Diagnostic and epidemiological studies of staphylococci in bovine mastitis

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Abstract

Mastitis is the most common disease in dairy cows and is often caused by staphylococcal infections. The genus *Staphylococcus* is divided in coagulase-negative (CNS) and coagulase-positive (CPS) staphylococci. The CPS *S. aureus* is the most prevalent udder pathogen in Swedish dairy cows. For successful mastitis control accurate diagnostics and good understanding of the bacterial epidemiology is essential. This thesis describes methods for species differentiation within CNS and CPS, examines genotypic diversity of *S. aureus* isolates within Sweden, and identifies potential sources of *S. aureus* in herds with mastitis problems. First, three phenotypic tests (P-agar with acriflavin, β-galactosidase, and haemolytic reaction in chocolate agar) of eight biochemical tests evaluated were found useful for differentiation between the CPS species, *S. aureus*, *S. hyicus* and *S. intermedius*. The proportions of each species among bovine milk isolates were 97%, 1%, and 2%, respectively. Then, species identification of CNS using the Staph-Zym™ test was compared with sequencing of part of the *tuf* gene. Staph-Zym™ correctly identified 61%, but gave an incorrect species name in 28% of the milk isolates. Supplementary tests were frequently needed when using Staph-Zym™. In the next study, *S. aureus* isolates from a national survey on acute clinical mastitis were genotyped using pulsed-field gel electrophoresis (PFGE), and 25 pulsotypes (PTs) were identified. Three of the PTss accounted for over 50% of the isolates and were found all over the country. The distribution of PTs was different in the southern region than in the northern and middle regions of the country. Finally, *S. aureus* PTs in quarter milk samples, body samples (BS), and environment samples (ES) from various animal groups were studied in five herds with *S. aureus* mastitis problems. Herd differences were found, but all herds had one predominant unique milk PTs. In three farms this PTs was often found in BS and ES of lactating cows, and occasionally in samples from the other groups.

Keywords: Bovine mastitis, dairy cows, clinical mastitis, *Staphylococcus aureus*, CNS, CPS, phenotypic, genotypic, Staph-Zym™, *tuf* gene sequencing, PFGE

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Dedication

To my son Paolo and my wife Cecilia

Människor borde alltid bete sig mot varandra som om deras möte vore det sista.
Vilhelm Moberg

Contents

List of Publications

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

I Capurro, A., Concha, C., Nilsson, L., & Östensson, K. (1999). Identification of coagulase-positive staphylococci isolated from bovine milk. *Acta Veterinaria Scandinavica* 40: 315-321.

II Capurro, A., Artursson, K., Persson Waller, K., Bengtsson, B., Ericsson Unnerstad, H., & Aspán, A. (2008). Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. *Veterinary Microbiology* (In press).

III Capurro, A., Aspán, A., Artursson, K., & Persson Waller, K. Genotypic variation among *Staphylococcus aureus* isolated from clinical mastitis in Swedish dairy cows. (Submitted for publication).

IV Capurro, A., Aspán, A., Ericsson Unnerstad, H., Persson Waller, K., & Artursson, K. Identification of potential sources of *Staphylococcus aureus* in dairy herds with mastitis problems. (Manuscript).

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Abbreviations

AFLP	Amplified fragment length polymorphism fingerprinting
BS	Body samples
BT	Binary-typing
CAMP-like	Synergistic hemolysis test
CFU	Colony-forming unit
CNS	Coagulase negative staphylococci
CPS	Coagulase positive staphylococci
ES	Environment samples
MBP	Modified Baird-Parker
MLEE	Multi-locus enzyme electrophoresis
MLST	Multilocus sequence typing
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PTs	Pulsotype(s)
S.	*Staphylococcus*
Sa+	Presence of *S. aureus*
SCC	Somatic cell count
VJ	Vogel-Johnson

1 Introduction

Mastitis is one of the most prevalent and most costly production diseases affecting the dairy cattle industry worldwide (Seegers *et al.*, 2003).

Most cases of bovine mastitis are of bacterial origin, and bacteria of the genus *Staphylococcus* are among the most prevalent agents causing mastitis in many parts of the world. Historically, the genus *Staphylococcus* is divided by the coagulase test into coagulase-negative (CNS) and coagulase-positive (CPS) species. The understanding of CNS mastitis is complicated by the heterogeneity of this group of bacteria as it contains a large number of different species. So far, 16 CNS species or subspecies have been diagnosed in bovine mastitis. In general, CNS is normally not identified at the species level in routine diagnostics, and has often been considered to be of minor importance as an udder pathogen. However, recent studies on mastitis prevalence have revealed that CNS may be of major importance in some countries (Pyorala & Taponen, 2008). Moreover, CNS species have also been shown to vary in their abilities to cause different types of mastitis (Thorberg, 2008; Taponen *et al.*, 2006). Thus, species-specific knowledge of the impact and epidemiology of CNS intra-mammary infections is needed. Among CPS isolated from bovine mastitis are *Staphylococcus* (*S.*) *aureus, S. intermedius* and *S. hyicus*. However, in routine diagnostics all CPS strains are diagnosed as *S. aureus*. *S. aureus* is the most common udder pathogen in bovine mastitis in Sweden (Ericsson Unnerstad *et al.*, 2008; Anonymous, 2007) and is an important udder pathogen also in many other parts of the world (Olde Riekerink *et al.*, 2008; Gianneechini *et al.*, 2002; Waage *et al.*, 1999; Elbers *et al.*, 1998). Recent studies have revealed that many different *S. aureus* strains exist in bovine mastitis, and that those strains may vary in virulence and epidemiology (Fournier *et al.*, 2008; Haveri *et al.*, 2007; Dingwell *et al.*, 2006; Zecconi *et al.*, 2006b; Sommerhauser *et al.*, 2003; Zadoks *et al.*, 2000). These findings may result in the need to update current

mastitis control programs. Thus, further studies on the bacterial epidemiology of *S. aureus* are warranted.

In investigations on bacterial epidemiology of staphylococcal mastitis accurate bacteriological diagnostics of CNS and CPS, both at species and strain level, is essential. Therefore, diagnostic methods should constantly be reevaluated and up-dated.

1.1 Identification of the genus *Staphylococcus*

Members of the genus *Staphylococcus* have historically been classified and differentiated on the basis of a variety of phenotypic characteristics such as morphology, and biochemical reactions. Pigment was the first criterion used to identify staphylococcal species, and in 1885, Rosenbach identified members of the genus *Staphylococcus* based on the colour of colonies. Rosenbach called staphylococci forming orange-yellow colonies *S. aureus* (or *S. pyogenes aureus*), while staphylococci forming white colonies were named *S. albus* (or *S. pyogenes albus*) (Kloos, 1980). It was not until almost 45 years later (1930) that another characteristic was described for differentiation between staphylococci, the coagulase test investigating the ability of staphylococcal species to clot blood plasma (Kloos, 1980). This test resulted in the separation of staphylococci into two groups, the CPS (formally *S. aureus*) and CNS. Later on, during the 1960s, Baird-Parker conducted some of the most comprehensive studies of the genus *Staphylococcus*. He examined different phenotypic characteristics of a large number of isolates of staphylococci, and proposed a sub-division into six sub-groups I-VI (Baird-Parker, 1963). *S. aureus* was placed in sub-group I, and sub-groups II-VI contained different types of CNS. These sub-groups were later called the *S. epidermidis* group. However, during the following years other approaches were used to identify members of the genus *Staphylococcus*, which resulted in the discovery and naming of new species and subspecies of CPS, but mainly of CNS. Most of this work was performed during the 70s and 80s (Devriese *et al.*, 1985; Kloos *et al.*, 1976; Kloos & Schleifer, 1975a; Schleifer & Kloos, 1975). Based on the characteristics used in those studies, 37 species and 17 subspecies can today be identified in the genus *Staphylococcus* (Bannerman & Peacock, 2007).

1.1.1 Species identification of CNS

In humans and animals, CNS have mainly been considered to be saprophytic and only rarely pathogenic. Currently, 30 species and subspecies of CNS are listed (Bannerman & Peacock, 2007), and today some of those

species are recognized as potential pathogens to both humans and animals (Pyorala & Taponen, 2008; Layer *et al.*, 2006). The species that most frequently cause diseases in humans are *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus* (Cunha Mde *et al.*, 2004), while *S. simulans, S. chromogenes, S. hyicus* and *S. epidermidis* seem to be the most common in bovine mastitis (Luthje & Schwarz, 2006; Taponen *et al.*, 2006; Birgersson *et al.*, 1992). The identification of CNS is, however, complicated by the heterogeneity of this group of bacteria. Therefore, a large number of biochemical tests are needed for phenotypic differentiation between CNS species (Bannerman & Peacock, 2007; Martineau *et al.*, 2001; Kloos & Wolfshohl, 1982). Identification of CNS can, however, also be made using genotypic methods.

Phenotypic methods

Different procedures involving a varying number of biochemical tests have been proposed (Bannerman & Peacock, 2007; Cunha Mde *et al.*, 2004; Bannerman, 2003; Thorberg & Brändström, 2000; Devriese *et al.*, 1994; Devriese *et al.*, 1985; Kloos & Schleifer, 1975a) for identification of CNS isolates from humans and animals. These procedures are mostly not suitable for routine use as they are laborious due to the large number (3-36) of biochemical tests required. Moreover, several tests are rather time-consuming. In addition, some of the procedures were not able to identify some of the most important CNS (Bannerman & Peacock, 2007; Cunha Mde *et al.*, 2004; Thorberg & Brändström, 2000) For routine identification of CNS isolates, ease of testing and time needed for the tests are critical variables.

Trying to solve these problems different commercial identification systems were developed to identify members of the genus *Staphylococcus*, and in particular those of the CNS group. Around twenty different commercial systems are available (Bascomb & Manafi, 1998) and approximately six of them have been tested on CNS isolates from animals (Ruegg, 2008; Watts & Yancey, 1994). Most commercial identification systems are based on a limited number of biochemical reactions, and were mainly developed for identification of human isolates. The accuracy, i.e. the level of agreement between the commercial system and a given reference method, reported for the commercial systems was very variable ranging from 11% to 99% (Bascomb & Manafi, 1998; Watts & Yancey, 1994)). Commercial systems like Staph-Zym™ (Rosco, Taastrup, Denmark) and API Staph ID 32 (API test, bioMérieux, France) have been used for phenotypic identification of CNS in human diagnostic laboratories as well as in laboratories diagnosing bovine mastitis (Bascomb & Manafi, 1998; Watts & Yancey, 1994).

However, when comparing Staph-Zym™ and API Staph ID 32 for identification of bovine CNS isolates against conventional methods, the level of agreement varied markedly (Sampimon *et al.*, 2008; Thorberg & Brändström, 2000). In addition, Thorberg & Brändström, (2000) found that additional tests were frequently needed when using Staph-Zym™, which adds extra labour time and costs. One important general problem with methods based on phenotypic characteristics is the fact that expression of metabolic activities and/or morphological features may be variable within each species (Layer *et al.*, 2006).

Genotypic methods

Genotypic methods use DNA as the basis for CNS identification. In general, such methods have higher discriminatory power, reproducibility and typeability than conventional phenotypic methods (Zadoks & Watts, 2008; Stepan *et al.*, 2004). A number of PCR amplicon sequencing-based methods for identification of CNS have been reported, e.g. targeting the 16S rRNA, *sodA*, *tuf*, *rpo*B, *dna*J, *cpn*60 (also called *hsp*60 or *gro*EL) or the *gap* genes (Mellmann *et al.*, 2006; Fontana *et al.*, 2005; Becker *et al.*, 2004; Kwok & Chow, 2003; Drancourt & Raoult, 2002; Martineau *et al.*, 2001; Yugueros *et al.*, 2000). Among these, PCR sequencing of the *tuf* gene has been proposed as a reliable and valuable approach for identification of CNS (Ghebremedhin *et al.*, 2008; Heikens *et al.*, 2005; Martineau *et al.*, 2001). According to Martineau *et al.* (2001), this assay is suitable for differentiation between 27 CNS species.

Various other DNA-based methods such as 16S rRNA sequencing (Becker *et al.*, 2004), ribotyping (Svec *et al.*, 2004; De Buyser *et al.*, 1992), PCR-restriction fragment length polymorphism analysis (Becker *et al.*, 2004; De Buyser *et al.*, 1992), amplified fragment length polymorphism fingerprinting (AFLP) (Taponen *et al.*, 2007), internal transcribed spacer PCR (Fujita *et al.*, 2005), tRNA intergenic spacer length polymorphism analysis (Maes *et al.*, 1997) and whole-genome DNA-DNA hybridization analysis (Svec *et al.*, 2004) have also been described for species identification of the genus *Staphylococcus*. None of these methods is today considered suitable for routine use due to problems with differentiation between some staphylococci, and interpretation of results, or due to being too time-consuming and expensive.

1.1.2 Species identification of CPS

In the latest edition of the Manual of Clinical Microbiology six species of CPS other than *S. aureus* are listed, e.g. *S. delphini, S. intermedius, S. lutrae*, *S pseudointermedius, S. schleiferi* subs. *coagulans*, and the coagulase variable *S. hyicus* (Bannerman & Peacock, 2007). *S. aureus*, being pathogenic to both humans and animals, is considered the most important of the CPS species whereas *S. hyicus* is less diagnosed and *S. intermedius* are frequently diagnosed in hund (Griffeth *et al.*, 2008; Saadatian-Elahi *et al.*, 2008; Calzolari *et al.*, 1995). In bovine mastitis, *S. aureus* is also considered to be the most relevant CPS, as *S. hyicus* and *S. intermedius* have only rarely been identified (Rampone *et al.*, 1993; Roberson *et al.*, 1992). However, few studies have investigated the prevalence of those CPS in mastitis, and today all bovine CPS diagnosed in routine laboratories are considered as *S. aureus* (Hogan *et al.*, 1999). When species identification of CPS is attempted, phenotypic and genotypic methods have been used.

Phenotypic methods

Colony morphology on blood agar, pigmentation and hemolysis are not sufficient for species identification of CPS (Bannerman & Peacock, 2007; Hogan *et al.*, 1999; Roberson *et al.*, 1992). There are, however, a few tests that have been used with some success for differentiation between *S. aureus*, *S. hyicus* and *S. intermedius*, e.g. P-agar supplemented with acriflavin, anaerobic fermentation of mannitol, production of acetoin from glucose, and expression of β-galactosidase (Roberson *et al.*, 1992; Raus & Love, 1983; Devriese, 1981). Recently, however, conventional tests failed to distinguish between *S. aureus*, *S. intermedius* and *S. pseudintermedius* (Sasaki *et al.*, 2007b).

Several commercial identification systems have been used to improve the identification accuracy of CPS (Bascomb & Manafi, 1998; Watts & Yancey, 1994; Lammler, 1989). However, the differentiation accuracy varied markedly (38%-91%) both when human and animal isolates were tested (Bascomb & Manafi, 1998; Watts & Yancey, 1994; Watts & Washburn, 1991; Watts & Nickerson, 1986b; Watts *et al.*, 1986a).

Genotypic methods

In the last decade, several genes, e.g. 16SrRNA, 23SrRNA, *nuc*, *coa* and *tuf*, have been used for identification of *S. aureus* and other clinically important CPS using PCR-based methods. Among those methods, real-time PCR is increasingly adopted for diagnoses of CPS in humans due to its high sensitivity and specificity, and low time-consumption (Espy *et al.*, 2006;

Riffon *et al.*, 2001). For example, a PCR targeting the *nuc* gene is now becoming established in clinical microbiology laboratories for detection of *S. aureus* in blood cultures in humans (Hogg *et al.*, 2008). Similarly, Graber *et al.* (2007) developed a PCR test that allows specific detection of low concentrations of *S. aureus* in bovine milk samples. However, less encouraging results have been achieved when trying to identify CPS other than *S. aureus* (Becker *et al.*, 2005; Devriese *et al.*, 2005; Takahashi *et al.*, 1999). Other gene targets have also been proposed such as *sodA* and *hsp*60 (also called *cpn*60 or *gro*EL). They have been reported to be more divergent than 16S rRNA genes (Devriese *et al.*, 2005; Takahashi *et al.*, 1999) in staphylococcal identification, having sufficient discriminative power to differentiate between *S. delphini, S. intermedius*, and *S. pseudintermedius* (Sasaki *et al.*, 2007a). Nevertheless, as reviewed by Stephan *et al.* (2001) they are used to a lesser extent.

1.1.3 Sub-typing of *S. aureus*

It has been recognized that *S. aureus* contains a lot of different strains. Therefore, sub-typing of *S. aureus* isolates may be a necessary procedure when investigating outbreaks of disease, sources and routes of infection, and virulence factors. Over the last decade, various phenotypic and genotypic methods have been developed and explored for their efficacy in differentiation between *S. aureus* strains.

Phenotypic methods

Various phenotypic methods such as phage typing, and multi-locus enzyme electrophoresis (MLEE) have been used to differentiate between strains of *S. aureus*. Phage typing has several drawbacks, which confine its use to relatively few reference laboratories (Bannerman *et al.*, 1995). For example, when used in an outbreak situation, the main disadvantage was the high proportion of isolates which were non-typeable (Weller, 2000; Olive & Bean, 1999; Aarestrup *et al.*, 1995a). Even though MLEE patterns are relatively easy to read and interpret, the facts that strain comparison is difficult (application of sophisticated algorithms and computer software are needed), the technique is labour intensive, and that unrelated isolates were mistakenly included when epidemiologically linked *S. aureus* were evaluated have confined MLEE to the research laboratory (Tenover *et al.*, 1994; Mulligan & Arbeit, 1991).

Genotypic methods

Among genotypic methods that have been used to differentiate between *S. aureus* strains are for example pulsed-field gel electrophoresis (PFGE), ribotyping, binary-typing (BT), AFLP, and multilocus sequence typing (MLST).

PFGE is today considered to be the "gold standard", especially when typing bovine *S. aureus* (Stepan *et al.*, 2004; Olive & Bean, 1999) strains due to its excellent typeability, reproducibility (intra- and inter-laboratory), discriminatory power and easy interpretation (Zadoks *et al.*, 2002; Bannerman *et al.*, 1995). The costs involved are also moderate (Olive & Bean, 1999). The main disadvantage of PFGE is, however, that it is time consuming as it takes several days until the result is achieved.

Among other genotypic methods used, ribotyping exhibits excellent reproducibility and stability, but has lower discriminatory power than other techniques (Tenover *et al.*, 1994; Prevost *et al.*, 1992). As it is also time consuming and technically complicated, ribotyping has not been widely adopted for typing *S. aureus* (Weller, 2000). Binary-typing is a relatively new robust technique, with discriminatory power superior to that of PFGE, and will possibly become a powerful tool for strain characterization of bovine *S. aureus*, but is so far not widely used (Stepan *et al.*, 2004; Weller, 2000; Zadoks *et al.*, 2000). A disadvantage of the method is the time needed to complete the process (van Leeuwen *et al.*, 1999; van Leeuwen *et al.*, 1996). Amplified fragment length polymorphism fingerprinting is another method used which is easy to interpret, has high discriminatory power, good inter-laboratory reproducibility and results are obtained in two days (Olive & Bean, 1999). However, the costs are substantial, which may be prohibitive for many laboratories. Multilocus sequence typing provides a new approach to molecular epidemiology making it possible to track global spread of bacteria (Turner & Feil, 2007). The advantages of MLST are its high discriminatory power, and reproducibility, and that results can be compared between studies and between laboratories. However, it has the disadvantage of being expensive and highly work demanding as reviewed by Stepan *et al.* (2004).

1.2 Staphylococcal bovine mastitis

As already mentioned, staphylococci are probably the most important etiological agents in bovine mastitis, and may cause both clinical and sub-clinical mastitis. In studies from different parts of the world staphylococci have been isolated from 27% to 69% of the mastitic cases (Ericsson

Unnerstad *et al.*, 2008; Bradley *et al.*, 2007; Ferguson *et al.*, 2007; Osteras *et al.*, 2006; Reksen *et al.*, 2006; Pitkala *et al.*, 2004; Makovec & Ruegg, 2003; Gianneechini *et al.*, 2002; Edinger *et al.*, 1999; Myllys *et al.*, 1998; Wilson *et al.*, 1997). For example, in a Swedish nation wide study on the microbial aetiology of cases of acute clinical mastitis the genus staphylococci represented 27.5% of all bacteriological diagnoses (Ericsson Unnerstad *et al.*, 2008).

1.2.1 CNS mastitis

As already mentioned, CNS is normally not identified at the species level in routine diagnostics. It has often been considered to be of minor importance as an udder pathogen (Schukken *et al.*, 2008), and the epidemiology of CNS mastitis has therefore not been studied in detail. However, recent studies on mastitis prevalence have revealed that CNS may be of major importance in some countries (Pyorala & Taponen, 2008).

The prevalence of CNS mastitis varies between studies as reviewed by Taponen, (2008). In clinical mastitis, the proportions of CNS among bacterial diagnoses range from 3.4% to 19.5% (Ericsson Unnerstad *et al.*, 2008; Olde Riekerink *et al.*, 2008; Hoe & Ruegg, 2005; Schällibaum, 2001; Hogan *et al.*, 1984). In a recent Swedish study, CNS was isolated in 6.2% of all cases of clinical mastitis (Ericsson Unnerstad *et al.*, 2008). In sub-clinical mastitis, the proportions of CNS positive quarters ranged from 4% to 35% (Thorberg, 2008; Bradley *et al.*, 2007; Pol & Ruegg, 2007; Tenhagen *et al.*, 2006; Chaffer *et al.*, 1999; Wilson *et al.*, 1997; Aarestrup *et al.*, 1995b; Birgersson *et al.*, 1992). A national survey on etiological cases in sub-clinical mastitis has so far not been performed in Sweden, but it is estimated that CNS accounts for approximately 25% of bacteriological findings in these cases (Anonymous, 2007).

Large surveys on prevalence of different CNS species in clinical and sub-clinical mastitis are rare. Among studies made, *S. chromogenes*, *S. epidermidis, S. hyicus,* and *S. simulans*, seem to be the most common CNS isolated from intra-mammary infections in spite of some variation between herds, countries, and methods used (Taponen *et al.*, 2008; Luthje & Schwarz, 2006; Taponen *et al.*, 2006; Birgersson *et al.*, 1992).

Although some studies have been performed to identify the reservoirs of CNS involved in bovine mastitis, the epidemiology of this group of udder pathogens is not well known. Bovine CNS have traditionally been considered as skin flora opportunists (Hogan & Pankey, 1987). In line with this, a wide range of CNS species have been isolated from different body sites in cows, heifers and calves, but CNS have also been isolated from the

cows´ environment (Taponen *et al.*, 2008; Thorberg *et al.*, 2006; Matos *et al.*, 1991; Trinidad *et al.*, 1990; White *et al.*, 1989; Devriese & De Keyser, 1980). The identification of different CNS on body sites and in the environment is, however, based on the use of different methods making interpretation sometimes difficult. Using conventional or commercial biochemical methods, *S. chromogenes* was frequently isolated from the teat skin, and teat canal, but also from extra-mammary sites like nares, hair coat and vagina in heifers (De Vliegher *et al.*, 2003; White *et al.*, 1989). According to Matos *et al.* (1991), however, *S. cohnii*, *S. saprophyticus*, *S. sciuri,* and *S. xylosus*, were the most common in the cows' environment (e.g. alfalfa hay, straw and bedding). Moreover, Taponen *et al.* (2008) found that *S. equorum* and *S. sciuri* were the predominant CNS species in extra-mammary samples when using phenotypic methods, but when using ribotyping, *S. succinus* and *S. xylosus* were the predominant CNS species. Using PFGE, Taponen *et al.* (2008) found that *S. chromogenes* isolates identified from udder skin and in milk were of the same PT. Thorberg *et al.* (2006), also using PFGE, found the same *S. epidermidis* PT in samples from humans as in milk samples in the same herd. *S. simulans* was not a common finding in samples from teat canal or the bovine udder skin (Taponen *et al.*, 2008).

Results from different studies indicate that some CNS, such as *S. chromogenes* and *S. simulans*, are more prone to cause persistent infections than other species (Thorberg, 2008; Taponen *et al.*, 2007; Taponen *et al.*, 2006; Matthews *et al.*, 1992). Other studies also reported that *S. chromogenes* and *S. simulans* may cause more severe mastitis than other CNS (Zhang & Maddox, 2000; Birgersson *et al.*, 1991). Taponen *et al.* (2006) found no significant differences in the severity of the clinical signs or in the bacterial cure between *S. chromogenes* and *S. simulans*. Few studies have, however, investigated virulence factors in different CNS isolated from mastitis. According to Anaya-Lopez *et al.* (2006) *S. epidermidis* isolates were able to invade bovine mammary epithelial cells *in vitro* at similar levels as the *S. aureus* control strain. Biofilm production has not only been found in *S. aureus,* but also in *S. epidermidis*, *S. chromogenes*, *S. hyicus* and *S. xylosus* isolated from bovine mastitis (Oliveira *et al.*, 2006; Cucarella *et al.*, 2004). More studies are needed to clarify the epidemiology of different CNS in dairy herds.

Very little information is available about prevention of CNS mastitis, reflecting the fact that mastitis control measurements have usually been targeted against other udder pathogens. Control measurements such as post-milking teat disinfection have been suggested to reduce CNS infections and

CNS mastitis (Taponen, 2008; Lam *et al.*, 1997; Eberhart *et al.*, 1983). Dry cow therapy is generally accepted to be an effective tool for mastitis control, but the information on its effect against CNS mastitis is scarce (Lam *et al.*, 1997; Eberhart *et al.*, 1983). According to preliminary studies of Rajala-Schultz *et al.* (2008) CNS may persist after dry-cow therapy. Moreover, Robert *et al.* (2006) performed a meta-analysis evaluating the general efficacy of dry cow therapy, and found that no clear trend was observed in the difference in incidence between untreated and treated quarters of CNS intra-mammary infections. Antimicrobial treatment of heifers before parturition reduced the number of clinical cases, was considered economically beneficial, but did not protect the heifers from new infections (Borm *et al.*, 2006; Middleton *et al.*, 2005; Oliver *et al.*, 2004; Oliver *et al.*, 2003).

1.2.2 CPS mastitis with emphasis on *S. aureus*

Little information is available on bovine mastitis due to *S. hyicus* and *S. intermedius,* but the proportions of *S. aureus*, *S. hyicus* and *S. intermedius* found were rather similar despite differences in methods and material used (Roberson *et al.*, 1996; Calzolari *et al.*, 1995; Rampone *et al.*, 1993; Botha & Brand, 1987). As an example, Roberson *et al.* (1996) found 82.1% *S aureus*, 17.7% *S. hyicus*, and 0.2% *S intermedius* among 487 CPS isolates studied. Due to the predominance of *S. aureus* among CPS in bovine mastitis, the remaining part of this section will concentrate on *S. aureus* mastitis.

S. aureus is mostly considered as a contagious udder pathogen, commonly spread within and between cows at milking (Fox & Gay, 1993). Due to its contagious nature it has become a major udder pathogen in many parts of the world. It may cause both clinical and sub-clinical mastitis, responds poorly to antimicrobial therapy, and often results in long-lasting chronic infections (Sol *et al.*, 2000; Fox & Gay, 1993; Bramley & Dodd, 1984).

The prevalence of *S. aureus* in clinical mastitis range from 3.3% to 40% (Ericsson Unnerstad *et al.*, 2008; Olde Riekerink *et al.*, 2008; Bradley *et al.*, 2007; Ferguson *et al.*, 2007; Waage *et al.*, 1999; Elbers *et al.*, 1998). In a recent nation wide Swedish study on the microbial aetiology of cases of clinical mastitis in Sweden *S. aureus* accounted for 21.3% of all bacteriological diagnoses (Ericsson Unnerstad *et al.*, 2008). In sub-clinical mastitis, the proportion of *S. aureus* isolates has been studied in some countries and regions, and ranged between 3.2% and 63% (Bradley *et al.*, 2007; Tenhagen *et al.*, 2006; Pitkala *et al.*, 2004; Gianneechini *et al.*, 2002). In Sweden, a national survey on sub-clinical mastitis has not been performed

so far, but it is estimated that *S. aureus* constitutes approximately 40% of bacteriological findings in these cases (Anonymous, 2007).

Following the general realization that different strains of *S. aureus* exist several reports have revealed the presence of different strains of *S. aureus* in bovine mastitis. According to those studies a few predominant strains of *S. aureus,* often widely distributed among herds across geographic regions, were associated with most of the udder infections (Mork *et al.*, 2005; Smith *et al.*, 2005; Sabour *et al.*, 2004; Buzzola *et al.*, 2001; Aarestrup *et al.*, 1997; Rivas *et al.*, 1997; Kapur *et al.*, 1995; Matthews *et al.*, 1994; Musser *et al.*, 1990). Regional differences in the distribution of strains have, however, also been described (Sabour *et al.*, 2004). Moreover, in a specific herd it is common to find a predominant strain, which tends to be unique for the herd (Joo *et al.*, 2001; Rivas *et al.*, 1997).

The probability of udder infection increases if the host has direct contact with the reservoirs of pathogens, or indirect contact via fomites (Zecconi *et al.*, 2006a). Reservoirs and fomites of pathogens like *S. aureus* can be traced by pheno- and genotyping of the bacteria. A number of studies investigating potential reservoirs and fomites of *S. aureus* in dairy farms have been (Haveri *et al.*, 2008; Piccinini *et al.*, 2008; Jorgensen *et al.*, 2005; Middleton *et al.*, 2002a; Zadoks *et al.*, 2002; Larsen *et al.*, 2000; Roberson *et al.*, 1998; Roberson *et al.*, 1994b; Fox *et al.*, 1991; Matos *et al.*, 1991; Davidson, 1961; Spencer & Lasmanis, 1952). Most of these studies investigated extra-mammary sites associated with spread of infection at milking such as teat skin of lactating cows, teat and udder skin lesions, milking liners, milkers` hands and nostrils. Only a few studies investigated the presence of *S. aureus* isolates on other body sites of the lactating cow, in other age groups of cattle, in the environment of the animals, in humans, or in bulk milk and raw-milk products (Jorgensen *et al.*, 2005; Roberson *et al.*, 1998; Roberson *et al.*, 1994b; Matos *et al.*, 1991). In the studies of Roberson *et al.* (1994b; Matos *et al.* (1991) and Roberson *et al.* (1998) different body sites of heifers and cows were sampled and found *S. aureus* positive in varying proportions. As an example, samples from nares, hair coat, teat canal, vagina and perineum in heifers, and from wounds on udders and teats of lactating cows, were frequently found positive for *S. aureus* (Matos *et al.*, 1991). In the study of Roberson *et al.* (1998), possible sources of *S. aureus* in heifer colostrum were found mainly in milk from lactating cows, but also in various body and environment samples. The proportions of positive samples varied between sites and between herds. In many of the above-mentioned studies identification of *S. aureus* was made on species level and not on strain level.

For correct identification of reservoirs and fomites for udder infections identification on strain level is essential as several *S. aureus* strains may occur in the same herd (Piccinini *et al.*, 2008; Sommerhauser *et al.*, 2003). Among studies trying to find *S. aureus* strains associated with intra-mammary infections in extra-mammary sites only some (Haveri *et al.*, 2008; Piccinini *et al.*, 2008; Jorgensen *et al.*, 2005; Middleton *et al.*, 2002b; Zadoks *et al.*, 2002) used PFGE, which today is considered the gold standard for typing of bovine *S. aureus* (Zadoks *et al.*, 2002; Bannerman *et al.*, 1995). In those studies, *S. aureus* strains of the same genotype as in milk from bovine mastitis were found in samples from teat skin, teat canal, milkers' hands and nostrils, and teat and udder wounds, as well as in bulk milk, and various raw-milk products. To our knowledge, no study has investigated hock lesions as a potential reservoir of *S. aureus*. Hock lesions is a common finding in some housing systems (Rutherford *et al.*, 2008; Fulwider *et al.*, 2007; Weary & Taszkun, 2000), and an association between hock lesions and high milk somatic cell count (SCC) has been reported (Fulwider *et al.*, 2007).

According to several recent studies, strains of *S. aureus* may vary in virulence, resulting in varying severity of mastitis, ability to spread in and between cows and herds, ability to cause persistent udder infections, and ability to survive in extra-mammary sites (Fournier *et al.*, 2008; Piccinini *et al.*, 2008; Haveri *et al.*, 2007; Dingwell *et al.*, 2006; Zecconi *et al.*, 2006b; Brouillette *et al.*, 2003; Sommerhauser *et al.*, 2003; Larsen *et al.*, 2000; Zadoks *et al.*, 2000; Dziewanowska *et al.*, 1999). It is very likely that *S. aureus* strains, which are predominant in a herd, are well-adapted for colonization and survival in both intra-mammary (Fournier *et al.*, 2008; Mork *et al.*, 2005; Smith *et al.*, 2005; Buzzola *et al.*, 2001; Kapur *et al.*, 1995) and extra-mammary sites (Haveri *et al.*, 2008; Piccinini *et al.*, 2008; Jorgensen *et al.*, 2005). These strains are likely to express virulence factors of great importance. For example, it has recently been observed that the presence of genes encoding for protein A, fibronectin-binding proteins, haemolysin, leukocidins and pyrogenic toxin superantigen facilitate the invasion into and the persistence of *S. aureus* in the bovine mammary gland (Piccinini *et al.*, 2008; Haveri *et al.*, 2007; Brouillette *et al.*, 2003; Dziewanowska *et al.*, 1999). In line with this, some relation have been found between the virulence gene patterns of the *S. aureus* strains and sub-clinical mastitis (Fournier *et al.*, 2008; Zecconi *et al.*, 2006b). In the study of (Fournier *et al.*, 2008) the predominant *S. aureus* strain was only observed in herds having severe udder health problems, e.g. high prevalence of infections and often more than one quarter infected per cow. In the study of Haveri *et al.* (2005) inflammation of the udder varied between PTs, which

supported the notion of an association between PTs and severity of symptoms. Recently, Anderson & Lyman, (2006b) and Anderson *et al.* (2006a) also reported that antimicrobial resistance and persistence of infection were associated with particular *S. aureus* strain isolated from herds having problems due to high SCC and decreased milk production.

Various programs to control *S. aureus* mastitis have been introduced over the years. Important measures like post-milking teat disinfection, use of individual towels for udder cleaning, and properly functioning milking technique were recommended to stop new infections. Elimination of mammary infections by dry cow therapy, or culling of chronic cases were other important measures taken (Bramley & Dodd, 1984; Philpot, 1984; Philpot, 1979; Neave *et al.*, 1969). Later on, other recommendations have been added such as strict milking order and segregation of the infected animals.

Although a great deal of progress in mastitis control has taken place over the years, resulting in a reduction in *S. aureus* infections in many herds, in other herds it has not been possible to eradicate the problem (Roberson *et al.*, 1998). As an example, some farms in USA experienced an outbreak of *S. aureus* mastitis despite excellent control practices (Middleton *et al.*, 2001; Smith *et al.*, 1998). Moreover, despite the application of standard mastitis control practices, several *S. aureus* strains persisted for a long time, and were associated with herd problems, with increased milk SCC and decreased milk production (Anderson & Lyman, 2006b). There may be different reasons for the failure to eradicate *S. aureus* despite the use of above-mentioned control measures. For example, control measures for mastitis do little to control *S. aureus* infections in prepartum heifers (Roberson *et al.*, 1998). Also, cure rates for dry-cow therapy have been low, and are ineffective in prevention of new *S. aureus* infections in the late dry period, and at calving (Eberhart & Buckalew, 1977). Another factor of importance is the risk of introducing *S. aureus* into the herd by purchasing infected replacement animals. Lately, the possibility of other sources of infection has been suggested, as Sommerhauser *et al.* (2003) found that several *S. aureus* strains showed a pattern described as characteristic of environmental pathogens, with low or no tendency to spread within a herd. Hence, these strains could not be efficiently controlled by the contagious control program, which at the same time was proven efficient against *S. aureus* types having a contagious pattern. This supports the notion (Sommerhauser *et al.*, 2003; Kapur *et al.*, 1995) that control programs must consider the particular epidemiological features of the *S. aureus* udder pathogens, especially in prevention of new infections.

2 Aims of the study

The general aim of the thesis was to improve the knowledge of bacterial diagnostics and epidemiology of staphylococcal mastitis in Sweden.

More specifically the main aims were:

- To study the relative occurrence of *S. aureus*, *S. intermedius*, and *S. hyicus* among CPS isolated from cases of bovine mastitis.
- To establish a relevant and accurate typing scheme for differentiation between *S. aureus, S. intermedius,* and *S. hyicus*.
- To compare some phenotypic and genotypic methods for species identification of CNS isolated from bovine mastitis.
- To examine the genotypic diversity among *S. aureus* isolated from cases of acute clinical mastitis, and to investigate genotypic differences between geographical regions.
- To identify potential sources of infection relevant for *S. aureus* mastitis within problem herds by sampling body sites and the environment of different animal groups.

3 Material and methods

A detailed description of material and methods are given in Papers I-IV. Here, a short summary of the most important parts is presented.

3.1 Bacterial isolates

3.1.1 Reference strains

In Paper I, five reference strains (2 *S. aureus,* 1 *S. intermedius,* 2 *S. hyicus*) were used. In Paper II, 24 reference strains of 18 different staphylococcal species were included, and 1 *S. aureus* reference strain was used in Papers III-IV.

3.1.2 Field isolates

In Paper I, a total of 414 CPS isolates were included. All isolates were obtained from bovine milk samples sent to the Mastitis laboratory at the National Veterinary Institute. The majority of the isolates (n=327) were from sub-clinical cases of mastitis while 87 originated in cases of clinical mastitis. The strains were divided in two groups, material A consisting of 177 isolates irrespective of type of hemolysis reaction, and material B consisting of 237 isolates, with no hemolysis reaction or α-hemolysis on bovine blood agar.

In Papers II and III, staphylococcal isolates originating in milk samples collected in a national survey on prevalence of udder pathogens in cases of acute clinical mastitis conducted in 2002-2003 were used. Sixty-four presumptive CNS isolates were included in Paper II and 82 *S. aureus* isolates in Paper III. Twenty CNS isolates from cases of sub-clinical mastitis sent to the Mastitis laboratory were also included in Paper II.

In Paper IV, 361 *S. aureus* isolates originating in five herds with *S. aureus* mastitis problems were included. They consisted of 82 milk isolates, 187

body sample isolates, 80 isolates from environmental samples close to the animals, and 12 isolates from other areas. Each herd was visited two or three times within a period of one to two weeks between December 2006 and March 2007. In the herds, 755 quarter milk samples were collected once from 192 lactating cows. In addition, 1906 samples were collected once from body sites (teat skin, groin, vagina, nares/muzzle, hock skin, teat/udder skin, and other skin wounds (if present) and umbilical area (calves only)) of one side of 192 lactating cows, 48 dry cows, 30 late pregnant heifers (<3 months before calving), 49 young heifers 4-12 months old, and 52 heifer calves 0-3 months old. Samples (n=866) were also taken from the close environment of the animals (housing (stanchion bars and cubicle surfaces), water cups, under the rubber mats (when present), feed bunks, feed and bedding) close to each group of animals. Some samples (n=283) were also taken from companion animals present in the herd (nose, under paws), from milkers (nares, hands, boots), and from some general areas (air, flies, storage of feed and bedding, tools, milking equipment). Information on management and housing in each herd was collected using a specially designed questionnaire. Hock skins of lactating cows were scored as intact, with hair loss or with wounds.

3.2 Bacteriological methods

3.2.1 Cultivation methods and phenotypic characterisation (Papers I-IV)

Quarter milk samples (Papers I-IV)

In all studies, growth of staphylococci in milk was evaluated after culturing 10µl milk on 5% bovine blood agar with 0.05% esculine and incubation at 37°C for 16-24 h. In paper IV the culturing of milk on blood agar was preceded by 16 h. incubation at 37°C. *S. aureus* was identified by typical colony morphology, and αβ hemolysis. When only α-hemolysis or no hemolysis zones were present coagulase reaction was performed. Coagulase-positive isolates were tested further (Paper I) or considered as *S. aureus* (Papers III-IV). A milk sample was classified as positive if at least one colony-forming unit (CFU) of *S. aureus* per 10µl was isolated.

In Paper I, CPS were differentiated using the following tests; ability to grow on P-agar supplemented with acriflavin, acetoin test, anaerobic and aerobic fermentation of mannitol, culture on purple agar with 1% maltose, β-galactosidase test, hemolytic reaction on chocolate agar, and synergistic hemolysis test (CAMP-like). In addition, the Accuprobe® *S. aureus* culture

identification test was used on a few isolates each of *S. aureus*, *S. intermedius* and *S. hyicus*.

CNS were identified by phenotypic appearance, and negative coagulase reaction, and by the Staph-Zym™ system (Paper II). Supplementary tests were performed according to the manufacturer's instructions.

Other samples (Paper IV)

Swab samples from body sites and environmental sites were stored sterile in test tubes containing liquid Vogel-Johnson (VJ) medium, refrigerated over night, adjusted to room temperature for one to two hours, and incubated at 37°C for 4 h. Then, 10µl of sample was cultured on 5% bovine blood agar with esculine and on modified Baird-Parker agar (MBP). The blood and MBP agar plates were incubated at 37°C for 16-24 h and 48 h, respectively. Bedding and feed samples were frozen after sampling, thawed in room temperature and processed by mixing 90 ml of VJ medium with 10g material in a stomacher for 120 sec. The bags were incubated at 37°C for 4 h, and 10µl of the sample mix were inoculated on 5% bovine blood agar with esculine and MBP agar. Flies and air filters were thawed in room temperature. The flies were placed into sterile test tubes containing VJ medium. The air filter was cut into small pieces and placed into a sterile test tube containing VJ medium. The fly and air filter samples were processed as described for body and environment samples in VJ.

Growth of *S. aureus* on blood agar was identified as described for milk samples. Reverse Camp reaction and growth in P-agar was also evaluated. Growth of *S. aureus* on MBP agar was evaluated as follow. From each MBP agar plate, a maximum of 4 CFU with black, dark gray, or shiny brown appearance, with or without clear zones, were also cultured on 5% bovine blood agar with esculine. Growth on blood agar was evaluated as described for milk samples.

Storage of isolates (Papers I-IV)

Staphylococcal isolates were stored in trypticase soy broth containing 15% glycerol at -20°C (Papers I-III) or at –70 °C (Paper IV).

Antimicrobial susceptibility (Papers II-III)

Isolates were tested for antimicrobial susceptibility by determination of minimum inhibitory concentrations using a microdilution method (Paper II) and/or examined for β-lactamase production by the clover-leaf method (Papers II-III).

3.2.2 Genotypic methods (Papers II-IV)

Genotypic identification of CNS species (Paper II) was performed using *tuf* gene sequencing after DNA extraction and amplification. The *tuf* gene sequences were translated to amino acid sequences and aligned with ClustalW Multiple Sequence Alignment tool and a rooted phylogenetic tree was constructed.

In Papers III and IV, PFGE of *S. aureus* isolates was carried out according to the Harmony protocol. Macrorestriction patterns were analysed by visual inspection (Paper IV), or by using visual inspection and Gelcompare software (BioNumerics, version 5.0; Applied Maths BVBA) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages clustering (Paper III). Isolates with identical restriction profiles were assigned the same PFGE PT.

3.3 Statistics

In Paper III, differences in distribution of PTs between regions were evaluated using Fisher's exact test. The same test was used to evaluate differences in breed, parity, and presence of teat lesions between the three most common PTs, and between regions. In Paper IV, associations between presence of *S. aureus* in milk and on hock skin, and between presence of *S. aureus* on hock skin and presence of hair loss or wounds on hock skin were evaluated using logistic regression.

4 Results

Here, a summary of the most important results obtained in Papers I-IV is given. A detailed description is given in the papers.

4.1 Species identification of CPS (Paper I)

CPS isolates (n=403) positive on P-agar with acriflavin, and in anaerobic and aerobic mannitol fermentation tests were determined as *S. aureus*. These strains were negative in the β-galactosidase test, and CAMP-like test and showed no hemolysis on chocolate agar. Eight isolates positive in the aerobic mannitol fermentation test and the β-galactosidase test were considered as *S. intermedius*. These isolates were negative on P-agar with acriflavin, the anaerobic mannitol fermentation test, and the CAMP-like tests, and showed no hemolytic reaction on chocolate agar. Three isolates were determined as *S. hyicus*. They were positive in the CAMP-like test, gave a hemolytic reaction on chocolate agar, and were negative in the other tests. Among the 21 CPS strains tested all *S. aureus* strains (n=10) were positive in the Accuprobe® test while the other CPS strains were negative.

In both materials A and B the proportions of *S. aureus*, *S. intermedius* and *S. hyicus* were 97%, 2% and 1%, respectively. The species distribution was similar within isolates from clinical and sub-clinical cases of mastitis.

4.2 Species identification of CNS (Paper II)

4.2.1 Prevalence of CNS species and antimicrobial resistance

Among the 82 CNS milk isolates, nine species were identified using tuf gene sequencing. *S. chromogenes* (27%) and *S. simulans* (24%) were the most common findings followed by *S. haemolyticus* (15%), *S. epidermidis* (12%), *S. hyicus* (11%), *S. warneri* (5%), *S. xylosus* (2%), *S. aureus* (2%) and *S. lentus*

(1%). Antimicrobial resistance was found in 15 (18%) of the 82 isolates. Most (87%) of those cases were due to β-lactamase production. One *S. epidermidis* isolate was multi-resistant.

4.2.2 Comparison between *tuf* gene sequencing and Staph-Zym™

Staph-ZymTM identified 20 (83%) of the 24 reference strains correctly. Compared to tuf gene sequencing only 50 (61%) of 82 milk isolates were correctly identified by the Staph-ZymTM system. Using this system, supplementary tests were needed in 50% of the reference strains and 34% of the milk isolates. Two (8%) reference strains and 23 (28%) milk isolates were misjudged by Staph-ZymTM as a different CNS species.

4.3 *S. aureus* pulsotypes in acute clinical mastitis (Paper III)

Among the 82 *S. aureus* isolates investigated 25 PTs arbitrary designated A-Y were found. Pulsotype U (25.6%) was the most common type followed by PTs Q (15.9%) and E (11.0%). Most (72%) of the PTs were only found once or twice.

Alfa/beta hemolysis (αβ) or α-hemolysis was found in 54 (66%) and 28 (34%) of the *S. aureus* isolates, respectively. Among PTs with at least two isolates, the same hemolysis type (αβ or α) was found in all isolates of 6 PTs (A, E, N, T, U, V), while both hemolysis types (αβ and α) were found in 5 PTs (B, K, P, Q, W). All isolates of the most common PT (U) had αβ-haemolysis. β-lactamase production was found in 7 (9%) of all isolates. These isolates belonged to 7 PTs (B, E, I, J, M, W, Y). In PTs B, E and W the majority of the isolates did not produce β-lactamase. None of the isolates of the most common PT (U) produced β-lactamase.

In total, 15, 11 and 12 PTs were found in the northern, middle and southern region, respectively. Regional differences in the distribution of the three most common PTs (U, Q and E) were found. Pulsotype U was significantly less common in the southern region than in the middle region, while PT E was more common in the southern region than in the northern and middle regions. Regional breed differences were also observed with significantly more Swedish Holstein cows in the southern region than in the northern and middle regions. Parity and presence of teat lesions did not differ between regions.

The Swedish Holstein-breed was significantly more common than the Swedish Red-breed in PT E compared to in PT U. Parity and presence of teat lesions did not differ between these three PTs.

4.4 Identification of potential sources of *S. aureus* in herds with mastitis problems (Paper IV)

In the five dairy herds studied, the overall prevalence of milk samples positive for *S. aureus* (Sa+) was 11%. Among all lactating cows, 27% and 56% were Sa+ in milk and body samples, respectively. The proportions of Sa+ cows varied markedly between herds with the highest numbers in herds I-III. *S. aureus* was found in a varying frequency in all body sites sampled in lactating cows, but most frequently in hock skin samples. Overall, 53% of hock skin samples in this group were Sa+, but it was more common to find *S. aureus* if hock lesions were present than if the skin was intact. The prevalence of hock lesions in lactating cows was 54%, on average. It varied between herds, and was most common in herds I and II. It was most common, however, to find *S. aureus* in hock skin in herd III. Among other animal groups *S. aureus* was most frequently found in body samples of heifer calves. Skin wounds, umbilical cord and vagina were the sites having most Sa+ samples.

S. aureus was also found in environment samples, but not as often as in body samples. It was most common to find Sa+ samples in the environment of lactating cows, and stanchion bars and cubicle surfaces, bedding and water cups were the most common sites.

All isolated *S. aureus* strains were subjected to PFGE analysis, and in each herd, 4-6 unique PTs were found, of which one was predominant in milk samples of that herd. These PTs was also the most frequent finding in all samples of the different animal groups. In herds I, IV and V more than one PT was found in milk samples. Pulsotypes, not predominant in milk were rarely found in other samples with one exception, PT VC in herd V, which was the most frequently isolated PT in body samples in that herd. In herd IV the predominant PT in milk was not isolated from body sites of lactating cows, but was isolated from body sites of dry cows.

5 General Discussion

5.1 Species differentiation of staphylococci in bovine mastitis

Species identification of staphylococci is essential in bovine mastitis as this group of bacteria is the most prevalent udder pathogen in many parts of the world including Sweden. As mentioned, such differentiation can be performed using phenotypic and/or genotypic methods. The first criterion used for differentiation of staphylococci is the coagulase test separating the group into CPS and CNS. In most routine diagnostic laboratories today, bovine udder pathogens of these groups of bacteria are not identified at the species level. As new knowledge on staphylococcal mastitis is gathered such differentiation may, however be necessary in the future. The choice of methods to use for species differentiation may be affected by a number of factors, but economical issues and ease-of-use are of large importance especially in routine diagnostics. Independent of such factors the methods used should be reliable without risk for misidentification. In the present thesis, the aim was to differentiate between species within CPS and CNS using various approaches.

5.1.1 Differentiation of CPS

For differentiation of CPS a number of phenotypic tests were selected, which were considered appropriate at the time of the study. Three of those tests, P-agar with acriflavin, β-galactosidase, and hemolytic reaction in chocolate agar, were considered suitable for differentiation between *S. aureus*, *S. hyicus* and *S. intermedius* (Paper I). These tests were selected as they have high accuracy in identifying each species, are easy to read and perform, and have relatively low costs. Moreover, all species were positive only in one of the tests. Since the study was performed new information on CPS has become available and new techniques have been developed.

A new CPS species, *S. pseudintermedius,* has been identified in samples from various animals and birds (Devriese *et al.*, 2005), but has so far not been identified in bovine samples. This species is closely related to *S. intermedius*, making differentiation between these two species difficult (Sasaki *et al.*, 2007b). Previous studies have shown genotypic and phenotypic variation among *S. intermedius* strains (Becker *et al.*, 2005; Futagawa-Saito *et al.*, 2004; Bes *et al.*, 2002; Aarestrup, 2001), which may indicate that at least some of those isolates actually belonged to *S. pseudintermedius*. As *S. pseudintermedius* is negative and positive in anaerobic fermentation of mannitol and β-galactosidase, respectively (Sasaki *et al.*, 2007b), it is not possible to state if the isolates identified in the present thesis (Paper I) belonged to *S. intermedius* or *S. pseudintermedius*. Moreover, it is not known if *S. pseudintermedius* can grow on P-agar with acriflavin or not.

Our study also revealed the dominance of *S. aureus* among CPS in Swedish milk isolates from cases of bovine mastitis. This supports the routine diagnostic procedure used today, which assumes that all CPS are *S. aureus*. Nowadays a *nuc* gene based PCR specific for *S. aureus* is often used in routine diagnostics to differentiate *S. aureus* from other CPS species (Graber *et al.*, 2007; Kim *et al.*, 2001). However, when atypical colonies appear the use of P-agar with acriflavin is recommended as an easy and cheap method to differentiate *S. aureus* from other CPS. This test in combination with antimicrobial susceptibility testing may be sufficient in most situations. In research, however, more detailed species identification may be needed. The prevalence of different CPS in bovine mastitis may also vary between countries and regions. Thus, if the situation is not known the prevalence of various CPS should be investigated. The knowledge on virulence and epidemiology of CPS mastitis other than *S. aureus* is very limited. If the prevalence of mastitis due to CPS other than *S. aureus* increases such studies should therefore be performed.

5.1.2 Differentiation of CNS

The results generated in this thesis (Paper II) clearly show that *tuf* gene sequencing is superior to the commercial system used, Staph-Zym™, for differentiation among CNS from bovine milk. This is in line with recent findings of Sampimon *et al.* (2008) comparing two commercial systems, Staph-Zym™ and API Staph ID 32, with sequencing of *rpoB*. In both studies, the commercial system was sometimes not able to give a species name to an isolate, but more seriously, misidentification of isolates was common.

Commercial systems for differentiation of CNS are based on a selection of phenotypic methods. A weakness of such methods is that there is an inherent variability in expression of phenotypic features among isolates of the same species (Heikens *et al.*, 2005; Bannerman *et al.*, 1993). In addition, the lack of adequate reference strains in the accompanying databases of commercial systems reduces the chance to identify isolates tested (Renneberg *et al.*, 1995). Moreover, data on different species are based on very few reference strains of each species (Becker *et al.*, 2004). These isolates mostly originate in humans, and only occasionally in animals. As the phenotypic variation among field isolates may be substantial, given the selective pressure from the environment, it is not surprising that identification difficulties may occur. In addition, supplementary tests are commonly needed when using commercial systems (Sampimon *et al.*, 2008; Thorberg & Brändström, 2000). According to our results and other studies (Sampimon *et al.*, 2008; Burriel & Scott, 1997), the use of such tests does not, however, improve the accuracy of identification.

Given the results of this thesis, *tuf* gene sequencing could be recommended as a reference method for differentiation of bovine CNS, and used in research studies aiming at investigating specific species of CNS. Unfortunately, the method is expensive as it needs special resources making it less suitable for routine diagnostics. Based on knowledge on phenotypic methods available today it is not recommended to differentiate between CNS in bovine mastitis in routine diagnostics. In certain situations, such as herd problems with CNS mastitis and in countries having a high prevalence of CNS mastitis, there is, however, a need for an adequate and easy-to-perform method for reliable and rapid differentiation of at least the most pathogenic CNS. Such a system, based on a limited number of phenotypic tests, has been suggested for use on bovine milk isolates (Thorberg & Brändström, 2000). The system needs, however, to be validated using *tuf* gene sequencing.

5.2 Epidemiology of *S. aureus* mastitis

As mentioned earlier studies from different countries reported that few a predominant strains of *S. aureus,* were associated with most intra-mammary infections, both studies on variation between and within regions, and within dairy herds (Mork *et al.*, 2005; Smith *et al.*, 2005; Sommerhauser *et al.*, 2003; Buzzola *et al.*, 2001; Joo *et al.*, 2001; Aarestrup *et al.*, 1997; Kapur *et al.*, 1995). However, differences in the distribution of *S. aureus* strains between regions have also been reported (Sabour *et al.*, 2004). Other studies

have investigated the presence of *S. aureus* on extra-mammary sites within herds (Haveri *et al.*, 2008; Piccinini *et al.*, 2008; Jorgensen *et al.*, 2005; Larsen *et al.*, 2000; Roberson *et al.*, 1994b; Matos *et al.*, 1991; Davidson, 1961). In many studies identification was, however, made on species level and not on strain level. Identification on strain level is essential to make a correct evaluation of the spread of *S. aureus* within a region or herd. Today, PFGE is considered the gold standard for sub-typing of bovine *S. aureus* due to its excellent typeability, reproducibility, discriminatory power and easy interpretation (Zadoks *et al.*, 2002; Bannerman *et al.*, 1995).

5.2.1 Epidemiology of *S. aureus* in Swedish dairy herds

The results generated in this thesis (Paper III) revealed a rather high diversity at the country level among *S. aureus* PTs originating in acute cases of clinical mastitis, but it was also clear that some PTs were more common than others indicating spread between herds. When studying individual farms with udder health problems (Paper IV) a relatively low diversity of PTs was found within each herd, and the PTs were unique for each herd. In each herd, however, a predominant strain of *S. aureus* was found in milk, which is in concordance with other studies (Haveri *et al.*, 2008; Joo *et al.*, 2001).

The number of *S. aureus* strains in a herd may increase after importation of cattle (Haveri *et al.*, 2008; Middleton *et al.*, 2002b). Today many Swedish dairy herds increase their herd size markedly, which in many cases requires purchasing of animals from other herds and regions of the country. By doing so, they run a risk of introducing new strains of *S. aureus* udder pathogens, which can be spread throughout the herd despite good milking hygiene and mastitis control procedures (Smith *et al.*, 1998). Based on the results of paper IV, purchasing any type of cattle from herds with udder health problems due to *S. aureus* should be avoided as *S. aureus* may be present not only in the udder and body sites of lactating cows, but to a varying degree also in body sites of animals in other age groups. In general, it is recommended to buy heifers rather than cows, as udder infections are less common in heifers. In our study, the lowest risk of finding *S. aureus* on body sites occurred in 4-12 months old heifers, which was in line with Roberson *et al.* (1994a). In another study Matos *et al.* (1991), however, *S. aureus* was frequently found also in this age group. A draw-back with our study was that we did not sample udder secretion from heifers. Intra-mammary infections of *S. aureus* have been observed in other countries both in unbred and pregnant heifers (Trinidad *et al.*, 1990). Thus, such a reservoir cannot be excluded in our herds.

S. aureus mastitis control programs have had limited success in some herds indicating that the programs need to be updated. The results generated in this thesis (paper IV) clearly show important herd differences in bacterial epidemiology indicating that control strategies should be designed for each herd. In each herd, a unique predominant PT was found in milk, and in three herds this PT was commonly found in body and environment sites, especially in lactating cows. In another herd, it was, however, uncommon to find *S. aureus* in body and environment samples, while a different PT was often found in body sites than in milk in yet another herd. These differences suggest expression of different virulence factors in the PTs, and/or differences in herd management resulting in easier spread between animals in some herds than in others.

Thus, to develop successful control strategies for a herd, knowledge of important reservoirs for *S. aureus* in that herd is essential. Based on this knowledge it can be decided if control measures other than those related to spread of *S. aureus* at milking should be adopted. To gather such knowledge it may be worthwhile to take samples from selected body and environment sites of lactating cows, such as hock skin, and stanchion bars and cubicle surfaces, to find out if *S. aureus* is present or not. If so, PFGE should be performed to compare PTs found in such samples with PTs found in milk isolated from mastitic cows.

Segregation and culling of cows with intra-mammary infections are important control tool to stop spread of infections within herds. In some of the herds in our study, however, *S. aureus* was found in body samples of many lactating cows without intra-mammary infections. Hock lesions were by far the most important reservoir in those cows, but *S. aureus* was also found on intact hock skin. Thus, strategies for prevention of hock lesions and treatment of hock skin infections should be developed. Moreover, the findings of *S. aureus* in the environment of lactating cows highlights the need for good hygienic conditions in the cubicles. Thorough cleaning of the cubicles between animals is therefore an important measure to stop spread between animals for example when animals are moving between cubicles to achieve a better milking order in a tie-stall barn. The risk of contamination of bedding and housing, for example via milk leakage or infected hock lesions, should be considered also in free-stall barns. Transfer of bacteria between body sites and/or environment sites, or within and between cows, could also occur for example by licking or rubbing.

Following lactating cows, it was most common to find samples positive for *S. aureus* among heifer calves. In that group, *S. aureus* was mainly found in body sites such as skin wounds and the umbilical cord area, but *S. aureus*

was also sometimes found in environment samples, at least in some herds. It is likely that *S. aureus* was spread from lactating cows to calves via air or flies, or by feeding milk containing *S. aureus*. Spread of infection between calves could occur for example by inter-suckling. Whether presence of *S. aureus* on the body of calves is a risk for *S. aureus* mastitis later in life or not has been debated. A link between feeding milk from mastitic cows to calves and mastitis has been proven for *Streptococcus agalactiae,* but Barto *et al.* (1982) did not consider it as a major risk for *S. aureus* mastitis. Udder and teat canal colonization or infections with *S. aureus* have, however, been found in unbred heifers and primigravid dairy heifers (Trinidad *et al.*, 1990). Although our study could not prove such a link, findings of the same *S. aureus* strains on body and environment sites of calves as in milk from lactating cows support the recommendation that feeding of milk from cows with *S. aureus* mastitis to calves should be avoided. It is also recommended to house calves in a building separate from lactating cows, and to avoid spread of bacteria from cows to calves via hands and tools.

In future studies on *S. aureus* epidemiology on national and herd level, differences between PTs, and between isolates of the same PT in expression of important virulence factors should be investigated. Here, studies on factors involved in persistency and spread of infections are of utmost importance.

6 Conclusions

Overall, the results of this thesis contribute new knowledge on staphylococcal diagnostics and *S. aureus* epidemiology, which can be used to improve strategies for mastitis control.

In more detail, the main conclusions were as follows:

- The relative occurrence of *S. aureus*, *S. intermedius*, and *S. hyicus* among CPS isolated from cases of bovine mastitis were 97%, 2% and 1%, respectively.
- Three tests, P-agar supplemented with acriflavin, β-galactosidase, and hemolytic reaction on chocolate agar, enabled quick and accurate differentiation between the CPS species *S. aureus, S. intermedius,* and *S. hyicus*.
- The commercial test Staph-Zym™ was inferior to *tuf* gene sequencing for species identification of CNS isolated from bovine mastitis due to frequent misidentification of CNS species and frequent need for supplementary tests.
- A rather high genotypic diversity was found among *S. aureus* isolated from cases of acute clinical mastitis, but a few PTs dominated indicating spread between herds. The distribution of PTs was different in southern Sweden compared to other regions of the country.
- *S. aureus* isolates with genotypes indistinguishable from those found in milk was frequently found in body and environment samples in lactating cows, but also in other animal groups such as heifer calves in three of five herds with *S. aureus* mastitis problems. Among body sites, *S. aureus* was most frequently found in hock skin.

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