine milk (Trinidad et al., 1990a; Matthews et al., 1992; Nickerson et al., 1995; Aarestrup and Jensen, 1997; De Vliegher et al., 2003; Rajala-Schulz et al., 2004), was also isolated from the skin of the teat apex, teat canal and mammary gland of unbred heifers as early as from 10 months of age (Boddie et al., 1987; De Vliegher et al., 2003). *S. chromogenes* was also found to frequently colonize other body sites like nares, hair coat, vagina and teat canal of heifers (White et al., 1989). In the study of De Vliegher et al. (2003), 20% of heifers had at least one teat apex colonized by *S. chromogenes* and prevalence of the teat apex colonization with *S. chromogenes* increased with age. This was however not associated with intramammary infection by the same agent. Prepartum teat canal colonization and intramammary infection with *S. chromogenes* can persist into the first lactation (Boddie et al., 1987).

Most of the studies on the epidemiology of CNS were performed before feasible methods for strain typing were available. As far as we know, CNS strains from mastitis and other sources were compared in only one study: Thorberg et al. (2006) compared *S. epidermidis* isolates from bovine mastitis and milkers' hands using ribotyping and pulsed-field gel electrophoresis (PFGE). The proportion of cows infected or colonized with *S. epidermidis* in the two study herds was high (22% to 31%), as compared with data from many other studies (Jarp, 1991; Waage et al., 2000). The same strains that were isolated in bovine milk were also isolated from milkers' hands and bends of elbows, indicating that the *S.*

epidermidis strains causing mastitis could have originated from humans. Epidemiology of CNS infections could be compared with the major mastitis pathogen *S. aureus*. *S. aureus* strains isolated in milk samples and other sources in dairy herds have been investigated in several studies (Zadoks et al., 2002; Smith et al., 2005, Hata et al., 2008). In contrast to the findings for *S. epidermidis*, *S. aureus* seems to be predominantly host-specific. *S. aureus* strains of human and bovine origin have been shown to be mainly different. It seems that further studies are needed for any conclusions to be reached on the epidemiology of CNS infections in dairy herds.

5. Antimicrobial resistance of bovine CNS

CNS tend to be more resistant than *S. aureus* and easily develop multiresistance. The most common resistance mechanism is β-lactamase production, which results in resistance to penicillin G and aminopenicillins. The reported percentage of penicillin resistance for CNS isolated in mastitis was 32% in Finland (Pitkälä et al., 2004), 36% in Norway (NORM-VET, 2005), 25% in Denmark (DANMAP, 2001) and 28% in the Netherlands (MARAN, 2003). In Finland, 13% of CNS isolated from clinical mastitis were β-lactamase positive (Nevala et al., 2004). Methicillin resistance of CNS is not uncommon; in the Finnish survey material 10% of CNS were resistant (breakpoint for oxacillin >2 µg/ml). Some CNS isolates, but none of the *S. aureus* isolates, carried the *mecA* gene (Pitkälä, A., personal communication). In principle, CNS with MIC higher than 0.5 µg/ml of oxacillin should be tested for possible carriage of the *mec*A gene (Pitkälä et al., 2004). Resistance to macrolides and lincosamides was recently reported to be 6-7% in Germany (Lüthje and Schwarz, 2006), and 5-19% in the Netherlands (MARAN, 2003). Resistance to oxytetracycline of CNS isolated from mastitis was 9% in Finland (Pitkälä et al. 2004) and 12% in the Netherlands (MARAN, 2003). Fusidic acid is not widely used in mastitis treatment, but resistance was reported for CNS isolated from mastitis; it was recently demonstrated to be mediated by the *fusB* gene (Yazdankhah et al., 2006). Reports of this kind are important as CNS may represent a reservoir of this and other resistance genes that could be transmitted to other staphylococci, including those pathogenic to humans.

6. Virulence factors of CNS

Possible virulence factors of *S. aureus* and CNS have been investigated both by measuring phenotypic expression of substances assumed to be associated with virulence and by screening of genes encoding these. Various virulence factors, including production of hemolysins, leucocidins, exfoliative toxins, enterotoxins, toxic-shock syndrome toxin, and slime and biofilm formation were found in *S. aureus* strains isolated from bovine mastitis (Cucarella et al., 2004; Zecconi et al., 2006), but only few studies have focused on the search for such virulence factors from CNS isolated from mastitis. Adherence and internalization of CNS into mammary epithelial cells was studied in cell cultures (Almeida and Oliver, 2001; Anaya-López et al., 2006; Hyvönen et al., 2007); CNS were shown to be able to adhere to bovine mammary cells. The adhesive capacity of various CNS was almost equal to the adhesive capacity of *S. aureus*, but the invasive capacity of *S. aureus* was stronger than that of CNS strains. Cytotoxic activity in cell cultures of CNS and *S. aureus* isolated from mastitis, possibly caused by a metalloprotease, was reported by Zhang and Maddox (2000).

Most CNS isolated from caprine mastitis produced at least one type of haemolysin, DNAse, and elastase (Bedini-Madani et al., 1998; da Silva et al., 2005). Kuroishi et al. (2003) found that a high percentage of both *S. aureus* and CNS from bovine subclinical, chronic or acute mastitis produced staphylococcal enterotoxins and/or toxic shock syndrome toxin-1. Somewhat surprisingly, production of different staphylococcal enterotoxins and toxic shock syndrome toxin-1 were as common in CNS isolates as in *S. aureus* isolates, and as common in isolates originating from subclinical mastitis as in those from chronic or acute mastitis.

Various virulence factors of *S. aureus* were compared with clinical characteristics of mastitis, but no specific virulence factor or combination of factors was strongly associated with the severity of mastitis (Haveri et al., 2007). In contrast, it was shown that a biofilm-producing *S. aureus* strain decreased severity of mastitis but increased colonization capacity in the mammary gland (Cucarella et al., 2004). The ability of staphylococci to generate biofilm was studied by measuring biofilm formation phenotypically and by screening genes associated with its formation. Biofilm-associated proteins were found among bovine mastitis isolates, including *S. aureus*, *S. epidermidis, S. chromogenes, S. hyicus,* and *S. xylosus* (Cucarella et al., 2004). Oliveira et al. (2006) found 6 out of 16 *S. aureus* and 6 out of 16 *S. epidermidis* isolates from subclinical mastitis to be phenotypically positive for biofilm production. Fox et al. (2005) showed that biofilm formation was clearly more common in *S. aureus* isolated from milk than in *S. aureus* isolated from teat skin and milking unit liners.

Biofilm formation in staphylococci isolated from bovine mastitis or humans has been associated at least with gene loci *ica* (intercellular adhesion), *bap* (biofilm-associated protein), *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator), but isolates able to form biofilm do not necessarily carry all these genes, and other mechanisms may be involved in biofilm formation (McKenney et al., 1998; Cramton et al., 1999; Cucarella et al., 2001, 2004; Beenken et al., 2003; Vasudevan et al., 2003; Fox et al., 2005; Lasa and Penadés, 2006; Planchon et al., 2006). The *bap* gene was identified in mastitis causing staphylococci with marked ability to produce biofilm and which belonged to several species, including *S. epidermidis, S. chromogenes, S. xylosus, S. simulans* and *S. hyicus* (Tormo et al., 2005). Baselga et al. (1993) noted that the severity of ruminant mastitis decreased, but the capacity of bacteria to colonize the mammary gland increased when infection was caused by a mucoid (slime producer) rather than a non-mucoid *S. aureus* isolate. The mucoid isolate carried *bap* and *ica* genes, both involved in biofilm formation (Cucarella et al., 2004).

Leitner et al. (2003) studied virulence of *S. aureus* and CNS using a mouse model. Seven strains of *S. aureus* and one strain of each of *S. chromogenes* and *S. intermedius* isolated from chronic bovine mastitis were studied. Mice were experimentally infected in one limb and thereafter inspected for morbidity (arthritis, gangrene) and mortality. One *S. aureus* isolate producing α-haemolysin was the most virulent, followed by isolates producing α+β-haemolysin and β-haemolysin. The least virulent isolates were the non-haemolytic *S. aureus* strains, but even they were more virulent than the *S. chromogenes* and *S. intermedius* isolates tested. These two CNS isolates did not cause any morbidity or mortality in the mice.

AIMS OF THE STUDY

The overall aim was to study bovine intramammary infections caused by coagulase-negative staphylococci (CNS): epidemiology, clinical characteristics, treatment of CNS mastitis, and prevalence and identification of different CNS species associated with mastitis. The specific aims of the studies were as follows:

The aims of study I were 1) to examine the persistence of subclinical and clinical mastitis caused by CNS either treated with antimicrobials or left untreated, 2) to identify the most common CNS species causing subclinical and clinical mastitis, 3) to compare amplified fragment length polymorphism (AFLP) typing of CNS with phenotypic identification, and 4) to evaluate possible differences in clinical characteristics of mastitis caused by different CNS species.

The aim of study II was to evaluate the efficacy of intramammary treatment of mastitis caused by Gram-positive bacteria, including CNS. The efficacy of penicillin G alone was compared with that of a combination of penicillin G and neomycin, to assess the superiority of the combination in the intramammary treatment of penicillin-sensitive Gram-positive bacteria.

The aim of study III was to investigate the persistence of CNS infection in the udder of lactating cows over the entire lactation period using consecutive sampling and phenotyping and genotyping of the isolates. The influence of CNS infection on the milk SCC was also studied.

The aims of study IV were 1) to identify the CNS species isolated from skin, teat apex and streak canals of lactating dairy cows, milk samples from cows with subclinical or clinical mastitis, and samples from teat cup liners and hands of staff working with the research dairy herd of the University of Helsinki, and 2) to compare the CNS species and strains isolated from mastitic milk samples with CNS species and strains isolated from other sampling sites.

MATERIALS AND METHODS

1. Study designs

This thesis consists of four parts (I-IV). Two parts (I and II) refer to field studies conducted in commercial dairy herds in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, University of Helsinki, and the two other studies (III and IV) were conducted in the Viikki research dairy herd of the University of Helsinki. In studies I and II, the effect of antimicrobial treatment on mastitis was studied. The outcome of clinical or subclinical CNS mastitis and the effect of CNS species on cure rate and clinical characteristics were investigated in study I. This material was collected in connection with the daily farm visits, and mastitis treatments were conducted according to the routine practice of the Ambulatory Clinic. In total, 133 quarters with CNS mastitis in 95 cows from 59 dairy herds were included in the study; 78 of those quarters were treated with antimicrobials and 55 quarters were left without treatment. In study II, with a double blind design, the efficacy of two antimicrobial intramammary treatments (penicillin, or penicillin + neomycin) to treat mastitis caused by Gram-positive bacteria, CNS included, was evaluated. In total, 117 quarters in 96 cows from 68 dairy herds were treated. The bacterial species isolated from those quarters were as follows: 28 CNS, 19 *S. aureus*, 24 *Streptococcus dysgalactiae*, and 46 *Streptococcus uberis*.

In study III, the persistence of CNS intramammary infection and the influence of persistent CNS infection on milk somatic cell count (SCC) were studied using consecutive milk sampling every four weeks throughout the whole lactation. In total, 328 udder quarters of 82 dairy cows (30 primiparous, 52 multiparous) were followed from about two weeks prior to calving until the end of lactation, or until the cow left the herd. In study IV, CNS species and strains from mastitis and extramammary sites were compared. Extramammary swab samples were collected from skin sites of a random sample of 31 and 35 out of about 70 dairy cows in the research dairy herd in 1999 and 2002, respectively. Swab samples were also collected from milkers' hands and teat cup liners. During years 1998 to 2002, milk samples from quarters with subclinical or clinical mastitis in the same dairy herd were sampled by the herd staff and sent for microbiological analysis to the National Veterinary and Food Research Institute, Finland. In total, 69 CNS isolates from mastitic milk samples, mostly from subclinical mastitis, were stored.

2. Animals

The majority of the dairy cows studied during this thesis work were of the Finnish Ayrshire breed, some were Holstein Friesians and very few were of the Finnish Landrace breed. In studies I and II, the cows were located in private commercial dairy herds in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, University of Helsinki (studies I and II) and in the practice area of four municipal veterinarians in southern Finland (study II). The mean milk production of the herds in these field studies was not recorded, but the mean milk production in Finland during the time of the studies was approximately 8000 kg/cow/year (Finnish Milk Recording Data 1999-2002). In study I, the proportion of primiparous cows was 40%, and in study II, 21%. The proportion of primiparous cows in Finland in 2003 was 38.5% (Finnish Milk Recording Data 2003, Sinikka Tommila, personal communication). In studies III and IV the cows were owned by and located in the Viikki Research Dairy Herd of University of Helsinki. The average

milk production of the herd in 2004 was 11 263 kg/cow/year. The proportion of cows in first lactation was 37%. Of the 82 cows used in study III, 30, 21, 15, 5, and 11 cows calved for the first, second, third, fourth, and fifth or more times, respectively.

3. Classification of mastitis (I, II)

Mastitis was classified as subclinical if no local or systemic signs or alterations in milk appearance were detected. Mastitis was classified as clinical if any local or systemic signs or any alterations in milk appearance were detected. Clinical mastitis was further divided into three groups according to signs: 1) mild clinical mastitis = clots and flakes in the milk, no or minor swelling in the affected quarter and a normal body temperature, 2) moderate clinical mastitis = clots and flakes or other changes in the milk, swelling of the affected quarter and a body temperature of $39.0^{o}C$ to $40.5^{o}C$, and 3) severe clinical mastitis = marked changes in the milk appearance, severe swelling, firmness and soreness in the quarter and a body temperature of $>40.5^{o}C$ and/or severe anorexia and depression and/or recumbent. For statistical analyses in study I, 2 and 3 were grouped: 1 = mild signs and 2 & 3 = moderate/severe signs.

4. Indicators of inflammation

Milk somatic cell count (SCC) (III), California mastitis test (CMT), which is based on somatic cell count (I), and milk N-acetyl-β-D-glucosaminidase (NAGase) activity (II) were used as indicators of inflammation. NAGase is an intracellular, lysosomal enzyme, which is released in milk during intramammary inflammation from the activated or broken inflammation cells.   NAGase activity of the milk samples taken on the day of diagnosis and at the follow-up visit, 3 to 4 weeks post-treatment, were determined with a fluorometric assay (Pyörälä and Pyörälä, 1997). The CMT test was performed by the veterinarians inspecting the cows (I, II). The Nordic classification of five CMT classes was used (Klastrup, 1975). The milk samples for SCC analysis (III) were milked into 10 ml plastic tubes containing a pill of preservative (Bronopol®, D & F Control Systems, Inc., Dublin, CA, U.S.A.). The SCC samples were sent with the milk transporter to the laboratory of Valio Ltd., where the milk SCC was measured with a Fossomatic instrument (Foss Electric, Hillerød, Denmark).

5. Antimicrobial treatments (I, II)

Mastitis caused by CNS was treated with penicillin and mastitis caused by beta-lactamase positive staphylococci with cloxacillin. In study I, 59% of the quarter cases were treated with antimicrobials and the rest were left without antimicrobial treatment, according to the practicing veterinarian's decision. The veterinarians of the Ambulatory Clinic visiting the herds and prescribing the treatments were free to choose the treatment: the route (systemic, intramammary, or combined), duration of the treatment (3 to 5 days), and the medicinal preparation. The preparations used were commercial products available on the Finnish market with indication for mastitis treatment, and the doses used were those labeled by the manufacturers (Pharmaca Fennica Veterinaria 1998-99; Suomen eläinlääkkeet 2000, 2001, and 2002).

In study II, the contents of the two intramammaries, A and B, were as follows: the treatment A, Carepen® (Vetcare Oy, Salo, Finland) contained procaine penicillin G

600 000 IU, and treatment B, Neomast® (Pfizer GmbH, Freiburg, Germany) contained 500 000 IU procaine penicillin and 300 mg neomycin. The intramammaries were administered to each of the diseased quarters once daily for four consecutive days. Both treatments were supplemented once with procaine penicillin G (Penovet®, procaine penicillin G 300 mg/ml; Boehringer Ingelheim, Copenhagen, Denmark) 20 mg/kg body weight intramuscularly at the beginning of the treatment. Treatment was randomized according to cow identity number.

6. Criteria for bacterial cure after antimicrobial treatment (I, II)

Three to four weeks after the initial milk sample and diagnosis of mastitis, a follow-up milk sample was collected. The quarter was classified as cured if bacteria of the original bacterial species in the initial milk sample were not isolated. In study I, CNS were not identified at species level but treated as a group. In study II, the identification of CNS species in follow-up milk samples was based on API testing.

7. Bacteriological methods

7.1. Milk samples (I, II, III)

Milk samples for bacteriology were collected aseptically. The udder and especially the teat were cleaned of dirt with a textile cloth moistened with distilled water. After that the teat apex was cleaned with a cotton swab moistened with antiseptic solution (Neoamisept®, Berner, Pennsylvania). Before milking the sample into the vial, which was held almost horizontally, a couple of fore strips were milked out to rinse out the normal bacterial flora from the teat canal and orifice (Honkanen-Buzalski, 1995).

In the laboratory, ten microlitres of milk was streaked on blood agar and incubated at 37°C overnight (18-22 hours). Staphylococci were further identified based on colony morphology, Gram-staining, microscopy, and a catalase test (Hogan et al., 1999). CNS were distinguished from *Staphylococcus aureus* using a Slidex test (Slidex Staph-Plus, bioMérieux, France), which in studies II and III was confirmed later using an API Staph ID 32 test and AFLP. β-Lactamase production of the CNS isolates was determined using a nitrocephin test (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). The CNS isolates were frozen (Protect Bacterial Preservers, Technical Service Consultants Ld., England) and stored at -80°C.

7.2. Extramammary samples (IV)

The perineum, udder skin and teat apex of cows, hands of milkers and teat cup liners were sampled using sterile swabs (Technical Service Consultants Ltd., Heywood, UK). Streak canals were sampled with ultrafine sterile swabs (Deltalab S.A, Barcelona, Spain). Each sample was placed into a test tube with 4 ml of phosphate-buffered rinse solution containing sodium citrate. Prior to sampling, swab heads were moistened in the rinse solution. For skin samples, the swab was rotated on the skin 360° or more. For streak canal samples, teat apices were first cleaned with cotton moistened with antiseptic solution. The ultrafine swab was carefully inserted 2 to 3 mm into the distal end of the streak canal and rotated 360°. After taking each sample, the swab was broken off into the test tube

containing rinse solution and the test tube was placed on ice for immediate transport to the laboratory.

In the laboratory, the test tubes were shaken and 100 µl of rinse solution from every tube was spread onto selective *Staphylococcus* medium in 110 agar plates supplemented with 0.05 g sodium azide/L, which inhibits growth of competing skin microbiota without inhibiting *Staphylococcus* species (Difco TM Staphylococcus medium 110, Becton, Dickinson and Company, Sparks, MD, USA) (White et al., 1988). Plates were incubated at 37°C for 24 h. After incubation, 1 to 5 colonies were selected from every plate with bacterial growth based on dissimilar colony morphology and pigmentation. From plates with more bacterial growth, more colonies were picked up than from plates with less growth, but on the whole selection of colonies was random. Colonies were transferred onto blood agar plates and further incubated at 37°C for 24 h. The colonies were then identified as staphylococci based on catalase reaction, Gram staining, microscopy, and lysostaphin and lysozyme susceptibility. CNS were distinguished from *Staphylococcus aureus* using a Slidex test (Slidex Staph-Plus, bioMérieux, France), which later was confirmed using an API Staph ID 32 test and ribotyping. The CNS isolates were frozen (Protect Bacterial Preservers, Technical Service Consultants Ltd., England) and stored at -80°C.

8. Phenotypic identification of coagulase-negative staphylococci (II, III, IV)

CNS isolates were phenotyped with an API Staph ID 32 test (bioMérieux, Marcy l'Etoile, France), a commercial kit with 32 different biochemical reactions. According to the phenotypic profiles, the isolates were identified to the species level using the Software apiweb (https://apiweb.biomerieux.com). The isolates with API results indicating >90% probability of belonging to a certain CNS species were assigned names at species level. The isolates with API results of <90% probability were assigned as *Staphylococcus* sp.

9. Genotyping of coagulase-negative staphylococci (II, III, IV)

Altogether three different methods of genotyping CNS isolates were used during the studies. The amplified fragment length polymorphism (AFLP) method was used for identification of CNS species and strains (II, III). In the AFLP method, the whole bacterial genome is digested (cut) with two restriction enzymes. Some of the resulting restriction fragments are then selectively amplified with the polymerase chain reaction (PCR) using two primers complementary to the adaptor and restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either using autoradiographic or fluorescence methodologies. AFLP analysis allows creation of very high density DNA marker maps, which can be used to differentiate closely related organisms at the species and strain level in epidemiological, genome evolution and taxonomic studies (Kokotovic, 2001). In the identification of CNS species, the AFLP patterns of CNS isolates were compared in a numerical similarity analysis with the AFLP patterns of 48 *Staphylococcus* type and reference strains. Isolates clustering together within a type strain were considered to belong to the species of the type strain (II, III).

The 16S and 23S rRNA gene restriction fragment length polymorphism (RFLP) method, commonly termed ribotyping, was used for identification of CNS species (IV). Ribotyping is not as discriminatory as AFLP and was expected to be more suitable for identification

of isolates from miscellaneous skin and environmental *Staphylococcus* populations in study IV. In ribotyping, the total genomic DNA is digested with a restriction enzyme into smaller fragments that are then separated by gel electrophoresis. The fragments are transferred from the gel onto a membrane, and hybridized using a labeled universal probe targeting the specific conserved domains of ribosomal 16S and 23S rRNA encoding genes. After hybridization, the label in the probe is visualized to show the fragments where the probe has hybridized as bands (Koort, 2006). These banding patterns, termed ribotypes, were compared in a numerical similarity analysis with the ribotypes of 46 *Staphylococcus* type and reference strains in study IV. Isolates clustering together within a type strain were considered to belong to the species of the type strain.

The CNS isolates belonging to different ribotype clusters that were detected both in mastitis milk and extramammary site samples, were further studied using pulsed field gel electrophoresis (PFGE) analysis (IV). In PFGE, the entire bacterial DNA is digested with a restriction enzyme. The restriction fragments are then separated by gel electrophoresis, in which the direction of the voltage is periodically reversed to make each band of DNA run in the opposite direction for a set time. This is necessary for separation of fragments larger than 15-20kb, which in a standard gel electrophoresis with the voltage in one direction would migrate together in a size-independent manner. Isolates with PFGE fingerprints of up to three band shifts were considered to be closely related and of the same strain (pulsotype).

For more detailed information about the genotyping methods used in studies II, III, and IV, the reader should refer to the original papers included at the end of the thesis.

10. Criteria for persistent intramammary infection (III)

The persistence of CNS intramammary infection was studied using consecutive milk samplings throughout the lactation (III). Two weeks prior to expected calving and on the day of calving, a milk sample was taken aseptically from each udder quarter for bacteriological examination. All quarters were then sampled regularly for bacteriological analysis and for determination of the SCC every four weeks until the end of lactation or until the cow left the herd. The SCC was not determined from the samples before and at calving, and the regular sampling started from two to four weeks after calving. The samples were taken in the afternoon before the evening milking.

CNS infection was termed persistent if CNS growth was detected in at least three consecutive or almost consecutive samplings (one bacterially negative sample was accepted between two samples with growth of the same CNS strain), and the isolates from the samplings possessed highly similar AFLP patterns (corresponding similarity level of patterns with the level of the internal control). Under these circumstances the isolates were considered to represent the same clonal lineage.

11. Statistical analyses (I, II, III)

Logistic regression analyses were used to test the effects of different variables on the bacterial cure rates (I, II) and clinical characteristics (II). Because of the small number of farms or cows that appeared more than once in the material, cows from the same farm, different quarters from the same cow and treatment by the same veterinarian, were

generally handled as if they were independent observations. In study I, 25 of the 95 cows had more than one quarter infected with CNS. For statistical purposes, only one quarter per cow was retained in the analysis, and the other quarters, 38 in total, were randomly excluded. A quarter was excluded five different times, and each analysis was run five times to confirm that excluding quarters did not alter the results. Results from these five analyses did not differ from each other. In study II, 22 of the 95 cows had more than one inflamed quarter. The statistics were initially run on the complete dataset. Most cows had one inflamed quarter, but some had several. The data were treated independently although multiple inflamed quarters in a single cow are unlikely to be independent of each other. The statistics were then run again with data for single inflamed quarters per cow, randomly eliminating data for multiple infections per cow. There were no statistically significant differences between the two approaches (Beaudeau et al., 1996). In testing the effect of two different treatments (A or B) on cure rate (I), the following variables were included in the model: the treatments (A or B), parity (first or subsequent), infecting organism (*S. aureus,* CNS, *Str. dysgalactiae,* or *Str. uberis*), and stage of lactation (1-60 days post partum or >60 days post partum). The effects of adding clinical signs (score mild or moderate/severe, presence of elevated body temperature or milk NAGase value) to the model were then assessed using a likelihood ratio test. Finally the model was further reduced with non-significant variables (stage of lactation). Parity, even when non-significant, was left in the model because cure rates for *S. aureus* mastitis differ between first and subsequent parities (Taponen et al., 2003), and in the final model, treatment, parity and infecting organism were included. Statistical differences between the treatment groups in the cure rates of mastitis caused by different bacteria were tested using Fisher's exact Chi-square test. The similarity of the two treatment groups was tested using a Chi-square test. The groups were not statistically different.

In testing the effect of CNS species on cure rate and clinical characteristics (II), the following variables were included in the model: CNS species (identified with API Staph ID 32) or AFLP cluster, antimicrobial treatment compared with no treatment, duration of the antimicrobial treatment, clinical status of the cow (clinical or subclinical mastitis), parity (first or subsequent lactation), and the ability of the CNS isolate to produce β-lactamase. The model included bacterial cure as the response variable and CNS species/AFLP cluster, type of mastitis (clinical/subclinical), lactation (first/subsequent), antimicrobial treatment (treatment/no treatment), duration of the antimicrobial treatment and β-lactamase production of the isolate as study variables. The effect of β-lactamase production was tested separately for treated and untreated cases having bacterial cure as the response variable and β-lactamase production of the isolate as the study variable. The effect of the two most common CNS species/AFLP clusters on the type of mastitis (clinical/subclinical) was tested with the clinical status of the cow as the response variable and CNS species/AFLP cluster as the study variable. The effect of the two most common CNS species/AFLP clusters on the severity of clinical mastitis was tested with clinical signs as the response variable and CNS species/AFLP cluster as the study variable.

For SCC, means and medians were calculated (III). A geometric mean of SCC was calculated for each quarter. For quarters infected with CNS during lactation, geometric means were also calculated from samplings at which CNS were isolated. A mean and a median of the geometric means of quarters were calculated for the following groups: 1) quarters with no bacterial growth during the entire lactation, 2) quarters with a transient CNS infection during lactation, 3) quarters with a persistent CNS infection during

lactation: geometric mean of the entire lactation, 4) quarters with a persistent CNS infection during lactation: geometric mean of the samples with CNS growth, and 5) quarters with CNS growth prior to calving, at calving, or both, but no bacterial growth during the lactation. For quarters with a transient CNS infection, a mean and a median of the samplings with CNS growth were calculated.

RESULTS

1. CNS species in bovine mastitis (I, III, IV)

The two CNS species most commonly isolated from mastitis in studies II, III and IV, irrespective of the identification method used, were *S. chromogenes* and *S. simulans*. The respective proportions of these two principal species slightly differ according to the identification method used. In study I, carried out in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, University of Helsinki, *S. chromogenes* and *S. simulans* were isolated in 23% and 44% of CNS mastitis samples, according to API test results. According to the AFLP analysis, the proportions for the two species were slightly different: 20% and 27%, respectively (Figure 1). *S. simulans* may be underrepresented in the AFLP results because only 99 of the total number of 133 isolates from mastitis were analyzed with AFLP, and a considerable number of isolates excluded from the AFLP analysis were *S. simulans* in the API test. In study III, in one dairy herd, *S. chromogenes* and *S. simulans* were, when the API test was used, isolated from 49% and 23% of the intramammary CNS infections before or at calving. According to AFLP, the same figures were 53% and 23%. In CNS infections during lactation, *S. chromogenes* and *S. simulans* represented 27% and 16% of the isolates diagnosed with the API test. In the AFLP assay the proportions were 35% and 14%, respectively. In study IV, in which ribotyping was used, *S. chromogenes* and *S. simulans* represented 50% and 31% of CNS causing mastitis in one herd.

Other CNS species were isolated in mastitic milk samples much less frequently than the predominant species *S. chromogenes* and *S. simulans*. Some of the isolates and isolate clusters had genotypes that were not similar to any of the genotypes of *Staphylococcus* type strains, and thereby remained unidentified with the genotypic methods. Genotyping failed to classify 7% to 18% of the isolates, and the API test from 11% to 41% of the isolates. In Table 1 CNS species isolated in mastitic milk samples are listed and were identified based on genotype, either with AFLP or ribotyping.

In first lactation cows, *S. chromogenes* was as common a mastitis causing species as *S. simulans,* but during later lactations *S. simulans* was the most common isolate from CNS mastitis (II). *S. chromogenes* and *S. simulans* respectively caused 56% and 19% of persistent mastitis in cows in first lactation (III).

Table 1. CNS species isolated from milk samples and identified based on genotype in studies II, III, and IV.

CNS species	Study II		Study III		Study IV
	AFLP		AFLP		Ribotyping
	Mastitis during lactation	Infection before or at calving	Persistent infection during lactation	Transient infection during lactation	Mastitis during lactation
S. chromogenes	28	30	12	10	29
S. cohnii	0	2	1	0	0
S. epidermidis	5	0	4	2	2
S. equorum	1	0	0	0	0
S. fleurettii	1	0	0	0	0
S. haemolyticus	3	6	3	11	0
S. hyicus	2	0	0	0	0
S. sciuri	2	0	0	0	0
S. simulans	36*	13	6	3	18
S. succinus	0	0	0	0	1
S. warneri	3	0	1	1	0
S. xylosus	0	1	0	2	0
S. sp	18	5	2	4	8
Total	99	57	29	34	58

* Number of *S. simulans* isolates is underrepresented because not all isolates identified as *S. simulans* in API test were analyzed with AFLP.
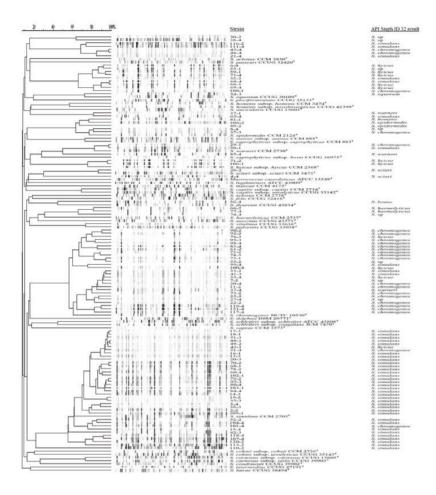
Figure 1. Dendrogram obtained from a cluster analysis of AFLP patterns of 99 CNS isolates from bovine clinical and subclinical mastitis and the type strains used in the comparison (study I).

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2. CNS species on extramammary sites (IV)

The distribution of CNS species isolated in extramammary sites and identified by ribotyping is shown in Table 2. The most numerous CNS species isolated in extramammary samples and identified with ribotyping were *S. succinus* and *S. xylosus*. Other species isolated in several sampling sites were *S. chromogenes* and *S. saprophyticus*. The isolates unidentified by ribotyping, 26% of the isolates, were distributed in 9 clusters and four single isolates. The predominant species was *S. equorum*, as identified with >90% probability with the API Staph ID 32 test. This was followed by *S. sciuri, S. saprophyticus* and *S. xylosus.* The API test failed to identify 43% of the extramammary isolates.

CNS species identified by ribotyping and isolated both in mastitis and in extramammary sites were *S. chromogenes*, *S. epidermidis*, *S. simulans*, and *S. succinus* subsp. *succinus* (Table 2). Three unidentified ribotype clusters contained isolates originating from mastitis and from skin (Figure 2). *S. chromogenes* isolates were divided into 10 different PFGE profiles (pulsotypes) (Table 3). Five pulsotypes were found both from mastitis and skin samples. The four *S. epidermidis* isolates were divided into three pulsotypes, of which one was found both from mastitis and from udder skin. *S. simulans* isolates were divided into five pulsotypes, of which two were found from mastitis, udder skin and teat canals. *S. succinus* subsp. *succinus* was very heterogeneous, consisting of 11 pulsotypes. The pulsotype of the mastitis isolate was not established for isolates from other sampling sites. One of the unidentified ribotype clusters was divided into eight pulsotypes, and the few isolates of the two other unidentified ribotype clusters were found to include only one pulsotype. Except for one *S. chromogenes* and one *S. succinus* subsp. *succinus* isolate, the isolates from hands of the herd staff were of different CNS species than those isolated from mastitis (Table 2). The *S. chromogenes* and *S. succinus* subsp. *succinus* isolates from hands of the herd staff were of different pulsotypes to those from mastitis and cow skin.

Table 2. The distribution of CNS species identified with ribotyping in samples collected in 1999 and 2002 from the perineum (P) and udder skin (U), teat apex (A) and streak canal (C) of lactating cows and hands of staff (H), and teat cup liners (L) of the research dairy herd of University of Helsinki, and in mastitis milk samples collected from the same herd in 1998-2002.

	Extramammary							Mastitis
	Tot.	H	P	U	A	C	L	
S. arlettae	7		1	5	1			0
S. chromogenes	18	1	3	8	3	3		29
S. epidermidis	2			1	1			2
S. gallinarum	5	1		1	3			0
S. hominis sp. *hominis*	2	1	1					0
S. kloosii	3		1	2				0
S. pasteuri	3	1	1		1			0
S. saprophyticus	13	1	1	4	7			0
S. sciuri subsp. *sciuri*	4	1	1	2				0
S. sciuri sp. *carnaticus*	6		1	1	3	1		0
S. simulans	3			1	1	1		18
S. succinus sp. *succinus*	13	1		2	9	1		1
S. succinus sp. *casei*	13		2	3	8			0
S. warneri	1	1						0
S. xylosus	23	4	5	1	10	2	1	0
S. sp. cluster 1	2			2				0
S. sp. cluster 2	2			2				0
S. sp. cluster 3	9		2	6	1			1
S. sp cluster 4	0							2
S. sp cluster 5	6		2	3	1			0
S. sp cluster 6	3		1		2			0
S. sp cluster 7	1		1					1
S. sp. cluster 8	13	1	5	3	3	1		0
S. sp. cluster 9	1				1			3
S. sp	4		1		3			1
Total	159							58

Cluster	Species	Strain	Origin
I		02/674	Mastitis milk
		02/678	Mastitis milk
		02/695	Mastitis milk
		02/689	Mastitis milk
	Staphylococcus simulans	CCUG 46177	
		52a-2002	Teat canal
		02/653	Mastitis milk
		127g-2002	Teat apex
	Staphylococcus simulans	DSM 20322^T	
II		02/644	Mastitis milk
		67b-1999	Teat apex
		02/675	Mastitis milk
		02/647	Mastitis milk
		32a-1999	Udder skin
		113a-2002	Perineum
		62a-1999	Udder skin
III		65b-2002	Teat apex
		138c-2002	Udder skin
		67a-1999	Teat apex
		82b-2002	Udder skin
		74d-2002	Udder skin
		30b-1999	Udder skin
IV		73a-2002	Perineum
		02/645	Mastitis milk
		02/652	Mastitis milk
		89c-1999	Teat apex
		27b-1999	Teat apex
		48b-1999	Teat canal
		7e-2002	Teat apex
		137el-1999	Teat apex
		42a-2002	Udder skin
		23a-1999	Teat apex
V		119e-2002	Teat apex
	Staphylococcus succinus subsp. succinus	DSM 14617^T	
		7c-1999	Teat apex
		28a-1-2002	Teat canal
		29a-1-2002	Perineum
		119el-1999	Teat apex
	Staphylococcus succinus subsp. casei	DSM 15096^T	
		114b-2002	Udder skin
	Staphylococcus epidermidis	DSM 20044^T	
VI		51a-2002	Teat apex
		02/684	Mastitis milk
		02/692	Mastitis milk
		7b-1999	Teat apex
		171a-1999	Perineum
		140b-1999	Udder skin
		149be-2002	Hand
VII		02/700	Mastitis milk
		02/701	Mastitis milk
		82a-2002	Udder skin
		133a-2002	Perineum
		02/699	Mastitis milk
	Staphylococcus chromogenes	DSM 20454^T	

Scale: 40 50 60 70 80 90 100%

Figure 2. A sample of isolates in CNS ribotype clusters containing isolates originating from both bovine mastitis and skin (study IV).

Table 3. The distribution of CNS strains, typed by PFGE, from CNS species identified with ribotyping in samples collected in 1999 and 2002 from the perineum (P) and udder skin (U), teat apex (A) and streak canal (C) of lactating cows and hands of staff (H) of the research dairy herd of the University of Helsinki, and in mastitic milk samples collected from the same herd in 1998-2002.

Species	strain	H	P	U	A	C	Mastitis
S. chromogenes	A					1	1
	B		1	1			
	C	1					
	D			3	1	1	6
	E		2	2	2	1	10
	F						1
	G			1			3
	H						2
	I			1			4
	J						1
S. epidermidis	A				1		
	B			1			1
	C						1
S. simulans	A				1		
	B					1	12
	C			1			1
	D						1
	E						4
S. succinus subsp. *succinus*	A			2	1	1	
	B				1		
	C				1		
	D	1					
	E				1		
	F				1		
	G				1		
	H				1		
	I				1		
	J				1		
	K						1
S. sp CL 3	A			1			
	B		1	2			
	C		1				
	D			1			
	E			1			
	F			1			
	G						1
	H				1		
S. sp CL 7	A		1				1
S. sp CL 9	A				1		3

3. Agreement of CNS identification of phenotypic and genotypic methods (I, III)

For some isolates the species identification result with >90% probability of the API Staph ID 32 test differed from the genotypic identification result. The agreement of API Staph ID 32 test and AFLP identification results were 75% (study I) and 71% (study III). The agreement of the API Staph ID 32 test and ribotyping results for mastitis isolates were 66% and for extramammary isolates 47%.

The contradictory identification results from the API Staph ID 32 tests and AFLP analyses in study III are shown in Table 4.

Table 4. Agreement between coagulase-negative staphylococci (CNS) species identification results from API Staph ID 32 and AFLP analyses for isolates in study III. The criteria for species identification were probability of the API test result >90% and similarity level of the AFLP pattern with the AFLP pattern of CNS type strain >50%.

| CNS species identification in API | CNS species identification in AFLP | | | | | | | | | | Agreement % |
	S. capitis	*S. chromogenes*	*S. cohnii*	*S. epidermidis*	*S. equorum*	*S. haemolyticus*	*S. simulans*	*S. warneri*	*S. xylosus*	*S.* subsp.	
S. capitis										1	0
S. chromogenes	32									1	97
S. cohnii			1								100
S. epidermidis				3							100
S. equorum									1		0
S. haemolyticus						4					100
S. simulans							16			1	94
S. warneri						4		2		2	25
S. xylosus									1		100
S. subsp.		6	1	3		6				6	27
Agreement %	84	50	50			29	100	100	50	55	

4. Bacterial cure rates (I, II)

In study I, some (55) of the CNS intramammary infections diagnosed were not treated with antimicrobials, and spontaneous elimination rate of CNS infections can be determined. The spontaneous cure rate for CNS mastitis four weeks after the diagnosis was 45.5% in this study In quarters with subclinical mastitis, the spontaneous cure rate was 39.5% and in quarters with clinical mastitis it was somewhat higher at 58.8%. The difference between the groups was not statistically significant. No difference in spontaneous cure rate between mastitis caused by β-lactamase-negative or β-lactamase-positive isolates was recorded (47.4% and 41.2%, respectively, p=0.670).

In the treatment trial (study II), the bacteriological cure rate for 28 cases of clinical mastitis caused by β-lactamase-negative CNS and treated with intramammary therapy was 78.6%. In study I, the bacterial cure rate for clinical or subclinical CNS mastitis, caused by 69 β-lactamase-negative and 9 β-lactamase-positive isolates and treated with penicillin G or cloxacillin, was 85.9%. The cure rate using antimicrobial treatment was significantly higher than the spontaneous cure rate of 45.5% in the group with no antimicrobial therapy (p<0.01). The cure rates for mastitis caused by β-lactamase-negative or β-lactamase-positive isolates and treated with antimicrobials were 88.4% and 66.7%, respectively. This difference in cure rates was not significant (p=0.095), but could indicate a trend towards better cure rates for mastitis due to existence of penicillin-susceptible strains.

5. Persistence of CNS infection (III)

In study I, 45.5% of the CNS infections were eliminated spontaneously, but the rest, i.e. more than half of the infections, remained persistent. This finding was confirmed in study III, in which 46% of CNS intramammary infections (29 of 63) detected during lactation persisted. CNS infection persisted in 13.3% of quarters of the primiparous cows and 6.3% of quarters of multiparous cows. During a persistent infection the same CNS strain was isolated from the same quarter between 3 to 12 times (in 10 of 29 quarters ≥10 times, in 24 of 29 quarters ≥5 times). Most infections persisted from the detection of the infection to the end of lactation or culling of the cow.

6. Clinical signs of CNS mastitis (II)

Fifty-one percent of the 133 quarter cases were clinical (49% of the quarters of primiparous cows and 53% of the quarters in older cows). In the majority of cases mastitis was mild: in 72% of clinical cases the only clinical signs detected were changes in the milk appearance, such as clots and flakes. Elevated body temperature was not a common sign: seven out of 95 cows had a body temperature >39.5°C and four of these >39.9°C.

7. SCC during CNS infection (III)

In study III, quarter milk SCCs were monitored in all cows of one herd, once a month over lactation, and were related to infection status of the quarters. The mean and median of geometric means of SCC of quarters with no bacterial growth throughout lactation were 65 000 cells/ml (95% confidence interval (CI) 44 700 – 85 300 cells/ml) and 34 300 cells/ml (lower quartile 21 700, upper quartile 61 700 cells/ml), respectively. The mean and the median of geometric means of SCC of quarters with persistent CNS infection

during the infection were 657 600 cells/ml (95% CI 223 400 – 1 091 800 cells/ml) and 355 400 cells/ml (lower quartile 180 500, upper quartile 765 600 cells/ml), and for the whole lactation 317 500 cells/ml (95% CI 209 500 – 425 500 cells/ml) and 202 800 cells/ml (lower quartile 63 000, upper quartile 440 500 cells/ml), respectively. The mean and the median of SCC of quarters with transient CNS infection during the infection were 619 100 cells/ml (95% CI 0 – 1 267 300 cells/ml) and 133 500 cells/ml (lower quartile 59 300, upper quartile 315 400 cells/ml), respectively, and the mean and the median of geometric means for the whole lactation 292 200 cells/ml (95% CI 7600 – 576 700 cells/ml) and 50 400 cells/ml (lower quartile 26 600, upper quartile 162 200 cells/ml), respectively. The mean and the median of geometric means of SCC of quarters with CNS growth prior to calving, at calving, or both, but with no growth during lactation, were 72 700 cells/ml (95% CI 46 600 – 98 900 cells/ml) and 41 800 cells/ml (lower quartile 29 400, upper quartile 84 800 cells/ml), respectively.

8. The effect of CNS species on clinical characteristics or persistence of mastitis (II, III)

The two most common AFLP clusters or CNS species, identified with API, had no effect on the severity of clinical signs (p=0.383 and 0.287, respectively) or on the elimination of the infection (p=0.163 and 0.297, respectively). *S. simulans* was slightly more often (in 57%) associated with clinical mastitis than *S. chromogenes* (in 45%), but this difference was not statistically significant (II). The same CNS species and isolates with similar AFLP patterns were found in both persistent and transient infections (III).

9. Prevalence and incidence of CNS intramammary infection (III)

In the Viikki research herd, the prevalence of CNS infection in 328 quarters of 82 cows before parturition, at parturition, or both, was 17.4%. The prevalence was 37.5% in quarters of the cows in their first lactation and 5.8% in quarters of cows in later lactations. Forty-nine percent of the infections before parturition, at parturition, or both were detected later during lactation. The incidence of CNS mastitis of 328 quarters during lactation was 19.2%. The incidence was 25.0% for quarters in primiparous cows and 15.9% for quarters in multiparous cows.

10. Penicillin-susceptibility (I, III)

Nineteen percent of the CNS isolates from the initial milk samples from different dairy herds were β-lactamase-positive (I). The proportion of isolates that produced β-lactamase was slightly higher in CNS isolated from subclinical mastitis (23%) than in CNS isolated from clinical mastitis (16%) (p=0.318). In the Viikki research dairy herd, the proportion of β-lactamase-positive CNS strains isolated from quarters with persistent or transient infection was 30% (III).

DISCUSSION

1. CNS species in bovine mastitis (I, III, IV)

The CNS species most commonly isolated from mastitic milk were *S. chromogenes* and *S. simulans*. *S. chromogenes* was clearly concentrated in heifers prior to or at calving and during first lactations. These results agree with those of earlier studies in which *S. chromogenes* was the major CNS species in subclinical mastitis in heifers and in primiparous cows (Trinidad et al., 1990a; Rajala-Schulz et al., 2004). *S. chromogenes* has been isolated from the udders of unbred, pregnant, or freshly calved heifers (Trinidad et al., 1990a; Matthews et al., 1992; Nickerson et al., 1995; Aarestrup and Jensen, 1997; De Vliegher et al., 2003). In a Danish study conducted on 180 heifers on 20 herds, the most common CNS species in heifers, *S. chromogenes*, was isolated from 15% of quarters before calving (Aarestrup and Jensen, 1997). Other species commonly isolated around calving in that study were *S. epidermidis* and *S. simulans*, which were both isolated from 2% of quarters. The majority of CNS isolates in milk samples from lactating cows during a three year period in a research dairy herd in Kentucky, USA, were *S. chromogenes* (Matthews et al., 1991).

In studies in which CNS isolates originate from diagnosed clinical or subclinical mastitis from the field, the predominant CNS species is usually *S. simulans* (Jarp, 1991; Waage et al., 1999). *S. simulans* was also the predominant CNS species in our field material, especially in multiparous cows, but was also common in cows during first lactation. In the study of Jarp (1991), 45% of 148 isolates from milk samples collected by veterinary surgeons from clinical (45%) and subclinical (55%) CNS mastitis were *S. simulans*. In clinical mastitis in heifers around parturition and at early lactation in Norway, *S. simulans* (54%) was the most prevalent CNS species, followed by *S. chromogenes* (15%) and *S. hyicus* (15%) (Waage et al., 1999). In an earlier Finnish study, Myllys (1995) reported that *S. simulans* and *S. hyicus* were the most frequently isolated CNS species in heifers around parturition, and were especially associated with clinical mastitis after parturition. The predominant CNS species of 298 isolates in milk samples from subclinical mastitis in Germany were *S. chromogenes* (33%) and *S. simulans* (23%) (Lüthje and Schwarz, 2006). Rather et al. (1986) isolated *S. simulans* from 42% of milk samples from two herds during a six month study period. They reported that *S. simulans*, similarly to *S. aureus*, but in contrast to other staphylococci, was often present in pure culture with heavy growth, indicating the ability of this species to cause mastitis. *S. chromogenes* was not identified in that study, which could reflect the problems in identification of CNS species.

In addition to the predominant species, various other CNS species are less frequently isolated from milk samples. *S. hyicus, S. epidermidis* and *S. haemolyticus* were detected in some samples (Devriese and De Keyser, 1980; Jarp, 1991; Aarestrup et al., 1995; Chaffer et al., 1999; Rajala-Schultz et al., 2004; Lüthje and Schwarz, 2006), but *S. warneri, S. sciuri* and *S. xylosus,* and several other CNS species usually only occur seldom (Rather et al., 1986; Aarestrup et al., 1995; Waage et al., 1999; Lüthje and Schwarz, 2006). Obviously the predominant CNS species are well adapted to the udder environment, but also many other CNS species are occasionally and opportunistically able to invade the mammary gland and cause mastitis.

It is somewhat surprising that the proportion of *S. hyicus* isolated from mastitis in our studies is very low. *S. hyicus* was isolated only in study I, and according to results from the API test and AFLP, the number of isolates was 11 and two, respectively. *S. hyicus* was reported to be a frequent isolate in CNS mastitis in several studies (Jarp, 1991; Waage et al., 1999; Rajala-Schultz et al., 2004). In the previous studies in Finland, in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, University of Finland, *S. hyicus* was reported to be one of the predominant CNS species in mastitis (Honkanen-Buzalski et al., 1994; Myllys, 1995). It is possible that the phenotypic identification methods easily classified some other CNS as *S. hyicus*. Of the 11 isolates identified as *S. hyicus* by API in study I, three were reassigned to *S. chromogenes,* one to *S. simulans,* and five to an unidentified cluster in AFLP analysis (Figure 1). In study IV, four isolates forming the ribotype cluster II (Figure 2) were identified as *S. hyicus* in the API test with 99.9% probability. It is very interesting to see in future studies if these hyicus-like isolates from different materials genotyped using different methods are genetically similar.

2. CNS species on extramammary sites (IV)

A wide variety of CNS species have been isolated from cows' skin, teat canal, and other extramammary sites (Devriese and De Keyser, 1980; Boddie et al., 1987; White et al., 1989; Trinidad et al., 1990a). The predominant CNS species in cows' beddings and environment were reported to be *S. xylosus*, *S. sciuri*, and *S. saprophyticus* (Matos et al., 1991). The same species are frequently isolated from cows' haircoat, nares, teat skin, and other sites (Devriese and De Keyser, 1980; Boddie et al., 1987; White et al., 1989). Many other species, including *S. chromogenes*, *S. warneri*, and *S. epidermidis*, are also isolated from cows' skin (Devriese and De Keyser, 1980; Boddie et al., 1987; White et al., 1989). Although the same CNS species are frequently isolated from teat skin, teat apex, and teat canal (Devriese and De Keyser, 1980; White et al., 1989; Trinidad et al., 1990a), the CNS species isolated from milk are mainly different. Devriese and De Keyser (1980) found that the distribution of CNS species in milk samples and on teat skin of dairy cows differed: *S xylosus, S. sciuri* and *S. haemolyticus* predominated in the samples obtained from teat skin and apex. Three other species, *S. epidermidis, S. chromogenes, S. simulans* and a group of unidentified staphylococci called group M were frequently isolated from milk samples. The same was shown by Trinidad et al. (1990a). In that study, seven different species of staphylococci were isolated from teat canals, but not from mammary gland secretion samples.

The predominant species in milk and extramammary samples are mainly different, but *S. chromogenes* is an exception. It seems to be adapted both to conditions on the skin and in the udder. We isolated *S. chromogenes* from several extramammary samples, and five out of ten pulsotypes were found both from mastitis and extramammary isolates. Our results agree with those of earlier studies. *S. chromogenes* was isolated from the skin of the teat apex, streak canals and mammary glands of unbred heifers as early as at 10 months of age (Boddie et al., 1987; De Vliegher et al., 2003). *S. chromogenes* was also found to frequently colonize other body sites like nares, hair coat, vagina and streak canal of heifers (White et al., 1989). Recovery of *S. chromogenes* from the teat apex and other body sites increases with increasing age of the heifers (White et al., 1989; De Vliegher et al., 2003). In the study of De Vliegher et al. (2003), 20% of heifers had at least one teat apex

colonized by *S. chromogenes*. This was however not associated with intramammary infection with the same agent.

In contrast to the frequent isolation of *S. chromogenes* outside the mammary gland, *S. simulans* is seldom isolated from extramammary sites. Few *S. simulans* isolations from extramammary samples, mainly from teat canals, have been reported (Devriese and De Keyser, 1980; Boddie et al., 1987; Trinidad et al., 1990a). These findings are in accordance with ours. *S. simulans* was isolated from extramammary sites only three times, once from udder skin, once from the teat apex, and once from the streak canal, which may be an indication of *S. simulans* being a specific mastitis pathogen. One of the five *S. simulans* pulsotypes predominated, and was also isolated from one teat canal. It is, however, possible that *S. simulans* from the udder contaminated the teat canal or the skin, rather than vice versa.

Coagulase-negative staphylococci are commonly considered to be teat skin opportunists that normally reside on the teat skin and cause mastitis via ascending infection through the streak canal (Radostits et al., 2007). Our results indicate that not all CNS species fit well with this definition. We found that CNS species other than the dominant mastitis species, *S. chromogenes* and *S. simulans,* were more numerous on the skin of the cows, but were uncommon or totally absent as causes of mastitis. CNS strains from milk and extramammary sites have, to our knowledge, previously been compared only by Thorberg et al. (2006). In that study, the same *S. epidermidis* strains were found both in bovine intramammary infections and the milkers' hands in two herds with a high incidence of CNS mastitis, indicating that *S. epidermidis* intramammary infections can originate from humans. Of the three *S. epidermidis* pulsotypes detected in our study, one was isolated both from mastitis and udder skin. The only CNS species isolated both from mastitis and from milkers' hands were *S. chromogenes* and *S. succinus* subsp. *succinus.* The isolates from the hands were, however, of different pulsotypes than the mastitis isolates.

3. Agreement of CNS identification using phenotypic and genotypic methods (I, III)

The agreement of CNS identification results using phenotypic and genotypic methods varied from 47% to 75%. In general, the agreement between the test results was better for the mastitis isolates (66% to 75%) than for the skin isolates (47%). API test identification results agreed with identification results of AFLP or ribotyping for the majority (>90%) of *S. epidermidis, S. gallinarum, S. saprophyticus, S. sciuri,* and *S. simulans* isolates. For *S. chromogenes* isolates, 68% to 80% of the identification results agreed. Phenotypic and genotypic identification results correlated better for *S. chromogenes* isolates from milk than for *S. chromogenes* isolates from skin. For *S. succinus, S. warneri* and *S. xylosus* isolates, the agreement between the API test and the genotypic methods was poor.

Genetic fingerprinting of CNS isolates and comparison of the fingerprint patterns with a library of fingerprint patterns of *Staphylococcus* type strains appeared to be a useful means to identify CNS species. With both fingerprinting methods used in this thesis, AFLP and ribotyping, the banding patterns formed clear clusters. Clusters including a type strain of a CNS species were considered to represent that species. Clusters without a type strain indicate possible existence of new CNS species. The similarity levels of clusters of AFLP patterns were >50%. In AFLP, Pearson correlation, based on curves instead of bands, is usually used in numerical analyses. This method is very sensitive to variation, which is

seen as the rather high variability percentage of the AFLP patterns of the internal control strain and the species-specific clusters. This has to be taken into account when the similarities of AFLP patterns of species and strains are evaluated. In the numerical analysis of ribotype patterns, a band-based Dice coefficient was used, and the similarity levels of clusters of ribotype patterns were >70%.

It seems that identification of CNS species with ribotyping or AFLP is more accurate than identification with the API Staph ID 32 test. Some genotypic clusters contained isolates that were identified with API as belonging to a certain species, but the type strain of that species was not included in that cluster. Some other genotypic clusters contained a type strain, but the isolates in those clusters had inconsistent API identification results. This indicates that the API test may give incorrect results for identification of species. This is not a surprise: the API test is designed for identification of pathogens of human origin, and the number of pathogens of veterinary origin in the reference databases of API and other commercial identification schemes is limited (Bes et al., 2000). However, phenotypic misidentification of CNS isolates originating from humans has also been reported (Couto et al., 2001; Heikens et al., 2005; Skow et al., 2005). Here the performance of the API test varied among CNS species: for some species the results of the API test correlated very well with the results of AFLP and ribotyping, but for some other CNS species results were inaccurate.

The conclusion from our results is that identification of CNS species is currently somewhat problematic. Most *Staphylococcus* species are defined based on phenotypic characteristics. The only commonly accepted standard for genotypic differentiation of two species is the DNA-DNA reassociation test (Wayne et al., 1987). Sequencing of the 16S rDNA gene has also been considered to be a reference method, but is not always be adequate for differentiating closely related species (Patel, 2001; Heikens et al., 2005). To date there are no gold standards for accurate CNS identification using genotypic tests. In the future, species identification of CNS, based on DNA-sequence of several bacterial house-keeping genes, may be the most accurate method because of existence of large reference databases, which can quickly be updated after identification of new species (Zadoks, 2007).

4. Bacteriological elimination rates for CNS intramammary infections (I, II)

The spontaneous elimination rate of CNS mastitis is generally regarded as high. Some studies have reported spontaneous elimination rates of about 60-70% (McDougall, 1998; Wilson et al., 1999). Markedly lower rates, 15%-44%, have also been reported (Rainard and Poutrel, 1982; Timms and Schultz, 1987; Deluyker et al., 2005). In Finland and in the other Nordic countries, the policy is to avoid unnecessary use of antimicrobials in animal husbandry (Anon., 2003). Subclinical and mild clinical mastitis caused by CNS is usually left untreated, the rationale being that CNS will be eliminated spontaneously. The spontaneous cure rate in our study (<50%) is lower than generally expected. Although we cannot exclude the possibility of elimination and reinfection with the same species, we assume that the CNS infections remained persistent in the udder.

The bacterial cure rates for CNS mastitis treated with antimicrobials were quite high in our study. In quarters with mastitis caused by β-lactamase-negative CNS, infection was eliminated in 80% to 90% of the quarters, but the elimination rate was somewhat lower,

less than 70%, in quarters with mastitis caused by β-lactamase-positive CNS. In the study of Pyörälä and Pyörälä (1998), the elimination rates for intramammary infections due to penicillin-resistant CNS were also lower (58%) than for penicillin-susceptible CNS (79%). The phenomenon of cure rates for mastitis caused by penicillin-resistant isolates, even when the isolate is sensitive to the antimicrobial used *in vitro*, is known for *S. aureus* mastitis (Sol et al., 2000; Taponen et al., 2003). The bacteriological cure rates reported from studies in which CNS mastitis was treated with penicillin G, were 70-90% (McDougall, 1998; Pyörälä and Pyörälä, 1998; Waage et al., 2000; McDougall et al., 2007). The elimination rate for 96 quarters infected with CNS and treated with cloxacillin was 87% (Rainard et al., 1990). Somewhat lower cure rates, 78% and 53% after pirlimycin treatment for two and eight days, respectively, were reported by Deluyker et al. (2005). Paradoxically, better results were achieved with the shorter treatment regimen. Antimicrobial susceptibility of isolates was not tested. The usual treatment time for CNS mastitis in Finland is from 3 to 5 days. Optimum duration of treatment for CNS mastitis is not known, but according to the study of Deluyker et al. (2005), extending treatment to 8 days does not improve cure rates of CNS mastitis, as compared with a 2-day treatment. In that study, the elimination rate of infection in the quarters with CNS mastitis and left untreated was 44%, which did not differ significantly from the treated groups. We did not study the effect of age on the elimination rates of CNS intramammary infection, but previous research showed that first-lactating cows have higher cure rates as compared with older cows (Pyörälä and Pyörälä, 1998). This was shown for *S. aureus* mastitis as well (Barkema et al., 2006).

5. Persistence of CNS infection (III)

We found that about half of the CNS infections persisted for long periods during lactation. Most of the quarters with persistent infections remained infected, from detection of the infection until the end of the lactation. Similar results were reported in a Norwegian study, in which all quarters of dairy cows from four herds were sampled monthly (Mørk et al., personal communication). In our study, based on the AFLP patterns, one clone was often isolated from the quarter before and after calving, but about half of the quarters became infected for the first time later during lactation. Multiparous cows in general became infected with CNS in later lactation, whereas primiparous cows were usually infected already at the beginning of lactation. Gröhn et al. (2004) also showed the same. In our study, infection was defined as persistent when CNS with identical AFLP patterns were isolated from a quarter at least three times, i.e. the infection had persisted for two months or longer. In 41% of the transient infections, isolates considered to represent the same clonal lineage were detected twice, the infection thus lasting at least one month.

Some earlier studies reported that CNS can cause chronic intramammary infection (Rainard et al., 1990), and at least some of the CNS infections are able to persist (Timms and Schultz, 1987; Aarestrup and Jensen, 1997; Laevens et al., 1997a; Chaffer et al., 1999). Timms and Schultz (1987) found that only 15.5% of CNS infections were eliminated spontaneously, and many CNS infections had a long duration. In the study of Rainard et al. (1990), which was carried out in France in a research dairy herd of 45 cows, 76% of the 184 CNS infections during 1983 to 1986 persisted until cessation of milking, and the mean duration of infections was 236 days. Chaffer et al. (1999) followed 24 primiparous cows during lactation. After parturition, 27% of the quarters were infected with CNS, and infection remained persistent in 85% of the quarters. Preliminary results of

the ongoing study of Rajala-Schultz et al. (2007) indicate that CNS infections can persist over the dry period, even after antimicrobial dry cow treatment.

6. Clinical signs of CNS mastitis (II)

It is widely known that CNS mastitis is usually subclinical or mildly clinical. Clinical CNS mastitis is associated with mild local signs, such as slight swelling of the quarter, and changes in the milk appearance, such as clots or flakes in the milk. Systemic signs, including fever, are uncommon. In a Norwegian study, Jarp (1991) reported that CNS mastitis can produce severe local and systemic signs, but no details were provided in that article. In our study, half of the IMIs were clinical, but in the majority of cases, the clinical signs were mild. Elevated body temperature was detected only in 7% of cows with CNS mastitis. In the treatment trial of Pyörälä and Pyörälä (1998), the local inflammatory reaction in quarters with clinical CNS mastitis, determined as milk NAGase activity, was found to be significantly stronger in early lactating cows than in cows in later lactation.

Very few studies have reported on clinical signs of mastitis caused by CNS. One study (Bleul et al., 2006) reported on three cases of toxic mastitis caused by staphylococci other than *S. aureus*. In that study, no information about coagulase production of the isolates was given. In a pilot study, in which five lactating cows were experimentally infected with *S. chromogenes*, the concentrations of different inflammation parameters in milk were 10 to 100 times as high as recorded for experimentally induced *E. coli* mastitis, and the clinical signs were very mild (Simojoki et al., 2007).

7. Milk SCC during CNS infection (III)

Compared with infections caused by so-called major Gram-positive mastitis pathogens, such as *S. aureus* and streptococci, milk SCC in quarters infected with CNS is rather low. It is, however, about 10-fold higher than SCC of healthy quarters, which typically remains under 50 000 cells/ml (Nickerson et al., 1995; Barkema et al., 1999). We found that in a healthy quarter, SCC remains between 10 000 and 40 000 cells/ml both in primiparous and multiparous cows, whereas in quarters infected with CNS, milk SCC is clearly elevated. Milk SCC in quarters with persistent CNS infection varied considerably from sampling to sampling. The geometric means of SCC of quarters with CNS infection also varied considerably. Even a transient CNS infection caused a temporary increase in milk SCC, which is consistent with the report of Laevens et al. (1997b). In our study, the mean and median of geometric means of SCC in quarters infected with CNS, 657 600 cells/ml and 355 400 cells/ml, respectively, agree with the results of Rainard et al. (1990). In that study, SCC was >500 000 cells/ml in 38% of milk samples taken from quarters infected with CNS, 200 000 to 500 000 cells/ml in 42% of the quarters, and <200 000 cells/ml in 20% of them. Compared with results from the study of Djabri et al. (2002), the mean SCC of quarters with CNS infection was somewhat higher in our study. In that meta-analysis the geometric mean SCC in CNS infected quarters was 138 000 cells/ml. Nickerson et al. (1995) reported that the mean SCC of quarters infected with *S. chromogenes* or *S. hyicus* during the three first months of lactation were 168 000 cells/ml and 193 000 cells/ml, respectively. In the study of Simojoki et al. (2007), the mean peak SCC after the experimental challenge with *S. chromogenes* was 2 000 000 cells/ml. It can be concluded that CNS infection induces an immunological reaction in the udder and should not be considered merely teat canal colonization or "normal" for the bovine udder. However,

SCC levels discussed here represent SCC in the milk in the infected quarter, which is diluted with milk from other quarters during milking. The cow composite SCC thus remains at a considerably lower level. In the literature, 200 000 cells/ml has been suggested to be a practically feasible threshold for milk SCC from healthy vs. infected cows under field conditions (Schukken et al., 2003). SCC of composite milk from cows with one quarter subclinically infected with CNS can often fall below this point. On the other hand, if the number of infected cows is high in the herd, bulk milk SCC can be more affected and it may not be possible to reach the premium level of SCC.

8. The effect of CNS species on clinical characteristics and persistence of mastitis (II, III)

It is commonly assumed that differences between CNS species can exist in clinical characteristics and persistence of the intramammary infection. The fact that *S. chromogenes* is especially associated with heifers around parturition, whereas *S. simulans* predominates in samples originating from diagnosed mastitis cases, clearly supports this assumption. However, to date, only few studies have been able to detect any significant differences in clinical characteristics of mastitis caused by different CNS species. In our study, the proportion of *S. simulans* causing clinical mastitis was slightly higher than that of *S. chromogenes*, but *S. chromogenes* was associated with moderate clinical signs slightly more often than *S. simulans.* These differences, in severity of mastitis caused by the two most common CNS species or AFLP clusters, were not significant however. Jarp (1991) also found that *S. simulans* more often caused clinical mastitis than other CNS species. In the present study, local signs of acute inflammation in quarters with clinical CNS mastitis were most often associated with infections caused by *S. simulans, S. haemolyticus* and *S. xylosus.* These differences were, again, not significant. Waage et al. (1999) reported that a significantly (p<0.05) higher proportion of *S. hyicus* was isolated from cases with systemic signs than without systemic signs in clinical CNS mastitis in heifers. *S. hyicus* was found to be the most pathogenic CNS species also by Myllys (1995). It was most often isolated from quarters with clinical CNS mastitis, and milk NAGase activity in *S. hyicus* infected quarters was significantly higher than in quarters infected with other CNS species. *S. simulans* was also frequently isolated from clinical mastitis in that study.

Differences in the capacity to cause persistent infection in the udder among CNS species may exist: Aarestrup and Jensen (1997) monitored quarters of heifers from 4 weeks prepartum until 4 weeks after calving. They showed that infections caused by *S. chromogenes* disappeared shortly after parturition, and infections caused by *S. epidermidis* were transient. In contrast, infections caused by *S. simulans* were found to persist for longer. The persistence of the same *S. simulans* clone was later confirmed by ribotyping (Aarestrup et al., 1999). We did not detect such a clear difference in the persistence between the two dominant species, *S. chromogenes* and *S. simulans.* In our study, in which cows were followed-up over the whole lactation, *S. chromogenes* infections persisted like other CNS infections. Of the 22 *S. chromogenes* infections during lactation, 12 (54.5%) caused a persistent infection. Among 9 *S. simulans* infections, 6 (66.7%) persisted. *S. epidermidis* also caused four persistent infections. However, the limited number of infected quarters both in Aarestrup's study (Aarestrup et al., 1999) and ours does not allow for a valid comparison between species.

9. Prevalence and incidence of CNS intramammary infections (III)

The highest prevalence of teat skin colonization, as well as intramammary infection, with CNS was detected in heifers at parturition (White et al., 1989; Fox et al., 1995; De Vliegher et al., 2003). In primigravid heifers, CNS accounted for a large number of bacteria isolated from mastitis around parturition (Oliver and Sordillo, 1988). In intensive production, the prevalence of quarters of heifers infected with CNS precalving may exceed 50% (Trinidad et al., 1990a; Oliveira et al., 2006). The rapid development of the mammary gland during that time may be one predisposing factor (Fox et al., 1995). The time period from two weeks prior to calving to about two to three weeks after calving has been considered as the most critical period for the health of the mammary gland (Oliver and Sordillo, 1988). The innate immune system of periparturient cows is compromised (Sordillo, 2005). During colostrogenesis the transition period towards lactation, the susceptibility of the mammary gland to infections increases also because the teat canal starts to open and may leak mammary secretion. Hormonal changes, such as increase of cortisol and estrogen concentrations, occur around parturition and may affect the immune mechanisms of the cow (Sordillo, 2005). In multiparous cows, severe mastitis caused by coliforms is the main problem after calving, whereas in heifers, CNS mastitis is an increasing burden. CNS mastitis is not clinically severe, but very prevalent and can persist for longer periods in the udder.

The quarter prevalence of CNS infections around calving found in the Viikki research dairy herd of the University of Helsinki was 37.5% in quarters in cows in the first lactation and 5.8% in quarters of cows in later lactations. This is similar to the findings of Rajala-Schultz et al. (2004) in one research herd in Ohio, USA. In that study, the prevalence of CNS intramammary infection in 692 quarters within three days after calving was 36% in quarters in first lactation and 16% in quarters in later lactations. Higher prevalence was reported by Trinidad et al. (1990a). In their study, 97% of heifers and 75% of quarters of heifers in four dairy herds in Louisiana, USA, were infected before calving, and about 70% of those infections were caused by CNS. The quarter prevalence of CNS infections varied among herds from 42% to 69%. Lower prevalence was reported by Aarestrup and Jensen (1997), who found that 19% of quarters of 180 heifers in Denmark were infected with CNS at around parturition. Oliver and Mitchell (1983) reported that 16% of quarters of 32 heifers near parturition in the research dairy herd of the University of Massachusetts, USA, were infected with CNS. The prevalence was low also in Vermont, USA, where 11% of heifer quarters were infected with CNS within three days postpartum (Pankey et al., 1991). In the study by Davidson et al. (1992), the quarter prevalence of CNS infections around calving in seven dairy herds in Canada ranged from 10% to 20%.

The prevalence of CNS infections has been reported to decrease markedly after parturition. In the study of Matthews et al. (1992), the quarter prevalence of CNS infection at calving was 28% in primiparous and 12% in multiparous cows. Five weeks after parturition the prevalence was 15% in quarters of primiparous and 11% in multiparous cows. The quarter prevalence of CNS infection in heifers in the study of Oliver and Mitchell (1983) decreased from 22% before parturition and 19% at parturition to 8% at early lactation. Aarestrup and Jensen (1997) reported the quarter prevalence of *S. chromogenes* infections in heifers to decrease from 15% before parturition to 1% shortly after parturition, whereas 2% of quarters were consistently infected with *S. simulans* before and after parturition. In a recent survey from Estonia, 16% of the quarters of cows

of different ages positive for bacterial growth harbored CNS (Haltia et al., 2006). In the pasture-based grazing system in New Zealand, a prevalence of 16% for quarters of heifers infected with CNS was reported (Parker et al., 2007). In our study in the Viikki research dairy herd, the quarter incidence of persistent CNS infections was 8.9%, and the prevalence of CNS mastitis during lactation in the herd can be predicted to be about 9%. This result is well in line with results from other studies, although prevalence of CNS mastitis varies among herds.

10. Penicillin-susceptibility (II, III)

The most common mechanism of resistance in staphylococci is β-lactamase production, which results in resistance to penicillin G and aminopenicillins (Pitkälä et al., 2004; Erskine et al., 2006; Østerås et al., 2006; Olsen et al., 2006). The proportion of penicillin-resistant CNS causing bovine mastitis varies in different countries. The reported percentage of penicillin resistance for CNS isolated from milk samples was 32% in Finland (Pitkälä et al., 2004), 36% in Norway (NORM-VET, 2005; Østerås et al., 2006), 25% in Denmark (DANMAP, 2001) and 28% in the Netherlands (MARAN, 2003). The proportion of penicillin-resistant CNS isolates varies also depending on the origin of the isolates: penicillin-resistance seems to be more common in isolates originating from survey-type prevalence studies with randomly sampled cows and where mastitis is mainly subclinical, than in isolates originating from clinical mastitis. In the Finnish prevalence study, 32% of the CNS isolates were penicillin-resistant (Pitkälä et al., 2004), but in a study on clinical CNS mastitis in the practice area of the Ambulatory Clinic of the Faculty of Veterinary Medicine, only 13% of the isolates were resistant to penicillin (Nevala et al., 2004). Our results in the first study (I) of this thesis, which was carried out in the same practice area, were consistent with that: the proportion of penicillin-resistance among isolates from subclinical mastitis was 23%, but among isolates from clinical mastitis only 16%. Consequently, CNS from subclinical mastitis tend to be more often penicillin-resistant than CNS causing clinical mastitis. The same was also reported by Jarp (1991): 16% of isolates from subclinical, but only 7% of isolates from clinical mastitis, were resistant to penicillin. The proportion of penicillin-resistant isolates from quarters with persistent or transient, mainly subclinical, infections in the Viikki research dairy herd was 30% (III). This figure, although obtained from only one herd, is very comparable with the result from the study of Pitkälä et al. (2004). The possible reasons for the apparent higher resistance of the bacteria isolated from subclinical mastitis are unknown. It can be speculated that strains causing subclinical mastitis represent a population that is able to cause chronic infections and may thus possess some virulence factors that favor that development. In a study on *S. aureus* (Haveri et al., 2007), production of β-lactamase was linked to some other virulence factors and to persistence of intramammary infection. Rajala-Schultz et al. (2004) reported that 26% of CNS isolated from primiparous cows in one dairy herd were penicillin-resistant, but the figure was 39% for CNS isolated from multiparous cows. They suggested that the higher proportion of beta-lactamase producing isolates among older cows could be due to greater use of antimicrobial agents in the older cows. In that study, blanket dry cow therapy at drying-off was used, which may also increase the selection pressure for antimicrobial resistance.

CONCLUSIONS

- The predominant species causing bovine mastitis are *S. chromogenes* and *S. simulans*. *S. chromogenes* predominates in heifers around calving and in the first lactation, whereas *S. simulans* predominates in the subsequent lactations. No statistically significant differences between *S. chromogenes* and *S. simulans* in clinical characteristics or persistence of intramammary infections were found, although *S. simulans* tended to cause clinical mastitis slightly more often than *S. chromogenes*.

- Other CNS species than the most common ones causing mastitis predominate on the bovine skin. *S. succinus* and *S. xylosus* were found to predominate in samples from cows' skin. Other species isolated in several sampling sites were *S. chromogenes* and *S. saprophyticus*. Same strains of *S. chromogenes* were detected both from milk samples and from cows' skin. Only three isolates of *S. simulans* were found from samples of cows' skin, indicating that *S. simulans* is a more specific mastitis pathogen and adapted to udder conditions. In contrast, *S. chromogenes* is able to live on bovine skin, but can also infect the mammary gland and cause mastitis.

- Two identification methods based on genotyping of the bacteria, AFLP and ribotyping, were compared with the phenotypic API Staph ID 32 test for identification of CNS species. AFLP and ribotyping appeared to be more accurate than the API test. The agreement of identification results between genotypic and phenotypic tests varied from 47% to 75%. The agreement between the identification results was better for the isolates from milk than for the isolates from skin.

- CNS mastitis responded well to antimicrobial treatment. The cure rates for mastitis caused by β-lactamase negative isolates were 88% (study I) and 79% (study II). The elimination rate for β-lactamase positive isolates was somewhat lower. The spontaneous elimination rate for intramammary CNS infections without antimicrobial treatment was 46%. When all quarters of the cows in one herd were sampled during entire lactation, 46% of the intramammary infections caused by CNS persisted. Most often the infections persisted in the quarter until the end of lactation.

- CNS mastitis usually remains subclinical or mildly clinical. About half of the infections caused clinical signs, but they were mostly mild and only 7% of the cows with CNS mastitis showed systemic signs.

- Intramammary infection due to CNS causes a moderate increase in the milk SCC of the affected quarter. The mean milk SCC in the quarters with persistent CNS infection was 658 000 cells/ml, which is considerably higher than the mean SCC in the quarters without bacterial growth, 65 000 cells/ml.

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