Epidemiology and characterization of coagulase-negative *Staphylococcus* species from dairy farms

Epidemiologie en karakterisatie van coagulase-negatieve *Staphylococcus* species op melkveebedrijven

(met een samenvatting in het Nederlands)

Piessens Veerle

Dissertation submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University, 2011.

Promotors:

Prof. Dr. Sarne De Vliegher
Department of Reproduction, Obstetrics and Herd Health
Faculty of Veterinary Medicine, Ghent University

Dr. ir. Els Van Coillie
Food Science and Technology Unit
Institute for Agricultural and Fisheries Research (ILVO)
Promotor
Prof. dr. S. De Vliegher
Department of Reproduction, Obstetrics, and Herd Health,
Faculty of Veterinary Medicine, Ghent University, Belgium

Co-promotor
Dr. ir. E. Van Coillie
Institute for Agricultural and Fisheries Research (ILVO),
Technology and Food Science Unit, Melle, Belgium

Additional Members of the Examination Committee
Prof. dr. dr. h. c. A. de Kruijf
Faculty of Veterinary Medicine, Ghent University

Prof. dr. Y.H. Schukken
Cornell University, Ithaca, USA

Dr. S. Taponen
University of Helsinki, Finland

Prof. dr. R. Zadoks
University of Edinburgh, UK

Prof. dr. P. Butaye
Veterinary and Agricultural Research Center (CODA), Ukkel

Prof. dr. ir. L. De Vuyst
Vrije Universiteit Brussel

Prof. dr. M. Heyndrickx
Institute for Agricultural and Fisheries Research (ILVO)

Prof. dr. K. Hermans
Faculty of Veterinary Medicine, Ghent University
# TABLE OF CONTENTS

CHAPTER 1: Role of coagulase-negative staphylococci in bovine intramammary infections – Review of the literature ................................................................. 1

1.1. Introduction ................................................................................................................. 1

1.2. Identification of coagulase-negative *Staphylococcus* species ........................................ 4

1.3. Significance of coagulase-negative staphylococci for bovine udder health ................. 11

1.4. Epidemiology of coagulase-negative staphylococci .................................................. 20

1.5. Antimicrobial resistance of coagulase-negative staphylococci .................................. 25

1.6. Virulence factors of coagulase-negative staphylococci ........................................... 31

CHAPTER 2: Objectives ........................................................................................................ 35

CHAPTER 3: Validation of amplified fragment length polymorphism genotyping for identification of bovine associated coagulase-negative *Staphylococcus* species .... 37

3.1. Abstract ...................................................................................................................... 39

3.2. Introduction .................................................................................................................. 40

3.3. Materials and Methods ............................................................................................... 41

3.4. Results ......................................................................................................................... 47

3.5. Discussion .................................................................................................................... 54

3.6. Conclusions ................................................................................................................. 57

CHAPTER 4: Distribution of coagulase-negative *Staphylococcus* species from cows’ milk and environment differs between herds ......................................................... 59

4.1. Abstract ...................................................................................................................... 61

4.2. Introduction .................................................................................................................. 62

4.3. Materials and methods ............................................................................................... 63

4.4. Results ......................................................................................................................... 70

4.5. Discussion .................................................................................................................... 80

4.6. Conclusions ................................................................................................................. 84
CHAPTER 5: Intra-species diversity and epidemiology varies among different coagulase-negative *Staphylococcus* species causing bovine intramammary infections

5.1. Abstract
5.2. Introduction
5.3. Materials and methods
5.4. Results
5.5. Discussion
5.6. Conclusions

CHAPTER 6: Characterization of coagulase-negative *Staphylococcus* species from cows’ milk and environment based on the presence of *bap*, *icaA*, and *mecA* genes and phenotypic susceptibility to antimicrobials and teat dip products

6.1. Abstract
6.2. Introduction
6.3. Materials and methods
6.4. Results
6.5. Discussion
6.6. Conclusions

CHAPTER 7: General discussion and future perspectives

7.1. Introduction
7.2. Application of AFLP genotyping for species identification and strain typing
7.3. CNS species diversity in milk and the farm environment
7.4. Pathogenic potential and persistence in the mammary gland of CNS species causing IMI
7.5. Epidemiology of individual CNS species: udder-adapted (contagious) versus environmental (opportunistic)
7.6. Characteristics of CNS species causing IMI as compared to environmental CNS species
7.7. Future perspectives

References

Summary
### ABBREVIATION LIST

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion</td>
</tr>
<tr>
<td>BMSCC</td>
<td>bulk milk somatic cell count</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CLSI</td>
<td>clinical and laboratory standards institute</td>
</tr>
<tr>
<td>CM</td>
<td>clinical mastitis</td>
</tr>
<tr>
<td>CMT</td>
<td>California Mastitis Test</td>
</tr>
<tr>
<td>CNS</td>
<td>coagulase-negative staphylococci</td>
</tr>
<tr>
<td>CPS</td>
<td>coagulase-positive staphylococci</td>
</tr>
<tr>
<td>D</td>
<td>Simpson’s diversity index</td>
</tr>
<tr>
<td>D/E</td>
<td>Dey-Engley</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EF</td>
<td>enrofloxacin</td>
</tr>
<tr>
<td>GM</td>
<td>gentamicin</td>
</tr>
<tr>
<td>Ica</td>
<td>intercellular adhesin</td>
</tr>
<tr>
<td>IMI</td>
<td>intramammary infection</td>
</tr>
<tr>
<td>IRCM</td>
<td>incidence rate of clinical mastitis</td>
</tr>
<tr>
<td>ITS-PCR</td>
<td>internal transcribed spacer-PCR</td>
</tr>
<tr>
<td>LF/LH</td>
<td>left front/left hind quarter</td>
</tr>
<tr>
<td>MBC</td>
<td>minimal biocidal concentration</td>
</tr>
<tr>
<td>MCC</td>
<td>milk control centre</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MLSST</td>
<td>multi locus sequence typing</td>
</tr>
<tr>
<td>MR</td>
<td>methicillin resistance</td>
</tr>
<tr>
<td>MRS</td>
<td>methicillin-resistant staphylococci</td>
</tr>
<tr>
<td>MR-CNS</td>
<td>methicillin-resistant coagulase-negative staphylococci</td>
</tr>
<tr>
<td>MS</td>
<td>maximum similarity</td>
</tr>
<tr>
<td>MSA</td>
<td>mannitol salt agar</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NMC</td>
<td>national mastitis council</td>
</tr>
<tr>
<td>OX</td>
<td>oxacillin</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIA</td>
<td>polysaccharide intercellular adhesin</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophilic leukocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>QAC</td>
<td>quaternary ammonium compound</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplification of polymorphic DNA</td>
</tr>
<tr>
<td>rep-PCR</td>
<td>repetitive DNA element-PCR</td>
</tr>
<tr>
<td>RF/RH</td>
<td>right front/right hind quarter</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SAg</td>
<td>superantigen</td>
</tr>
<tr>
<td>SCC</td>
<td>somatic cell count</td>
</tr>
<tr>
<td>SE</td>
<td>staphylococcal enterotoxin</td>
</tr>
<tr>
<td>TAC</td>
<td>teat apex colonization</td>
</tr>
<tr>
<td>tDNA-PCR</td>
<td>transfer-RNA intergenic spacer PCR</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TSA</td>
<td>trypton soy agar</td>
</tr>
<tr>
<td>TSST-1</td>
<td>toxic shock syndrome toxin-1</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair group method with arithmetic mean</td>
</tr>
</tbody>
</table>
PREFACE

In the last twenty years the interest in coagulase-negative *Staphylococcus* (CNS) species has significantly increased, both in veterinary and human medicine. *Staphylococci* are a common cause of opportunistic infections and are particularly known for their ability to adapt to various conditions. Despite their overall low virulence, CNS are often resistant to various antimicrobials and biocides, and dispose of virulence factors such as biofilm formation, which might contribute to their spread and success as opportunistic pathogens. On many well-managed dairy farms where dry-cow therapy and post-milking teat disinfection is routinely used, CNS have become the most common cause of intramammary infections (IMI). To say the least, the CNS have some intriguing properties: on the one hand CNS increase the somatic cell count of milk from infected quarters, and occasionally give rise to (mild) clinical mastitis, on the other hand they have also been shown to protect against infections caused by major mastitis pathogens. The diversity of CNS species causing bovine IMI is large and still increasing, but little is known on the importance of the individual CNS species for udder health. Whether (and which) CNS species are harmful, advantageous, or simply unimportant as mastitis pathogens has not been equivocally established. Much of the confusion lies in the fact that CNS include numerous species, which might have different clinical impact, epidemiology, and characteristics. Most previous studies lack accurate CNS species information, and in this dissertation, it was attempted to answer some of the questions concerning the epidemiology, sources, and characteristics of the individual CNS species involved in bovine IMI.
CHAPTER 1

ROLE OF COAGULASE-NEGATIVE STAPHYLOCOCCI IN BOVINE INTRAMAMMARY INFECTIONS – REVIEW OF THE LITERATURE

1.1. Introduction

Bovine mastitis - or inflammation of the bovine mammary gland tissue - is the most common and costly disease in the dairy sector throughout the world. The main economic losses related to the disease can be attributed to reduced milk production and quality, veterinary fees, therapy costs, and culling and replacement of affected animals (Halasa et al., 2007). In most cases, mastitis is the result of bacteria migrating into the mammary gland through the teat canal. Invaded bacteria interact with the host tissue and activate the cow’s immune system, promoting neutrophil migration from the blood into the milk leading to an increase in somatic cell count (SCC) in the milk (Figure 1-1). An increase in milk SCC of an individual cow or quarter above a threshold is indicative for the presence of an intramammary infection (IMI), and is inversely correlated with milk production and quality (Jones et al., 1984). Mastitis cases can vary from (severely) clinical, with visible signs, to subclinical without signs but with an elevated SCC. Not only clinical mastitis (Gröhn et al., 2004), but also subclinical mastitis results in reduced milk production (Ott, 1999; Halasa et al., 2009).

In many countries, the quality of raw milk is monitored routinely and various parameters of the delivered milk such as total bacterial count and SCC have to meet minimal quality standards, and the presence of antimicrobial residues above a specific maximum residue limit is prohibited. In Flanders, the main reason for milk price penalties for the farmer is a bulk milk SCC (BMSCC) exceeding the upper acceptable [1]
limit, which is 400,000 cells per millilitre of milk in Europe (Annual reports 2008, 2009, 2010, Milk Control Centre Flanders, Lier, Belgium). The total bacterial count of bulk milk mainly depends on the conditions during the milking process and milk preservation, whereas the BMSCC depends on the infection status of the herd. Consequently, farmers attempt to maintain good milk quality by adopting hygienic milking procedures and by controlling the number of (sub)clinical infections in the herd. Good quality milk is not only essential for the farmer, but also for the dairy processing industry, because higher yields and longer shelf lives of manufactured products, such as yoghurt and cheese, can be obtained (Le Roux et al., 2003). Furthermore, consumers are concerned about animal welfare and demand dairy products produced by healthy cows. Thus, the growing quality demands and the issue of animal welfare increase the need to control mastitis, in Flanders as well as in many other regions in the world.

![Figure 1-1](http://www.ag.ndsu.edu/pubs/ansci/dairy/as1129w.htm)

**Figure 1-1.** The course of an intramammary infection: (A) bacterial pathogens (●) enter the mammary gland through the teat canal and colonize the mammary gland tissue, (B) in response to the bacterial infection large numbers of neutrophils (○) pass between milk producing cells into the lumen of the alveolus, thus increasing the somatic cell count (source: http://www.ag.ndsu.edu/pubs/ansci/dairy/as1129w.htm).

Many different microorganisms have been isolated from cases of bovine IMI, but the major causative agents are staphylococci, streptococci, and coliforms (Tenhagen et al., 2006; Bradley et al., 2007; Botrel et al., 2010). Mastitis pathogens are traditionally subdivided into three categories based on their reservoirs or source and means of
transmission: contagious, environmental, and skin opportunistic pathogens. As the different subgroups of pathogens have different ways of spreading, each subgroup requires specific control measures (Smith and Hogan, 2001). In many countries, the BMSCC has declined over the last decades as a result of the better control of clinical and subclinical mastitis caused by major contagious pathogens (*Staphylococcus aureus* and *Streptococcus agalactiae*). Simultaneously, there has been a shift towards an increased prevalence and incidence of IMI caused by environmental pathogens (coliforms and *Streptococcus uberis*) and coagulase-negative staphylococci (CNS). Currently, CNS are the leading cause of subclinical mastitis on most well managed dairy farms that have controlled contagious mastitis.

Several studies have suggested that CNS should be considered emerging mastitis pathogens that need attention in herds aiming for a low BMSCC (Davidson *et al*., 1992; Pyörälä and Taponen, 2009; Paradis *et al*., 2010). On the other hand, protective effects of preexisting CNS IMI against mastitis with major pathogens have been demonstrated, suggesting they might be beneficial to udder health (Matthews *et al*., 1991; Nickerson and Boddie, 1994). The understanding of CNS mastitis is fairly complicated by the large number of species and the heterogeneity of the group. The high prevalence of CNS with rare, aberrant, or poorly defined phenotypic characteristics makes it difficult to identify them to the species level using biochemical methods. Currently, it is acknowledged that the CNS group can no longer be regarded as a homogeneous group, but should be studied on the species level to correctly assess the clinical impact and epidemiology of CNS IMI. Therefore, the unreliable conventional identification methods should be abandoned and more accurate and robust molecular identification methods should be applied to clarify the contradictory findings on CNS infections regarding their protective or pathogenic behaviour, and to determine whether CNS species act as environmental or skin opportunists, or as udder-adapted, possibly contagious mastitis pathogens.
1.2. Identification of coagulase-negative *Staphylococcus* species

1.2.1. Coagulase-positive and coagulase-negative staphylococci

Staphylococci are Gram-positive spherical bacteria occurring in microscopic clusters resembling bunches of grapes, hence their name (Gr. *staphylos* = grape). They belong to the family *Staphylococcaceae* and are facultative anaerobic, catalase-positive, and highly salt tolerant (most strains survive in the presence of 10% NaCl). Staphylococci are part of the normal microbiota of the skin and mucous membranes of mammals, but are also ubiquitously distributed in very different niches in nature, including soil, water, and air, and in a variety of foodstuffs, such as meat, cheese, and raw milk (Irlinger, 2008; Schleifer and Bell, 2009; Coton *et al.*, 2010). Most species colonize specific animal species or niches and are rarely isolated from other than their typical sources (Watts and Yancey, 1994). However, in close contact situations, isolation of human-associated species from animals, and *vice versa*, can occur (Kloos and Bannerman, 1994). In Table 1-1, the primary host species or sources of a variety of CNS species is represented (Schleifer and Bell, 2009).

In 1985, only 15 CNS species had been recognized (Parisi, 1985). Since then, the number of described *Staphylococcus* species has increased dramatically, and especially in animals numerous new CNS species have been discovered in recent years (Vernozy-Rozand *et al.*, 2000; Spérgser *et al.*, 2003; Supré *et al.*, 2010; Riesen and Perreten, 2010; Taponen *et al.*, 2011). Currently, the *Staphylococcus* genus consists of 45 validated species and 24 subspecies, of which the majority is coagulase-negative (Euzeby, 2011). The latter are distinguished from coagulase-positive staphylococci (CPS) (*Staphylococcus aureus*, *S. delphini*, *S. intermedius*, *S. pseudintermedius*, *S. lutralae*, *S. schleiferi* subsp. *coagulans*, and some strains of *S. hyicus*) by their inability to produce the enzyme coagulase. This enzyme mediates the conversion of fibrinogen to fibrin resulting in the clotting of blood, and has been associated with pathogenicity. Whereas CPS, and especially *S. aureus*, are regarded as important pathogens in humans and animals, CNS are generally described as benign commensal organisms. In the last two decades, however, the interest in CNS species has significantly increased in both human and...
<table>
<thead>
<tr>
<th><em>Staphylococcus</em> species</th>
<th>Human</th>
<th>Primate</th>
<th>Bovine</th>
<th>Caprine</th>
<th>Ovine</th>
<th>Porcine</th>
<th>Equine</th>
<th>Canine</th>
<th>Feline</th>
<th>Avian</th>
<th>Rodent</th>
<th>Foodstuffs</th>
<th>Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. arlettae</em></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. carnosus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. felis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. fleurettii</em></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>S. lugdunensis</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. schleiferi</em></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td></td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>S. simiae</em></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
veterinary medicine. When gaining entry into host tissue (e.g. skin, internal organs, mammary glands), CNS can cause a wide variety of diseases. In humans, CNS species are the most isolated microorganisms from blood cultures, and are a major cause of nosocomial infections in individuals with compromised host defences or implanted medical devices, such as catheters and prostheses. Several studies have reported CNS as a cause of bacteremia, urinary tract infections, and infections of wounds, the peritoneum, bones, joints, etc. The species most frequently associated with human infections are *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus* (for a review, see: Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; von Eiff *et al.*, 2002; Piette and Verschraegen, 2009). Furthermore, CNS are the most common cause of IMI in small ruminants and cattle and are currently regarded as emerging mastitis pathogens (Pengov, 2001; Contreras *et al.*, 2007; Pyörälä and Taponen, 2009).

### 1.2.2. Phenotypic species identification

In veterinary diagnostic laboratories, a rough delineation of the most important mastitis pathogens can be obtained by a number of simple phenotypic tests, allowing rapid identification of the causative agent (Hogan *et al.*, 1999). Phenotypic species identification methods are generally based on the observation or testing of criteria such as colony morphology, growth characteristics, ability to metabolise various substrates, reaction with blood components, etc. Within the staphylococci, the major pathogen *S. aureus* can be discriminated from CNS by its pigmented, coagulase-positive, haemolytic, and DNase-positive phenotype, although some bovine *S. aureus* strains may require a long incubation time (24 h) to detect coagulase-production, and not all strains are haemolytic (Boerlin *et al.*, 2003). Further identification of individual CNS species is more laborious and requires a large number of microbiological tests, which are described in conventional or simplified identification schemes (Kloos and Schleifer, 1975; Devriese *et al.*, 1985; Watts *et al.*, 1991; Thorberg and Brändström, 2000; Cunha *et al.*, 2004).Although these conventional methods are accurate, they are too elaborate and time-consuming to be used in routine diagnostics. Consequently, bovine staphylococci are usually merely differentiated into *S. aureus* and other staphylococci.

Several commercial identification kits allowing more rapid diagnosis of CNS species in human clinical samples have been developed, such as the API Staph ID system...
bioMérieux, Marcy l’Etoile, France), and the Staph-Zym system (Rosco, Taastrup, Denmark). These kits combine a number of differential biochemical tests and perform relatively well in identifying human staphylococcal isolates (Kloos and Bannerman, 1994), although limitations in their precision have been reported (Bannerman et al., 1993; Renneberg et al., 1995; Cunha et al., 2004; Heikens et al., 2005). Because of their ease of use, these commercial systems have been commonly used for speciation of CNS isolates from animals as well. The API Staph ID test has even been recommended for CNS differentiation by the National Mastitis Council (Hogan et al., 1999). Only more recently, mastitis researchers have thoroughly investigated the ability of these commercial systems to accurately identify isolates from cows. A number of comparative studies have been performed to determine the level of agreement with a conventional or genotypic reference method (Thorberg and Brändström, 2000; Taponen et al., 2006; Taponen et al., 2008; Capurro et al., 2009; Sampimon et al., 2009c; Onni et al., 2010; Park et al., 2011a), and it appeared that the commercial systems performed poorly in identifying animal isolates correctly.

Agreement between API Staph ID and conventional methods in identifying mastitis isolates was 77% (Thorberg and Brändström, 2000). However, using rpoB sequencing as a reference method, levels of agreement as low as 41% have been reported (Sampimon et al., 2009c). In another study, 24% of bovine isolates were misidentified by the API Staph ID test using 16S rRNA gene sequencing as a reference, of which 76% were identified with high confidence levels (Park et al., 2011a). Misidentification of this nature could lead to interpretation errors and underestimation of the impact of specific species. However, the accuracy of identification results seems to be species-dependent. For the API Staph ID system, identification of S. chromogenes, S. haemolyticus, S. simulans, S. warneri, and S. epidermidis animal isolates in particular has been shown to be problematic (Thorberg and Brändström, 2000; Sampimon et al., 2009c; Onni et al., 2010; Park et al., 2011a). As these species are frequently associated with IMI, this system is regarded unfit to study CNS from cows’ milk (Park et al., 2011a).

For the Staph-Zym test, good agreement (94%) has been shown with conventional methods, although additional tests were necessary to identify 45% of strains, increasing time and cost (Thorberg and Brändström, 2000). In a study using tuf sequencing as the reference method, the Staph-Zym system gave 61% of milk isolates a correct species name (Capurro et al., 2009). In another study, only 31% of milk isolates were correctly
identified by the Staph-Zym system, and for 59% of the isolates no definite identification could be obtained due to ambiguous or lack of typing results (Sampimon et al., 2009c). Thus, next to their limited accuracy, the low sensitivity of these systems, i.e. the high level of unidentified isolates, is another shortcoming. This low sensitivity seems to be even more pronounced for extramammary CNS than for mastitis-associated isolates. For swab samples from extramammary sites (perineum and udder skin, teat apices, teat canals, hands of staff, and teat cup liners), only 57% (321/563) of CNS isolates could be identified with >90% of probability using API Staph ID, compared to 84% (58/69) of CNS mastitis isolates (Taponen et al., 2008). The poor performance of identification kits on animal isolates is not surprising, as these systems have been developed for the analysis of human pathogens, and contain limited animal strains in their database. The composition of the CNS population from dairy cattle, humans, or other niches differs considerably (Table 1-1). Moreover, strain differences can occur between isolates of the same species but originating from different host species (Zadoks and Watts, 2009; Supré et al., 2009). A major drawback of species identification based on a limited number of phenotypic traits is that expression can be highly variable among different strains within species, compromising correct interpretation and reproducibility of tests (Watts et al., 1984; Zadoks and Watts, 2009). Incorporating more animal strains and species in the databases of commercial systems should improve their accurateness and sensitivity, although the interpretation of weak or aberrant reactions remains difficult and subjective.

1.2.3. Molecular species identification

To overcome the constraints associated with phenotypic species identification, several molecular methods have been adopted for CNS differentiation. DNA sequencing of (housekeeping) genes has been commonly used in phylogenetic studies (Takahashi et al., 1999; Ghebremedhin et al., 2008), and is currently a generally used method for species identification of staphylococci and many other microorganisms (CLSI, 2007a; Zadoks and Watts, 2009). Several target genes have been used for genotypic species identification of Staphylococcus species, including the 16S rRNA gene, hsp60/cpn60/groL (heat shock protein 60), rpoB (beta subunit of RNA polymerase), sodA (superoxide dismutase A), gap (glyceraldehyde-3-phosphate dehydrogenase), and tuf (elongation
factor Tu) (Goh et al., 1996; Takahashi et al., 1997; Takahashi et al., 1999; Kwok et al., 1999; Poyart et al., 2001; Martineau et al., 2001; Drancourt and Raoult, 2002a; Kwok and Chow, 2003; Boerlin et al., 2003; Heikens et al., 2005; Ghebremedhin et al., 2008; Capurro et al., 2009). Gene sequences for a variety of CNS species are currently available in GenBank for reference, and have been used in several recent studies for the identification of CNS isolates from bovine milk samples (Takahashi et al., 1997; Boerlin et al., 2003; Capurro et al., 2009; Supré et al., 2009; Sampimon et al., 2009c).

Other genotypic methods involve the comparison of DNA fragments, usually restriction fragments, amplified sections, or combinations thereof, which are separated on the basis of their molecular size by gel electrophoresis, resulting in a bacterial fingerprint. The basic principle is to compare fingerprints of isolates to those of reference strains to come to a final species identification. In this respect, the composition of the reference library is of major importance. Several polymerase chain reaction (PCR) based methods using primers targeting specific regions of the bacterial genome have been optimized for rapid identification of CNS isolates from humans and animals, such as transfer-RNA intergenic spacer PCR (tDNA-PCR) (Maes et al., 1997; Supré et al., 2009), 16S-23S rDNA gene internal transcribed spacer PCR (ITS-PCR) (Mendoza et al., 1998; Bes et al., 2000), and (GTG)5-PCR (Braem et al., 2011). To increase the resolution of PCR fingerprinting, a restriction step can be added, as is done in PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. RFLP-PCR analysis of \( rrs, \) \( gap, \) and \( hsp60 \) has also been used to speciate CNS isolates from animals (Santos et al., 2008a; Onni et al., 2010; Park et al., 2011a). Ribotyping is another, non-PCR based method, in which genome restriction is followed by hybridization with a probe complementary to ribosomal DNA. This method can be automated and performs very well in discriminating CNS species (Bes et al., 2000; van Belkum et al., 2007; Taponen et al., 2008). In general, the fingerprints generated with the aforementioned methods contain a limited number of fragments and are highly species-specific. Their major advantage is that they are rapid, easy to perform, and cheap (except for automated ribotyping), but due to limitations in their discriminatory power, they sometimes fail to differentiate closely related CNS species (Mendoza et al., 1998; Onni et al., 2010; Park et al., 2011a).

Another method that has been applied for CNS species identification is amplified fragment length polymorphism (AFLP) genotyping. AFLP is a method based on (i) the
restriction of genomic DNA with two restriction enzymes (usually a “rare” and a “frequent cutter”), (ii) the ligation of oligonucleotide adapters to restriction fragments, and (iii) the selective amplification of a subset of the fragments with adapter-specific primers extended with (a) selective nucleotide(s) (Vos et al., 1995; Savelkoul et al., 1999). Usually one primer is fluorescently labelled and fragment separation is done by capillary electrophoresis, allowing for high-resolution and high-throughput genotyping of large numbers of isolates. Contrasting to the other described methods, AFLP has whole-genome coverage and maps polymorphisms in restriction sites and adjacent sequences, and insertions and deletions in amplified fragments, dispersed over the entire bacterial genome. Obviously, typing methods mapping polymorphisms at multiple sites are more discriminatory than are methods exploring variation at only one or a few loci. Due to its superior discriminatory power and broad taxonomic range, AFLP has been extensively used in phylogenetic and taxonomic studies, and has been described as a convenient method for delineation of species within various bacterial genera (Huys et al., 1996; Coenye et al., 1999; Kokotovic et al., 1999; Rademaker et al., 2000; On and Harrington, 2000; Keto-Timonen et al., 2003). The actual gold standard for species determination, total genomic DNA-DNA hybridization, is fairly laborious and unfeasible in many laboratories. However, AFLP analysis generally shows good agreement with DNA-DNA-hybridization results and is considered a good alternative (Coenye et al., 1999; Rademaker et al., 2000; van Belkum et al., 2007).

Another promising, advanced molecular identification method is matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis (MALDI-TOF), that generates fingerprints by fragmentation of the whole cell instead of the genomic DNA (Dubois et al., 2010). Both AFLP and MALDI-TOF have been proven useful for species identification of bovine CNS isolates (Taponen et al., 2006; Taponen et al., 2007; Huber et al., 2011). Whereas the MALDI-TOF identification method has been been well validated using a large number of CNS reference strains previously identified with gene sequencing, the performance of the AFLP method has not been validated by comparison with a reference method (Taponen et al., 2006; Taponen et al., 2007; Dubois et al., 2010). These highly discriminatory methods are more expensive and require special equipment and expertise, but their key benefit is that species identification and strain typing can be done concurrently, because their resolution exceeds the species level (see paragraph 1.4.2.).
1.3. Significance of coagulase-negative staphylococci for bovine udder health

1.3.1. Prevalence and incidence of CNS intramammary infections

In a number of countries, the prevalence and causative agents of bovine mastitis have been investigated in nationwide surveys (Pitkala et al., 2004; Osteras et al., 2006; Tenhagen et al., 2006; Bradley et al., 2007; Piepers et al., 2007; Sampimon et al., 2009a; Botrel et al., 2010). Considerable analogy in pathogen distribution has been observed between regions, and in most recent surveillance studies, the CNS are the most frequently isolated group of organisms causing IMI (Pitkala et al., 2004; Tenhagen et al., 2006; Bradley et al., 2007; Piepers et al., 2007; Sampimon et al., 2009a). Other common mastitis pathogens are S. aureus, Streptococcus sp. (Streptococcus uberis, Streptococcus dysgalactiae, and Streptococcus agalactiae), Corynebacterium bovis, and coliforms (Escherichia coli and Klebsiella sp.). When comparing recent data with surveys and studies from the past, some distribution changes in mastitis pathogens seem to have occurred. The major contagious pathogen S. agalactiae, which used to cause a considerable proportion of clinical cases many years ago, has been virtually eradicated in some countries (Hogan et al., 1989a; Pitkala et al., 2004; Piepers et al., 2007; Sampimon et al., 2009a). Prevalence of S. aureus infections has decreased as well, although this major pathogen is still a problem in many dairy herds (Pitkala et al., 2004; Osteras et al., 2006; Tenhagen et al., 2006). On the other hand, environmental pathogens, i.e. the non-agalactiae streptococci and coliform bacteria, have become increasingly important as causes of clinical mastitis (Hogan et al., 1989a; Bradley et al., 2007; Botrel et al., 2010).

Over the years, implementation of effective mastitis control measures has resulted in a general decrease of “contagious mastitis” and BMSCC. Nevertheless, the proportion of culture-positive milk samples has increased, which is explained by the fact that the minor pathogens, C. bovis and CNS, have become more common (Pitkala et al., 2004; Tenhagen et al., 2006). In herds that have controlled “contagious mastitis” effectively, as evidenced by a low herd SCC, prevalence and incidence of subclinical and clinical mastitis can still be high (Hogan et al., 1989a). In Flanders, 17% of quarters sampled in
the course of a 3-year survey were positive on culture, and over 50% of all IMI were caused by CNS (Piepers et al., 2007). In a survey in Finland, 33.5% of quarter milk samples were culture-positive, and about half of the bacteriological findings were CNS (Pitkala et al., 2004). In the Netherlands, the national prevalence of CNS infected quarters was 10.8% (Sampimon et al., 2009a). In some dairy herds, the prevalence of CNS infections is remarkably high for unknown reasons (Piepers et al., 2007; Schukken et al., 2009). The mean herd quarter prevalence of CNS in Flanders was 9.9%, although multiple farms with more than 50% of CNS infected quarters were found (Piepers et al., 2007). In an American study, an average of 15% of cows per herd was infected with CNS, ranging from 0 to 100% (Schukken et al., 2009).

Although CNS are rarely the major cause of clinical mastitis within herds, they have been isolated from quarters with elevated SCC and from (mild) clinical mastitis cases. In Flanders, CNS have been isolated in 41% of culture-positive quarters from cows with high SCC (geometric mean composite SCC of ≥250,000 cells/ml) (Piepers et al., 2007). In England and Wales, CNS were also the most common finding (15%) in quarters with high SCC (>200,000 cells/ml), and pure cultures of CNS were isolated from 8% of the clinical cases (Bradley et al., 2007). In a nationwide survey in France, CNS were isolated from 13.7% of subclinical and 9.5% of clinical mastitis cases (Botrel et al., 2010). Thus, while the majority of CNS infections in lactating cows are subclinical, some do result in (mild) clinical mastitis cases. As the control of major pathogens is improving, CNS might get relatively more important as clinical mastitis pathogens.

1.3.2. CNS species associated with bovine intramammary infections

Studies on CNS from bovine IMI show a wide variation with regard to the most isolated species: *S. chromogenes* is the predominant species in most studies, but other commonly isolated species are *S. epidermidis, S. hyicus, S. simulans, S. haemolyticus*, and *S. xylosus* (Matthews et al., 1992; Aarestrup and Jensen, 1997; Chaffer et al., 1999; Rajala-Schultz et al., 2004; Taponen et al., 2006; Thorberg et al., 2009; Sampimon et al., 2009b; Park et al., 2011a). A number of CNS species more rarely associated with IMI have been frequently recovered in some studies, such as *S. sciuri, S. capitis, S. intermedius*, and *S. hominis* (Davidson et al., 1992; Chaffer et al., 1999; Sampimon et al., 2009b). Some studies have indicated that the species distribution of CNS causing IMI in dairy herds
may be influenced by specific management practices, such as the use of teat disinfection (Hogan et al., 1987) and housing conditions (White et al., 1989), as well as by the parity of the cows (Taponen et al., 2006; Thorberg et al., 2009). Alternatively, the lack of consistency between studies in CNS species distribution found in bovine milk samples may be attributed to regional differences, or to the limited accuracy and reproducibility of the phenotypic identification methods generally used.

1.3.3. Coagulase-negative staphylococci and heifer mastitis

A great number of dairy heifers develop IMI in the period around calving. Considerable variation in quarter infection prevalence has been observed between studies, ranging between 29-75% prepartum and 12-46% at parturition (Fox, 2009). Mastitis in primiparous heifers is an economically important disease, as the presence of IMI caused by major pathogens in these young animals impairs mammary gland development and thus future performance. Moreover, the frequency of heifer mastitis is higher in well-managed herds with high mean production and low SCC (Myllys and Rautala, 1995). The pathogens most commonly associated with clinical mastitis in heifers are largely the same as those in mature cows, being S. aureus, Streptococcus sp., and coliforms (Waage et al., 1999; Tenhagen et al., 2006; Piepers et al., 2010), but the primary cause of subclinical IMI in heifers are CNS (Fox, 2009).

In contrast with most mastitis pathogens, CNS are isolated more frequently from heifers than from multiparous cows (Matthews et al., 1992; Tenhagen et al., 2006; Taponen et al., 2007; Schukken et al., 2009; Sampimon et al., 2009a). In an American study, as much as 75% of quarters of unbred and primigravid heifers were reported to be infected, and CNS accounted for 67% of the bacteria isolated (Trinidad et al., 1990b). Matthews et al. (1992) reported that 35.5% of colostrum samples were positive for staphylococci, with S. chromogenes, S. aureus, and S. simulans being the most isolated species.

In general, prevalence of CNS IMI is highest at calving and decreases in the course of first lactation (Matthews et al., 1992; Aarestrup and Jensen, 1997; Piepers et al., 2010). Matthews et al. (1992) reported a CNS quarter prevalence of 38.9, 27.8, 15.3, 14.6, 13.2, 15.3, and 14.6% prepartum, at parturition, and weeks 1 to 5 of lactation, respectively, in primiparous cows. In a Belgian study, as many as 80% of fresh heifers
Chapter 1

Review of the literature

had at least one subclinically infected quarter, of which 72% were due to CNS, but during the first week in lactation, more than half of these CNS isolated at the start of lactation could not be cultured again during the second sampling within one week (Piepers et al., 2010). In prepartum heifers in Denmark, *S. chromogenes* was isolated from 15% of all quarters, but its prevalence decreased to 1% after parturition (Aarestrup and Jensen, 1997).

Typically, CNS IMI in heifers are associated with subclinical or mild clinical mastitis, but *S. chromogenes*, *S. hyicus*, and *S. simulans* have been associated with clinical signs (Trinidad et al., 1990b; Waage et al., 1999). Infections with major pathogens are known to have a significant effect on future performance of the heifer. Although CNS cause the majority of IMI in heifers, they are not considered to have a significant impact on future milk production or udder health (Piepers et al., 2009a). On the contrary, CNS infections in freshly calved heifers have been associated with higher milk yields and a lower clinical mastitis incidence in first lactation (Piepers et al., 2010).

1.3.4. Clinical characteristics and impact on SCC and milk production of CNS

Mastitis caused by the major pathogen *S. aureus* has been associated with local and systemic clinical signs, serious lesions, and significant milk production losses (Trinidad et al., 1990a; Waage et al., 1999). By contrast, IMI with CNS are generally subclinical or mildly clinical, and cause only a moderate increase in SCC. Despite the mild nature of CNS IMI, harmful effects have been demonstrated. Trinidad et al. (1990a) reported that quarters of heifers infected with CNS exhibited greater leukocyte infiltration and more interalveolar stroma compared to uninfected controls, resulting in reduced secretory activity. Benites et al. (2002) studied the mammary parenchymas of slaughtered dairy cows culled due to mastitis and reported that presence of CNS was associated with chronic inflammatory response, mostly followed by repair, and mild tissue lesions.

Numerous studies report a significant but moderate increase in SCC in quarters with CNS IMI compared to uninfected quarters (Timms and Schultz, 1987; Hogan et al., 1987; Davidson et al., 1992; Chaffer et al., 1999; Taponen et al., 2007; Gillespie et al., 2009; Schukken et al., 2009; Sampimon et al., 2009b; Piepers et al., 2010; Paradis et al., 2010). Elevation of SCC is estimated to be 2- to 3-fold the number of somatic cells in milk from healthy quarters (Oliver and Jayarao, 1997), although increases up to 10-fold have
been reported (Taponen et al., 2007). Most studies indicate that at the cow-level, CNS infections might be relatively unimportant, but when prevalence of CNS infections in low SCC herds is high, they might significantly contribute to the BMSCC (Rainard et al., 1990; Davidson et al., 1992; Schukken et al., 2009; Piepers et al., 2009a; Sampimon et al., 2009b).

The migration of leucocytes into the parenchyma and the subsequent phagocytosis processes potentially have damaging effects on secretory tissue of lactating cows, and the negative effect of elevated SCC on milk production has been well established (Jones et al., 1984; De Vliegher et al., 2005). Timms and Schultz (1987) estimated that CNS infected animals had a 821 kg decrease (8.7%) of mature equivalent lactation milk production compared with uninfected animals. Still, Paradis et al. (2010) found that the presence of CNS infections in the first month of lactation had a negative effect on test-day SCC during the entire first lactation, but no significant effect on milk yield. In experimental CNS mastitis models, cows challenged with S. chromogenes developed mild clinical mastitis with local signs, and milk production decreased on average 16.3% during 7 days post-challenge (Simojoki et al., 2009).

Surprisingly, several researchers have observed higher milk production in CNS infected animals relative to uninfected animals (Schukken et al., 2009; Thorberg et al., 2009; Piepers et al., 2010). Furthermore, Grohn et al. (2004) reported that mature cows that developed clinical CNS mastitis during lactation are significantly higher producers before onset of mastitis than their uninfected counterparts. These findings suggest that high-producing cows are likely more prone to CNS infections, and that production losses due to CNS could have been previously underestimated.

There is debate about whether specific CNS species are more pathogenic than others. In some studies, differences in the milk SCC have been observed between CNS species (Sampimon et al., 2009b), but not in others (Hogan et al., 1987). Experimental challenge with S. simulans resulted in a slightly stronger innate immune response than for S. epidermidis (Simojoki et al., 2011). Furthermore, S. hyicus and S. chromogenes have also been described as being more pathogenic than other CNS species (Myllys, 1995; Zhang and Maddox, 2000). Thorberg et al. (2009) reported that the majority of persistent IMI with S. chromogenes, S. epidermidis, and S. simulans induce a mild to strong inflammatory reaction as measured by the California mastitis test (CMT), whereas IMI with other CNS species is mostly non-persistent and has a low CMT score.
Contrasting, Taponen (2006) did not find differences in severity of IMI caused by different CNS species.

1.3.5. Persistence of CNS intramammary infections

In many cases, CNS IMI disappear shortly after parturition (Myllys and Rautala, 1995; Aarestrup and Jensen, 1997), although prepartum CNS IMI can persist into lactation (Taponen et al., 2007). Spontaneous cure rates of CNS IMI are believed to be high (Harmon et al., 1986; Deluyker et al., 2005; Taponen et al., 2006). Nonetheless, many CNS infections have a long duration and often persist until the end of lactation (Timms and Schultz, 1987; Rainard et al., 1990; Chaffer et al., 1999; Taponen et al., 2007).

In a single American herd, a mean duration of 222 days has been found for 86 persisting CNS IMI, of which 55% were caused by S. chromogenes (Todhunter et al., 1993). Of 63 CNS IMI detected in a Finish herd, about half were found to persist, and no clear difference in duration between the two predominating CNS species, S. chromogenes and S. simulans, was reported (Taponen et al., 2007). In another study, prepartum S. simulans IMI seemed to persist more frequently into lactation than infections with S. chromogenes and S. epidermidis, suggesting S. simulans is better adapted to the bovine mammary gland (Aarestrup and Jensen, 1997). Thorberg et al. (2009) sampled cows twice, one month apart, and found that 58 to 76% of IMI with S. chromogenes, S. epidermidis, and S. simulans persist, whereas most IMI with other CNS species are eliminated within one month. Infections with S. epidermidis were mainly transient in one study (Aarestrup and Jensen, 1997), but persistent in others (Taponen et al., 2007; Thorberg et al., 2009).

The factors that lead to elimination or persistence of infection can be both host- and pathogen-dependent. Some cows seem to be able to easily eliminate CNS infections, whereas others are not, but the host factors involved are hard to determine. Alternatively, some CNS species or strains might be better adapted to the udder than others. Possible explanations for persistence of bacteria in the mammary gland are the adhesion to mammary epithelial cells, biofilm formation, and intracellular survival, which have been demonstrated in vitro for several CNS species (Almeida and Oliver,
2001; Tormo et al., 2005; Anaya-Lopez et al., 2006; Hyvonen et al., 2009). However, little is known about these mechanisms in vivo.

1.3.6. Protective potential of coagulase-negative staphylococci against mastitis with major pathogens

There is discussion on the importance of eliminating infections by minor pathogens, because several studies have indicated that quarters infected with C. bovis or CNS are less susceptible to infection by major pathogens (Pankey and Nickerson, 1985; Rainard and Poutrel, 1988; Matthews et al., 1990; Matthews et al., 1991; Nickerson and Boddie, 1994; Lam et al., 1997). Multiple studies have shown that naturally occurring CNS infections afford protection against challenge with major mastitis pathogens, although the effect differs depending on the species of the pathogen used for experimental challenge (Pankey and Nickerson, 1985; Nickerson and Boddie, 1994). It appears that CNS infected quarters are protected against challenge with S. aureus, but are at an increased risk to become infected with S. agalactiae (Matthews et al., 1990; Nickerson and Boddie, 1994). Under field conditions as well, reduced susceptibility to new infections has been observed in quarters with preexisting CNS infections (Rainard and Poutrel, 1988; Matthews et al., 1991). In addition, a significantly lower incidence rate of clinical mastitis has been observed during first lactation of CNS infected heifers at calving compared to non-infected heifers (Piepers et al., 2010).

Teat apex colonization (TAC) by CNS has also been suggested to exert a protective effect (De Vliegher et al., 2003; Piepers et al., 2011). Heifers with teat apices colonized by S. chromogenes have significantly lower odds of having a quarter milk SCC >200,000 cells/ml the first days after calving (De Vliegher et al., 2003). Prepartum TAC with CNS also protects heifers against IMI caused by major environmental and contagious pathogens in early lactation (Piepers et al., 2011).

Proposed mechanisms for the protection afforded by minor pathogens establishing TAC or IMI are: competitive exclusion of other mastitis pathogens, production of inhibitory substances, or the stimulation of the udders’ innate immunity (Woodward et al., 1987; Rainard and Poutrel, 1988; Nascimento et al., 2005; Piepers et al., 2009b). Bovine strains of S. chromogenes, S. epidermidis, S. simulans, S. saprophyticus, S. hominis, and S. arlettae have been shown to produce antimicrobial substances that inhibit growth
of some major mastitis pathogens, including *S. aureus* (De Vliegher *et al.*, 2004; Nascimento *et al.*, 2005). Heifers from which the teat apices are colonized by CNS prior to calving also have an increased number of viable milk polymorphonuclear neutrophilic leukocytes (PMN) in early lactation, indicating better defences against invading pathogens than for non-colonized teats (Piepers *et al.*, 2009b).

In other reports it is claimed that there is no benefit in having CNS IMI to prevent new major pathogen infections (Matthews *et al.*, 1991; Zadoks *et al.*, 2001), or that CNS infected quarters even have an increased risk for subsequent infections with major pathogens (Hogan *et al.*, 1988; Lam *et al.*, 1997; Green *et al.*, 2002). Increased rates of environmental streptococcal mastitis in quarters infected with CNS compared to uninfected controls have been reported (Hogan *et al.*, 1988). However, inconsistent results of repeated trials have been obtained even within studies (Pankey and Nickerson, 1985; Hogan *et al.*, 1988), and the role of CNS IMI in the development of new IMI by major pathogens is inconclusive. The observed discrepancies between studies may be attributed to differences in experimental challenge, study models, or CNS species involved.

### 1.3.7. Risk factors for CNS intramammary infections

In a Belgian risk factor study, poor heifer hygiene, lack of teat dipping prior to calving, and a non-clipped udder were found to increase the odds of IMI with CNS in heifers (Piepers *et al.*, 2011). Poor heifer hygiene in the latter study was a risk factor both for IMI with CNS and environmental major pathogens, suggesting the CNS involved are of environmental origin. In a Dutch study, significant herd-level risk factors for increased prevalence of CNS IMI in lactating cows include source of drinking water not being tap water, pasturing during the outdoor season, percentage of stalls contaminated with milk, and BMSCC >250,000 cells/ml (Sampimon *et al.*, 2009b). The nature of some of these risk factors also seems to indicate that the CNS infections in the herds originate from the environment.

Bacteria establishing IMI are believed to enter the mammary gland through the teat canal, and there is a likely association between numbers of organisms on teat ends and incidence of new infections. Surprisingly, TAC with CNS in prepartum heifers has not been found associated with an increased risk of CNS IMI in early lactation (Piepers *et al*., 2009b).
al., 2011). However, no species-specific information of the CNS involved in IMI and TAC was available. Similarly, no association has been found between TAC with *S. chromogenes* in prepartum heifers and IMI in early lactation with the same pathogen (De Vliegher *et al.*, 2003). These findings suggest that either TAC with CNS does not lead to IMI, or that other CNS species are primarily involved in TAC than in IMI.
1.4. Epidemiology of coagulase-negative staphylococci

1.4.1. Reservoirs of CNS in dairy farms

Coagulase-negative staphylococci not only cause IMI, but are also natural colonizers of teat and udder skin, teat canals, mucosa, and other bovine body sites (White et al., 1989; Trinidad et al., 1990b; Taponen et al., 2008). Furthermore, non-S. aureus staphylococci are among the most isolated organisms from teat apices and bedding samples (Rendos et al., 1975). In a few studies CNS species from extramammary reservoirs in dairy farms have been speciated. In Table 1-2, the main CNS species occurring in niches in the dairy farm that could act as sources are represented.

**Table 1-2.** Most commonly isolated coagulase-negative *Staphylococcus* species from extramammary reservoirs in dairy farms.

<table>
<thead>
<tr>
<th>Reservoir</th>
<th>Most isolated CNS species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streak canals</td>
<td><em>S. chromogenes</em>, <em>S. warneri</em>, <em>S. xylosus</em></td>
<td>(White et al., 1989)¹</td>
</tr>
<tr>
<td></td>
<td><em>S. chromogenes</em>, <em>S. hyicus</em></td>
<td>(Trinidad et al., 1990b)²</td>
</tr>
<tr>
<td></td>
<td><em>S. chromogenes</em>, <em>S. simulans</em>, <em>S. hominis</em>, <em>S. epidermidis</em></td>
<td>(Matthews et al., 1992)²</td>
</tr>
<tr>
<td>Teat skin</td>
<td><em>S. xylosus</em>, <em>S. sciuri</em>, <em>S. warneri</em>, <em>S. haemolyticus</em></td>
<td>(Devriese and Dekeyser, 1980)¹</td>
</tr>
<tr>
<td></td>
<td><em>S. xylosus</em>, <em>S. warneri</em>, <em>S. chromogenes</em></td>
<td>(White et al., 1989)¹</td>
</tr>
<tr>
<td>Udder skin</td>
<td><em>S. haemolyticus</em>, <em>S. xylosus</em>, <em>S. cohnii</em></td>
<td>(Baba et al., 1980)¹</td>
</tr>
<tr>
<td>Other body sites</td>
<td><em>S. xylosus</em>, <em>S. chromogenes</em>, <em>S. warneri</em>, <em>S. sciuri</em></td>
<td>(White et al., 1989)¹</td>
</tr>
<tr>
<td></td>
<td><em>S. equorum</em>, <em>S. sciuri</em>, <em>saprophyticus</em>, <em>S. xylosus</em></td>
<td>(Taponen et al., 2008)²,³</td>
</tr>
<tr>
<td>Bedding</td>
<td><em>S. xylosus</em>, <em>S. sciuri</em>, <em>saprophyticus</em>, <em>S. cohnii</em></td>
<td>(Matos et al., 1991)¹</td>
</tr>
<tr>
<td>Milkers' hands</td>
<td><em>S. epidermidis</em></td>
<td>(Thorberg et al., 2006)¹</td>
</tr>
<tr>
<td></td>
<td><em>S. xylosus</em></td>
<td>(Taponen et al., 2008)²,³</td>
</tr>
</tbody>
</table>

Species identification according to: ¹a modified biochemical identification scheme (Kloos and Schleifer, 1975; Devriese and Dekeyser, 1980; Devriese et al., 1985; Langlois et al., 1988); ²the API Staph-Trac system; ³Ribotyping.

*Staphylococcus xylosus*, *S. sciuri*, *S. saprophyticus*, and *S. cohnii* are found as predominating *Staphylococcus* species in bedding samples, and are described as “environmental staphylococci” (Matos et al., 1991). The same species are also isolated from various bovine body sites, such as teat and udder skin, the hair coat, and the nares (White et al., 1989). The CNS species occurring in milk mainly differ from those of
extramammary samples, although species common in milk are also frequently isolated from teat skin, teat orifices, and teat canals, especially *S. chromogenes* (Devriese and Dekeyser, 1980; White *et al.*, 1989; Trinidad *et al.*, 1990b; Matthews *et al.*, 1992; Taponen *et al.*, 2008). Conversely, several species generally regarded as environmental, such as *S. xylosus* and *S. sciuri*, are also isolated from bovine IMI, but usually in smaller numbers than those typically associated with IMI (Davidson *et al.*, 1992; Boerlin *et al.*, 2003; Thorberg *et al.*, 2009). Most studies on extramammary CNS reservoirs have been conducted before molecular identification methods were commonly available (Table 1-2). Consequently, reliable data on the CNS species distribution in extramammary sources based on accurate identification methods is lacking, which is yet indispensable for proper reservoir and source investigation.

### 1.4.2. Strain typing methods for coagulase-negative staphylococci

Strain typing is a valuable tool for the epidemiological investigation of bacterial infections. Strain typing of pathogenic species recovered from different time points or origins (*e.g.* infected glands, body or environmental sites, other animal species) can provide information on persistence of infections, population structure, infection sources, and transmission routes. Besides, the repeated isolation of a particular strain from an infection is clinically/epidemiologically more significant than the repeated isolation of the same species. Strain variation may also be associated with a relevant difference in virulence or epidemiology, and typing can contribute to a better understanding of bacterial pathogenicity and mechanisms for host-adaptation. Typing is ideally done by a polyphasic approach, using a combination of different geno- or phenotypic typing methods to draw final conclusions on intra-specific genetic diversity. The more “conventional” methods previously applied for epidemiological typing of *S. epidermidis*, such as phage typing, biotyping, serotyping, antibiogram, or plasmid profiling have been largely replaced by genotypic methods, which greatly differ in taxonomic resolution, discriminatory power, reproducibility, and applicability (Figure 1-2; Parisi, 1985; Savelkoul *et al.*, 1999; van Belkum *et al.*, 2007).
In paragraph 1.2.3., a number of genotypic methods were discussed that are mainly applicable for the differentiation of CNS at the (sub)species level (Figure 1-2). The fingerprinting methods with a broader taxonomic range, such as AFLP, random amplification of polymorphic DNA-PCR (RAPD-PCR), and pulsed-field gel electrophoresis (PFGE) can additionally be used for strain typing. AFLP and RAPD can be generally applied and allow fast and high-throughput analysis of isolates. Nonetheless, these PCR-based typing methods have some limitations regarding reproducibility (RAPD) and interpretation of complex fingerprints (AFLP). Contrasting, PFGE, which is based on macrorestriction analysis of the genomic DNA with a rare-cutting enzyme, has excellent discriminatory power and intra- and interlaboratory reproducibility, and clear guidelines for interpretation of polymorphisms have been described (Tenover et al., 1995). Although PFGE is technically demanding and expensive, it has become a standard method for comparative typing of many human and animal bacterial pathogens (van Belkum et al., 2007). Multiple locus sequence typing (MLST), a sequence based typing method that establishes the sequence variation in seven staphylococcal household genes, has also become a valuable epidemiological typing method for bacterial
Chapter 1

Review of the literature

pathogens (http://www.MLST.net). The great advantage of MLST is that sequence data are unambiguous and that the allelic profiles of isolates can easily be compared to those in a central database on the internet. This is in contrast to most other typing procedures, which involve the subjective comparison of DNA fragment sizes. However, a major drawback is that amplification schemes have to be optimized for each individual species, and at present only schemes for strain typing of S. aureus and S. epidermidis are available (Enright et al., 2000; Thomas et al., 2007).

1.4.3. Molecular epidemiology of coagulase-negative staphylococci from cattle

Several studies using highly discriminatory typing methods, including PFGE and MLST, have been conducted to study the epidemiology of S. aureus mastitis (Fitzgerald et al., 1997; Roberson et al., 1998; Zadoks et al., 2000; Zadoks et al., 2002; Smith et al., 2005a; Fournier et al., 2008). By contrast, studies determining the genetic diversity within CNS species from cattle are limited. An important prerequisite to conduct epidemiologic research is the accurateness of species identification. Unfortunately, in most studies in which bovine CNS isolates have been genotyped, unreliable phenotypic methods were used for species identification (Thorberg et al., 2006; Gillespie et al., 2009; Rajala-Schultz et al., 2009; Sawant et al., 2009). When isolates are falsely assumed to belong to the same CNS species and are subsequently genotyped, this will lead to misinterpretation of typing results.

In a few studies, bovine CNS isolates belonging to the most common species (S. epidermidis, S. hyicus, S. chromogenes, and S. simulans) have been investigated by molecular methods (Aarestrup et al., 1999; Thorberg et al., 2006; Gillespie et al., 2009; Rajala-Schultz et al., 2009; Sawant et al., 2009). Aarestrup et al. (1999) found two predominating S. simulans ribotypes causing IMI among isolates originating from 37 herds, indicating that the discriminatory power of ribotyping might be too limited to conduct epidemiologic studies. Among the S. epidermidis isolates from multiple cows in two herds, PFGE using Smal macrorestriction identified one to two predominating pulsotypes causing IMI per herd, in addition to a variety of other pulsotypes (Thorberg et al., 2006). This study indicated that specific S. epidermidis strains disseminate within these herds, and that a good discriminatory power was obtained by PFGE. The persistence of particular CNS strains in the mammary gland has been confirmed at the
strain level in several studies, in which identical CNS genotypes determined by PFGE, ribotyping, or AFLP have been repeatedly recovered from the same quarter for long periods (Aarestrup et al., 1999; Taponen et al., 2007; Gillespie et al., 2009; Sawant et al., 2009; Rajala-Schultz et al., 2009). In only two studies, CNS from IMI have been genotypically compared with those from extramammary sources in the dairy farm (Thorberg et al., 2006; Taponen et al., 2008). Using PFGE, the milker’s hands and bovine skin have been found as potential sources of *S. epidermidis* and *S. chromogenes* IMI, respectively (Thorberg et al., 2006; Taponen et al., 2008).

Given the niche-preference of particular CNS species, the transmission routes and potential sources of CNS causing IMI might differ among species (Table 1-2). At present, the epidemiology of CNS is poorly understood, making the recommendation of control measures difficult. Further study based on accurate species identification and molecular typing will be needed to improve our understanding of the sources and transmission routes of the different CNS species relevant to udder health.
1.5. Antimicrobial resistance of coagulase-negative staphylococci

1.5.1. Monitoring antibiotic resistance in mastitis pathogens

Mastitis is the number one reason for antimicrobial drug use in dairy cattle, as it is a major component both in mastitis prevention (dry-cow treatment) and therapy. Antimicrobial use in dairy cows has consequences in terms of residues in the milk, but has also been associated with the risk of selecting antimicrobial resistance. Nonetheless, the prevalence of resistant mastitis pathogens is generally low compared to human pathogens, and as of now, no evidence for an increasing trend in antimicrobial resistance in major mastitis pathogens has been established (Erskine et al., 2002; Pitkala et al., 2004; Erskine et al., 2004; Botrel et al., 2010). Still, the occurrence of resistant strains in food-producing animals is regarded as a potential risk both for public and animal health. Antimicrobial agents used to treat animals are essentially the same classes of compounds as those used in human medicine, and resistant bacteria can be transferred from infected or colonized animals to humans who are in close contact with them. Another concern is the potential transmission of resistant bacteria in the dairy food chain, e.g. via non-pasteurized milk. Furthermore, determinants conferring resistance might be exchanged from harmless commensals to bacteria that are more pathogenic to man or animals.

In many surveillance studies, agar disk diffusion (Kirby-Bauer method) has been used to evaluate resistance patterns of mastitis pathogens (Erskine et al., 2002; Gentilini et al., 2002; Osteras et al., 2006; Botrel et al., 2010). However, interpretation of inhibition zone sizes is not always consistent between studies and does not correlate well with minimal inhibitory concentration (MIC) values determined by standard dilution methods. Currently, determination of exact MIC values using broth dilution or agar diffusion (e.g. Etest) methods is preferred, as the MIC is a more meaningful quantitative measure allowing for valid interpretation and comparison among studies (Watts and Yancey, 1994; Kloos and Bannerman, 1994; Erskine et al., 2004).

To categorize bacteria as susceptible or resistant, either clinical break points or epidemiological cut-off values for the zone size or MIC can be used. Clinical breakpoints
are based on microbiological, clinical and pharmacological aspects, and are used to predict clinical outcome after treatment with a particular antimicrobial agent. However, for most bacterium-antibiotic combinations, no veterinary breakpoints have been determined, and those for human pathogens are the only ones available for use. However, their value in predicting efficacy of mastitis treatment has been questioned (Owens et al., 1997; Schwarz et al., 2010). Furthermore, susceptibility data of veterinary pathogens is used to monitor the prevalence of resistant strains, providing epidemiological information on the spread of antimicrobial resistance within bacterial species. Epidemiological cut-off values delineate wild-type strains of a certain species from strains that have acquired phenotypic resistance. The MIC distribution and epidemiological cut-offs for a large number of bacterium-antibiotic combinations from multiple sources and time periods has been made available by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org).

1.5.2. Antimicrobial resistance in coagulase-negative staphylococci from cows

A great part of the success of staphylococci as opportunistic pathogens comes from their extraordinary capacity to adapt to changing environmental conditions, and to cope with selective pressure by developing or acquiring resistance. The fact that CNS are an increasing problem in human nosocomial infections has been attributed in part to an increase in antimicrobial resistance (Kloos and Bannerman, 1994; Archer and Climo, 1994; Otto, 2004). The common use of antimicrobials might have led to the selection of more resistant CNS, and a similar trend might explain the increase in CNS causing IMI in cows as well. A direct correlation has been found between resistance patterns of CNS isolated from IMI, and the antimicrobial drug usage in the studied animals and herds, suggesting that selective pressure is an important factor in the development of resistant CNS in dairy farms (Rajala-Schultz et al., 2004). It has been shown that CNS isolates from multiparous cows that have received dry-cow treatment have higher odds of being resistant than isolates from primiparous cows never exposed to antimicrobials (Trinidad et al., 1990c; Rajala-Schultz et al., 2004). In addition, CNS isolates from conventional farms were more likely to be resistant to ampicillin, penicillin, pirlimycin, and tetracycline than those from organic herds (Pol and Ruegg, 2007).
Susceptibility studies on staphylococci isolated from bovine IMI have revealed considerable herd differences in resistance patterns (Owens and Watts, 1988; Trinidad et al., 1990c), but overall penicillin resistance is most common, with percentages ranging between 27 and 57% (Todhunter et al., 1993; Owens et al., 1997; Gentilini et al., 2002; Devriese et al., 2002; Pitkala et al., 2004; Rajala-Schultz et al., 2004; Botrel et al., 2010). Penicillin resistance of staphylococci is achieved primarily by the production of β-lactamase encoded by the blaZ gene that inactivates the drug. Resistance to ampicillin, tetracycline, erythromycin, and lincomycin has also been reported in bovine CNS isolates, but prevalence of resistance to other antimicrobials than penicillin is relatively low (Owens and Watts, 1988; Salmon et al., 1998; Botrel et al., 2010).

An important resistance mechanism in staphylococci is that encoded by the mecA gene, referred to as “methicillin resistance” (MR). Expression of mecA confers multi-resistance to all classes of penicillins, but also to cephalosporins, carbapenems, and monobactams. Phenotypic resistance to oxacillin is considered a good indicator for MR, but the standard method for MR detection is the confirmation of mecA carriage by PCR or DNA hybridization (Archer and Climo, 1994; Kloos and Bannerman, 1994; Fessler et al., 2010). Methicillin-resistant CNS (MR-CNS) not only are resistant to all β-lactam antibiotics, but have been shown to be more frequently resistant to antimicrobials of other classes as well (Archer and Climo, 1994; van Duijkeren et al., 2004; Moon et al., 2007). Moon et al. (2007) reported that MR-CNS isolates from bovine milk samples are significantly more resistant to kanamycin, gentamicin, and tetracycline than methicillin-susceptible CNS. Despite the low prevalence of MR-CNS originating from cows’ milk, ranging between 2.4 and 10% (Gentilini et al., 2002; Pitkala et al., 2004; Moon et al., 2007), colonization of food-producing animals by MR staphylococci is considered of major importance. Besides concerns that MR strains may disseminate to humans, IMI with MR-CNS may limit therapeutic options, if ever needed, and some researchers even recommend culling of animals colonized by MR-CNS (Gentilini et al., 2002).

Although CNS are less virulent than S. aureus, they generally exhibit higher antimicrobial resistance, and are more often multi-resistant (Owens and Watts, 1988; Archer and Climo, 1994; Costa et al., 2000; Martineau et al., 2000). Owens and Watts (1988) evaluated staphylococcal resistance patterns and found that percentages of bovine CNS strains resistant to ampicillin, erythromycin, oxacillin, and streptomycin are considerably higher than for S. aureus strains. In a Dutch study, 41% of CNS isolates
from milk expressed resistance to a single antimicrobial class, and 11% were resistant to multiple drug classes (Sampimon et al., 2011). Nevertheless, antimicrobial resistance in CNS infecting bovine udders is not regarded as a therapeutic problem. Whereas mastitis caused by *S. aureus* often responds poorly to antimicrobial therapy, infections with non-*aureus* staphylococci are generally easy to treat (Sears and McCarthy, 2003). However, failure to eliminate CNS IMI after dry-cow antimicrobial therapy has been reported (Harmon et al., 1986; Owens et al., 1997; Rajala-Schultz et al., 2009).

Some authors report no difference in antimicrobial susceptibility between CNS species (Rajala-Schultz et al., 2004; Capurro et al., 2009), whereas others have demonstrated significant differences in resistance patterns (Sawant et al., 2009; Sampimon et al., 2011). In one report, penicillin resistance was significantly more common in bovine *S. epidermidis* isolates than in other CNS species, as was the case for erythromycin resistance in *S. equorum*, and oxacillin resistance in *S. xylosus* (Sampimon et al., 2011). In several reports, multi-resistance has been found more frequently in *S. epidermidis* than in other CNS species (Owens and Watts, 1988; Sawant et al., 2009; Sampimon et al., 2011). Differences in the occurrence of the meca gene between CNS species have also been demonstrated, emphasizing the importance of accurate species identification of CNS (Fessler et al., 2010; Sampimon et al., 2011). In a recent study, *S. epidermidis* and *S. haemolyticus* represented the most frequently observed species among MR-CNS isolated from bovine mastitis (Fessler et al., 2010). Given the potential differences in susceptibility between CNS species, variations in response to therapy might occur, but this has not yet been demonstrated.

In addition to being a potential pathogen, resistant CNS could function as a donor of resistance genes to other CNS, or to the more virulent coagulase-positive species *S. aureus*, which shares the same habitat. Similar as for CNS, *S. aureus* has been isolated from IMI, bovine skin, and the environment (Roberson et al., 1998; Fox et al., 2005). The occurrence of identical resistance determinants within the staphylococcal group indicates that staphylococci share the same gene pool, and that horizontal gene transfer mechanisms are effectively used under in vivo conditions (Archer and Climo, 1994; Werckenthin et al., 2001; Schwarz and Chaslus-Dancla, 2001). However, the current knowledge on the role of staphylococci of bovine origin as reservoirs or recipients of resistance determinants is limited.
1.5.3. Biocide resistance in coagulase-negative staphylococci

Coagulase-negative staphylococci include normal skin microbiota of teats and the udder, and post-milking teat disinfection has been shown effective in reducing the incidence of new CNS IMI (Hogan et al., 1987; Oliver et al., 1990; Hogan et al., 1995). In natural exposure trials, implementation of chlorhexidine-based teat dips reduce the incidence of new CNS infections by 49 to 58% (Oliver et al., 1990; Hogan et al., 1995). It appears that teat dipping not only reduces the number of new CNS IMI, but also affects the distribution of CNS species involved (Hogan et al., 1987). Herds applying post-milking teat disinfection appear to have lower prevalence of S. epidermidis IMI, but a higher prevalence of IMI caused by S. hyicus. In herds dipping with chlorhexidine, S. xylosus IMI is overrepresented compared to herds not dipping, or using iodine or linear dodecyl benzene sulfonic acid teat dips. Possibly, susceptibility of individual CNS species to specific biocides might differ, but this has not been documented.

Increased tolerance to disinfectants in staphylococci is mostly conferred by integral drug transporter systems, and efflux mechanisms common in staphylococci are those encoded by the qac or smr genes (Russell, 1997; Hassan et al., 2007). In a Norwegian study, CNS resistant to quaternary ammonium compounds (QAC) have been isolated from 21% of bulk milk samples originating from 127 cattle herds. A variety of QAC-resistant CNS species have been identified, but the most prevalent are S. haemolyticus and S. warneri (Bjorland et al., 2005). Furthermore, one particular pulsotype of QAC-resistant S. warneri has been repeatedly recovered from quarter milk samples of a large proportion of cows in a single herd during a 3.5 year period, indicating that disinfectant resistance might play a role in the spread and persistence of staphylococci within herds (Bjorland et al., 2006). Although disinfectants are widely used in dairy herds, prevalence of biocide resistance in CNS associated with bovine IMI is hardly studied.

In many herds where post-milking teat disinfection is routinely implemented, the prevalence of CNS IMI remains high, indicating there are limitations to the effectiveness of this procedure in reducing the incidence of CNS IMI. Teat disinfection has no effect on duration of existing IMI, and has not been equally effective against all types of mastitis pathogens. Whereas new IMI by contagious pathogens such as S. agalactiae and S. aureus is effectively prevented by implementing teat disinfection (Boddie et al., 1997; Foret et
al., 2003), reduction of new IMI by environmental mastitis pathogens is less successful (Pankey et al., 1984; Hogan et al., 1995). This apparent ineffectiveness against environmental pathogens is assumed to be related to their epidemiological behaviour, rather than to insusceptibility for the used disinfectants (Pankey et al., 1984). Contagious pathogens are transmitted primarily during the milking process, and new infections can be effectively reduced by implementation of good milking hygiene procedures, including post-milking teat dipping. By contrast, environmental pathogens can come into contact with the teat skin between milkings, when the biocidal activity of teat dips has diminished (Pankey et al., 1984). The latter is also true for CNS, which are ubiquitous on the bovine udder and in the cows’ environment, which provide a constant source of bacteria. Whether resistance to disinfectants plays a role in CNS epidemiology remains to be determined.
1.6. Virulence factors of coagulase-negative staphylococci

1.6.1. Virulence potential of coagulase-negative staphylococci

The virulence potential of a bacterial strain is attributed to the combined effect of extracellular factors such as toxins, together with the adhesive and invasive properties mediated by components on the bacterial cell wall. A plethora of studies have been conducted on virulence traits and pathogenesis of *S. epidermidis*, which is accountable for most of the nosocomial CNS infections in humans (for review see: von Eiff *et al.*, 2002; Otto, 2004; Otto, 2009). The most important defence mechanism of CNS is to hide behind a layer of extracellular matrix by forming a biofilm. The genotypic basis of staphylococcal biofilm formation and its complex regulation have been studied most extensively for *S. aureus* and *S. epidermidis* (O’Gara, 2007). The biofilms of a few other CNS species associated with human infections (de Silva *et al.*, 2002; Fredheim *et al.*, 2009) and some food-related CNS species (Moretro *et al.*, 2003; Planchon *et al.*, 2006) have been studied to a lesser extent.

Literature concerning host-bacterium interactions and pathogenesis of CNS in the bovine mammary gland is scarce. However, the current knowledge on virulence of human CNS isolates can be used to analogously examine bovine CNS isolates.

1.6.2. Biofilm formation in bovine staphylococci

A biofilm is defined as a complex of adherent bacterial cells colonizing (biological) surfaces, encased by self-produced polymeric matrix, and biofilm formation is an important step in colonizing host tissue (Pace *et al.*, 2006). Better adherence to the mammary tissue might provide a selective advantage by protecting bacteria against the shear forces during milking. Furthermore, the biofilm components protect bacteria against phagocytosis and antimicrobials. It has been demonstrated that resistance to many therapeutic agents *in vitro* is strongly increased in staphylococci growing in biofilms (Cucarella *et al.*, 2004; Cerca *et al.*, 2005a; Melchior *et al.*, 2007).
For *S. aureus*, biofilm formation has been recognized as an important virulence factor in bovine mastitis, as strains that are associated with milk are more likely to produce a biofilm as compared to strains from extramammary sources (Fox et al., 2005). Low cure rates for chronic *S. aureus* IMI have been reported despite *in vitro* susceptibility, indicating that a protective mechanism is in play *in vivo* (Owens et al., 1997). Biofilm-producing *S. aureus* have an increased capacity to colonize the mammary gland, but clinical signs are generally less severe than for non-producing strains (Baselga et al., 1993; Cucarella et al., 2004). Consequently, low SCC cows can represent a reservoir of bacteria with biofilm-forming capacity and low early pathogenicity, making detection of infected quarters and eradication difficult. Biofilm formation of CNS from bovine mastitis has rarely been addressed, although some features suggest a biofilm mode of growth, *e.g.* the persistent nature of certain CNS causing IMI (Chaffer et al., 1999; Taponen et al., 2007) and the occasional failure of antimicrobial therapy (Owens et al., 1997; Rajala-Schultz et al., 2009).

Two surface components have been identified as important factors involved in staphylococcal biofilm formation: the polysaccharide intercellular adhesin (PIA) and the biofilm-associated protein (Bap). The icaADBC genes (intercellular adhesion operon) mediate the biosynthesis of the matrix exopolysaccharide PIA, which is composed of linear β-1-6-linked N-acetylglucosamine residues, and plays a major role in cell-cell adhesion. Its association with virulence in clinical *S. epidermidis* strains has been well established (Mack et al., 1996; Ziebuhr et al., 1997; Frebourg et al., 2000; Kozitskaya et al., 2005; Klingenberg et al., 2007). The ica operon has been identified in a variety of other CNS species associated with nosocomial infections (Cramton et al., 1999; de Silva et al., 2002) and food processing environments (Moretro et al., 2003), indicating a wide distribution of this gene cluster among CNS species. Furthermore, the ica operon has been found associated with methicillin resistance in *S. epidermidis* strains causing mastitis in dairy cows (Sawant et al., 2009), and in *S. caprae* strains from goat milk (Allignet et al., 2001). However, the role of PIA in pathogenesis of CNS from mastitis has not been studied.

Bap is a large surface protein (2,276 amino acids) first identified in strong biofilm-forming *S. aureus* strains from chronic bovine mastitis (Cucarella et al., 2001). *Staphylococcus aureus* isolates possessing the *bap* gene are strong biofilm formers and are more often associated with persisting bovine IMI (Cucarella et al., 2004). It is

[32]
speculated that the Bap protein is projected from the cell surface, promoting interaction with abiotic surfaces, host cell components, or other bacteria (Cucarella et al., 2001). Bap homologues are also identified in S. epidermidis, S. chromogenes, S. hyicus, S. simulans, and S. xylosus isolates from ruminant mastitis with hybridization using the bap gene of S. aureus as a probe (Tormo et al., 2005). Bap is implicated in primary attachment as well as in the following step of bacterial aggregation, possibly in cooperation with PIA (Cucarella et al., 2001; Cucarella et al., 2004). However, Bap can also mediate biofilm formation in the absence of PIA, indicating that expression of bap represents an alternative mechanism of biofilm formation (Tormo et al., 2005). Proteins of the Bap family typically contain a signal sequence for extracellular secretion and a core domain (C-region) with a variable number of C-repeats of 86 amino acids (Cucarella et al., 2004; Lasa and Penades, 2006). Even within strains, the number of these C-repeats has been shown to change during infection, and it is suggested that the existence of alternative forms of the Bap protein could also be related to evasion of the host immune system. In serum samples taken from animals with confirmed S. aureus infection, the presence of anti-Bap antibodies has been demonstrated, strongly indicating that Bap is produced during infection and plays a role in S. aureus pathogenesis (Cucarella et al., 2004).

It is clear that staphylococci may use different approaches to form a biofilm. In several studies, the presence of ica or PIA production did not fully correlate with in vitro biofilm production, indicating that other mechanisms are likely involved (Mack et al., 1996; Allignet et al., 2001; Vasudevan et al., 2003; Chokr et al., 2006). Other factors implicated in biofilm formation of S. epidermidis include the accumulative associated protein (Aap), autolysin (AtlE), teichoic acids, and other staphylococcal surface proteins (Gotz, 2002; de Araujo et al., 2006; O’Gara, 2007). Whether biofilm formation plays a major role in the pathogenesis of CNS species associated with bovine IMI is unknown.

1.6.3. Other virulence factors

CNS usually do not cause much damage to the host. However, several studies have indicated that CNS from humans and bovine mastitis produce factors that induce inflammatory responses or damage host tissue (Otto, 2004). Several toxins have been described in S. aureus, including staphylococcal enterotoxins (SE), exfoliative toxins
(ET), and toxic shock syndrome toxin-1 (TSST-1). These so called superantigens (SAg) have emetic activity after oral administration, leading to occasional food poisoning outbreaks caused by toxin-producing *S. aureus*. In addition to their importance for public health and food safety, SAg from bovine *S. aureus* isolates have an immunosuppressive role and likely favor the establishment of persistent IMI (Ferens *et al.*, 1998). Superantigens typically associated with the major pathogen *S. aureus* are also found in bovine CNS. In a large scale study investigating the prevalence and distribution of 19 SAg genes among CNS isolates from bovine IMI, it appeared that these are widely distributed (Park *et al.*, 2011b). Of 263 CNS tested, 31.2% had one or more SAg genes, of which the majority was *S. chromogenes*.

Production of elastase and haemolysins, as well as collagen- and lactoferrin-binding capacity by CNS from bovine IMI has been demonstrated (Watts and Owens, 1987; Watts *et al.*, 1990; Naidu *et al.*, 1990). A cytotoxic protein has been identified in CNS from clinical and subclinical mastitis, which is produced at high levels especially in *S. chromogenes* strains (Zhang and Maddox, 2000). Invasion and intracellular survival in mammary epithelial cells has also been demonstrated *in vitro* for CNS mastitis isolates (Almeida and Oliver, 2001; Anaya-Lopez *et al.*, 2006; Hyvonen *et al.*, 2009). All these factors could contribute to persistence in the host, degradation of host tissue, immune evasion, or spreading of infection, but their exact role in CNS mastitis should be further investigated.
CHAPTER 2

OBJECTIVES

The conflicting findings on the relevance of CNS infections indicate that certain CNS species might be considered true mastitis pathogens, whereas others might be harmless. Because of their increasing importance as subclinical mastitis agents, it is necessary to recognize these differences, and this can only be done by evaluating CNS as individual species. CNS have always been regarded as skin opportunistic pathogens, although more recently it has been indicated that CNS IMI might originate from environmental sources. However, information on CNS species living in environmental niches in the dairy farm is scarce. Furthermore, there is a great lack of knowledge regarding the contribution of known virulence factors of CNS to the pathogenicity of bovine isolates.

The main objectives of this thesis are:

1. to develop and validate a CNS species identification method based on amplified fragment length polymorphism (AFLP) genotyping (Chapter 3).
2. to establish the biodiversity of CNS species associated with bovine IMI and living freely in the dairy farm environment, as determined by AFLP (Chapter 4).
3. to study the epidemiology of IMI-causing CNS species by means of two genotypic typing methods, and assess the possible role of the environment as a source of CNS IMI (Chapter 5).
4. To evaluate the potential role of antimicrobial resistance and biofilm formation in CNS pathogenicity (Chapter 6).
CHAPTER 3

VALIDATION OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISM GENOTYPING FOR IDENTIFICATION OF BOVINE ASSOCIATED COAGULASE-NEGATIVE *STAPHYLOCOCCUS* SPECIES

V. Piessens\(^1\), K. Supré\(^2\), M. Heyndrickx\(^1\), F. Haesebrouck\(^3\), S. De Vliegher\(^2\), and E. Van Coillie\(^1\)

\(^1\)Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium; \(^2\)Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium; \(^3\)Department of Pathology, Bacteriology, and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

3.1. Abstract

In many countries, coagulase-negative staphylococci (CNS) are currently the most common cause of intramammary infections in lactating cows. To elucidate the importance of various CNS species in udder health and milk quality, further research conducted on the species level is required. Phenotypic identification of CNS species appears to be unreliable and more accurate and reproducible genotypic methods are needed. In the current study, the use of amplified fragment length polymorphism (AFLP) genotyping was validated for species identification of bovine associated CNS.

An initial reference library was generated with AFLP fingerprints of 52 different CNS type and reference strains. Next, 247 bovine CNS field isolates with known species identity were analyzed. These field isolates had been previously identified by gene sequencing and were randomly divided into two subsets, *i.e.* a training set and a validation set. The training set was identified against the initial reference library containing only type and reference strains, which resulted in a typeability of 80.5%. Accuracy of the AFLP identifications, being the correspondence with gene sequencing results, was 95.0%. Fingerprints of the training set were then added to the initial library and identification of the validation set was done by means of this extended library. By adding bovine CNS to the library, the performance of the AFLP identification method improved considerably. Final typeability and accuracy were 98.4% and 99.2%, respectively.

Numerical analysis of AFLP fingerprints proved to be an accurate genotypic method for identification of CNS from bovine origin. The constructed AFLP library provides a useful identification tool for field studies on the subject of CNS.
3.2. Introduction

In Flanders, Belgium and in numerous other countries, coagulase-negative staphylococci (CNS) are currently the most prevalent bacteria isolated from milk samples of both healthy cows and cows affected by mastitis (Osteras et al., 2006; Tenhagen et al., 2006; Piepers et al., 2007; Rajala-Schultz et al., 2009; Sampimon et al., 2009b). Though most CNS infections remain subclinical and are left untreated, CNS have also been associated with clinical mastitis and persistent intramammary infections (Taponen et al., 2007; Gillespie et al., 2009). In the past, CNS were considered a homogenous group of mildly pathogenic bacteria that occasionally caused mastitis. However, now that major mastitis pathogens are largely controlled as a result of mastitis prevention programs, CNS have emerged as a frequent cause of (mild) inflammation and increased milk somatic cell count (SCC) (Pyörälä and Taponen, 2009). In quarters infected with CNS, increase of SCC is generally moderate compared to quarters infected with major mastitis pathogens, but is significantly higher than in uninfected quarters (Gillespie et al., 2009; Schukken et al., 2009). Consequently, in herds with low bulk tank milk SCC, CNS infections may be an important contributor to the total SCC (Schukken et al., 2009).

To date, approximately 50 Staphylococcus species and subspecies have been characterized. Moreover, new bovine related previously un-described Staphylococcus spp. have recently been defined (Supré et al., 2010; Taponen et al., 2011). Many different CNS species have already been associated with bovine mastitis, but epidemiological studies of accurately identified individual CNS species have rarely been done. In many studies and in routine diagnostics, CNS are merely confirmed as non-aureus staphylococci. The lack of identification beyond the genus level results in a loss of information with regard to mammary pathogenicity of individual CNS species. Important differences may exist, but little is known about virulence factors of various CNS species. To add to the confusion, some protective aspects have been suggested for CNS (Matthews et al., 1990; De Vliegher et al., 2003; De Vliegher et al., 2004). However, studies are not conclusive, as some show a protective effect (Linde et al., 1980; Lam et al., 1997) and others do not (Hogan et al., 1988). The lack of accurate species differentiation could explain part of these conflicting findings. Since clinical relevance of
CNS is now expected to be more substantial than previously thought, their pathogenic and/or protective potential should be assessed further relying on profound species-specific research.

Several rapid phenotypic identification methods are commercially available for staphylococci, but these are designed for speciation of clinical human isolates rather than bovine associated CNS (Grant et al., 1994; Heikens et al., 2005). Their accuracy can be poor for some common bovine CNS species and identification results can vary between methods. To overcome these problems, genotypic methods with higher accuracy and reproducibility are necessary. Various DNA-based methods have been developed to study the phylogeny of *Staphylococcus* species and to improve their identification, including sequencing of housekeeping genes, such as *hsp60* (Goh et al., 1996), 16S rRNA gene (Takahashi et al., 1999), *rpoB* (Drancourt and Raoult, 2002b), *gap* (Ghebremedhin et al., 2008), *sodA* (Poyart et al., 2001) and *tuf* genes (Martineau et al., 2001), ribotyping (Bes et al., 2000; Taponen et al., 2008), tRNA-intergenic spacer PCR (Maes et al., 1997; Supré et al., 2009), and 16S-23S rRNA-intergenic spacer PCR (Mendoza et al., 1998; Bes et al., 2000). Most described methods are not designed to differentiate all known *Staphylococcus* species, but several have been proven useful in accurately identifying clinical CNS isolates from humans and animals (Maes et al., 1997; Mendoza et al., 1998; Heikens et al., 2005; Supré et al., 2009).

In the current study, amplification fragment length polymorphism (AFLP) genotyping was evaluated for species identification of bovine CNS isolates. The objectives of this study were to construct a staphylococcal AFLP library and to improve and validate the performance of a library-based AFLP identification method using bovine CNS field isolates.

## 3.3. Materials and Methods

### 3.3.1. Bacterial isolates

Fifty-four validly described type and reference strains, representing 49 staphylococcal species and subspecies (Table 3-1), were analyzed for the construction of an AFLP reference library. *Macrococcus caseolyticus* (Kloos et al., 1998) was also
included in the study, because it is a related species, previously classified as *Staphylococcus caseolyticus*, that is occasionally isolated from cattle. To validate the performance of the AFLP identification method, a total of 247 well characterized bovine CNS field isolates, including six *M. caseolyticus* isolates, were analyzed. These CNS field isolates belonged to 18 different species and were isolated from teat apices from heifers (n = 75) and from milk samples of heifers and cows with or without mastitis (n = 172) in the course of previous field studies (Supré et al., 2009). Accurate species identification of these field isolates has previously been done by sequencing of the *rpo*B gene and additional housekeeping genes if necessary (in the following order: *hsp60*, 16S rRNA gene, and *tuf*) (Supré et al., 2009). In the study presented here, species identification of the field isolates based on these gene sequences was considered as the reference method (CLSI, 2007a; Zadoks and Watts, 2009). All bacterial strains were kept as frozen stocks at –80°C in Brain Heart Infusion broth (Oxoid Ltd., Basingstoke, Hampshire, UK) with 15% (w/v) glycerol.

### 3.3.2. Genomic DNA extraction

Strains were cultured overnight at 37°C on Tryptone Soy Agar plates (Oxoid Ltd.) and checked for purity the next day. Bacterial DNA was extracted by the method described by Flamm et al. (1984) with slight modifications. Briefly, colonies were scraped from plates and suspended in 100 µl 0.01 M sodium phosphate buffer (pH 7.0) with 20% sucrose and lysozyme (4 mg/ml; Roche, Basel, Switzerland). To each cell suspension, 5 µl of lysostaphin (1 mg/ml; Sigma-Aldrich, St. Louis, MO) was added and cells were lysed during 45 min at 37°C. Next, 100 µl proteinase K (2.5 mg/ml; Promega Corporation, Madison, WI) was added in a mixture with 200 µl TE buffer (0.05 M Tris, 0.02 M EDTA) and 100 µl sarkosyl solution (5% in TE buffer; Sigma-Aldrich). Cell lysates were incubated at 37°C for 1 h and subsequently at 80°C for 5 min. Samples were deproteinized by phenol/chloroform extraction, after which remaining RNA in the DNA extract was degraded with 2.5 U DNase-free RNase (Stratagene, La Jolla, CA) for 1 h at 37°C. DNA was then precipitated with ethanol and resuspended in water. Nucleic acid concentration and purity were measured using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).
Table 3-1. Type and reference strains used for construction of the initial library.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus subsp. anaerobius</em></td>
<td>LMG 22203&lt;sup&gt;T&lt;/sup&gt;</td>
<td>sheep, abscess</td>
</tr>
<tr>
<td><em>S. aureus subsp. aureus</em></td>
<td>DSM 20231&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, pleural fluid</td>
</tr>
<tr>
<td><em>S. arlettae</em></td>
<td>LMG 19114&lt;sup&gt;T&lt;/sup&gt;</td>
<td>poultry, pleural fluid</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>ATCC 33753&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, ear</td>
</tr>
<tr>
<td><em>S. capitis subsp. capitis</em></td>
<td>CCM 2734&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. capitis subsp. urealyticus</em></td>
<td>ATCC 49326&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td>CCM 3573&lt;sup&gt;T&lt;/sup&gt;</td>
<td>goat, milk</td>
</tr>
<tr>
<td><em>S. carnosus subsp. carnosus</em></td>
<td>DSM 20501&lt;sup&gt;T&lt;/sup&gt;</td>
<td>dry sausage</td>
</tr>
<tr>
<td><em>S. carnosus subsp. utilis</em></td>
<td>DSM 11676&lt;sup&gt;T&lt;/sup&gt;</td>
<td>fermented fish sauce</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>NCTC 10530&lt;sup&gt;T&lt;/sup&gt;</td>
<td>pig, skin</td>
</tr>
<tr>
<td><em>S. cohnii subsp. cohnii</em></td>
<td>DSM 20260&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. cohnii subsp. urealyticum</em></td>
<td>ATCC 49330&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. condimenti</em></td>
<td>DSM 11674&lt;sup&gt;T&lt;/sup&gt;</td>
<td>soy sauce mash</td>
</tr>
<tr>
<td><em>S. delphini</em></td>
<td>DSM 20771&lt;sup&gt;T&lt;/sup&gt;</td>
<td>dolphin</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>LMG 10474&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, nose</td>
</tr>
<tr>
<td><em>S. equorum subsp. equorum</em></td>
<td>DSM 20674&lt;sup&gt;T&lt;/sup&gt;</td>
<td>horse, skin</td>
</tr>
<tr>
<td><em>S. equorum subsp. linens</em></td>
<td>DSM 15097&lt;sup&gt;T&lt;/sup&gt;</td>
<td>cheese</td>
</tr>
<tr>
<td><em>S. felis</em></td>
<td>ATCC 49168&lt;sup&gt;T&lt;/sup&gt;</td>
<td>cat, ear</td>
</tr>
<tr>
<td><em>S. fleurettii</em></td>
<td>CCM 4922&lt;sup&gt;T&lt;/sup&gt;</td>
<td>goat, milk</td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>CCM 3572&lt;sup&gt;T&lt;/sup&gt;</td>
<td>chicken, skin</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>CCM 2737&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. hominis subsp. hominis</em></td>
<td>DSM 20328&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, skin</td>
</tr>
<tr>
<td></td>
<td>CCM 2732</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. hominis subsp. novobiosepticus</em></td>
<td>DSM 15614&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, blood</td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td>CCM 2368&lt;sup&gt;T&lt;/sup&gt;</td>
<td>pig, epidermitis</td>
</tr>
<tr>
<td></td>
<td>DSM 19100</td>
<td>cow, mastitis</td>
</tr>
<tr>
<td></td>
<td>DSM 19101</td>
<td>cow, mastitis</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>CCM 5739&lt;sup&gt;T&lt;/sup&gt;</td>
<td>pigeon, nares</td>
</tr>
<tr>
<td><em>S. kloosii</em></td>
<td>DSM 20676&lt;sup&gt;T&lt;/sup&gt;</td>
<td>squirrel, skin</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>ATCC 29070&lt;sup&gt;T&lt;/sup&gt;</td>
<td>goat, udder</td>
</tr>
<tr>
<td><em>S. lugdunensis</em></td>
<td>ATCC 43809&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, lymph node</td>
</tr>
<tr>
<td><em>S. lutrae</em></td>
<td>LMG 22566&lt;sup&gt;T&lt;/sup&gt;</td>
<td>otter, mammary gland</td>
</tr>
<tr>
<td><em>S. muscae</em></td>
<td>DSM 7068&lt;sup&gt;T&lt;/sup&gt;</td>
<td>fly</td>
</tr>
<tr>
<td><em>S. nepalensis</em></td>
<td>DSM 15150&lt;sup&gt;T&lt;/sup&gt;</td>
<td>goat, nares</td>
</tr>
<tr>
<td><em>S. pasteuri</em></td>
<td>ATCC 51129&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, vomit</td>
</tr>
<tr>
<td><em>S. piscifermentans</em></td>
<td>SK03&lt;sup&gt;T&lt;/sup&gt;</td>
<td>fermented shrimp</td>
</tr>
<tr>
<td><em>S. pulvereri</em></td>
<td>CCM 4481&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, hip infection</td>
</tr>
<tr>
<td><em>S. saprophyticus subsp. bovis</em></td>
<td>DSM 18669&lt;sup&gt;T&lt;/sup&gt;</td>
<td>cow, nares</td>
</tr>
<tr>
<td><em>S. saprophyticus subsp. saprophyticus</em></td>
<td>LMG 13350&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, urine</td>
</tr>
<tr>
<td><em>S. schleiferi subsp. coagulans</em></td>
<td>ATCC 49545&lt;sup&gt;T&lt;/sup&gt;</td>
<td>dog, ear</td>
</tr>
<tr>
<td><em>S. schleiferi subsp. schleiferi</em></td>
<td>ATCC 43808&lt;sup&gt;T&lt;/sup&gt;</td>
<td>human, jugular catheter</td>
</tr>
<tr>
<td><em>S. sciuri subsp. carnaticus</em></td>
<td>DSM 15613&lt;sup&gt;T&lt;/sup&gt;</td>
<td>sliced veal leg</td>
</tr>
<tr>
<td><em>S. sciuri subsp. rodentium</em></td>
<td>CCM 4657&lt;sup&gt;T&lt;/sup&gt;</td>
<td>rat</td>
</tr>
</tbody>
</table>
Chapter 3

Validation of AFLP genotyping

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATCC/LMG/CCM/CNRS</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. sciuri</em> subsp. <em>sciuri</em></td>
<td>ATCC 29062</td>
<td>squirrel, skin</td>
</tr>
<tr>
<td><em>S. simiae</em></td>
<td>LMG 22723</td>
<td>monkey, faeces</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>CCM 2705</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. succinus</em> subsp. <em>casei</em></td>
<td>LMG 22186</td>
<td>cheese</td>
</tr>
<tr>
<td><em>S. succinus</em> subsp. <em>succinus</em></td>
<td>LMG 22185</td>
<td>plant and soil</td>
</tr>
<tr>
<td><em>S. vitulinus</em></td>
<td>CCM 4511</td>
<td>ground lamb</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>ATCC 27836</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>CCM 2738</td>
<td>human, skin</td>
</tr>
<tr>
<td><em>Macrococcus caseolyticus</em></td>
<td>CNRS E880066</td>
<td>cow, milk</td>
</tr>
<tr>
<td></td>
<td>CNRS E880071</td>
<td>animal</td>
</tr>
</tbody>
</table>

1LMG: Belgian Co-ordinated Collections of Microorganisms/Laboratory of Microbiology (BCCM/LMG), Ghent University, Belgium; CCM: Czech Collection of Microorganisms, Prague, Czech Republic; CNRS: Centre National de Références des Staphylocoques, Lyon, France; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ); Weringerode, Germany; NCTC: National Collection of Type Cultures, London, UK; T: type strain.

3.3.3. AFLP genotyping

The AFLP protocol and restriction site-specific adapter and primer sequences have been described by Keto-Timonen et al. (2003). Briefly, restriction was performed on 400 ng RNase-treated genomic DNA for 1.5 h at 37°C in a total volume of 20 µl, which consisted of both 15 U of HindIII and 15 U of MseI (New England Biolabs (NEB), Ipswich, MA), 2 µl of 10× NEB 2 buffer, 5 mM dithiothreitol (Promega) and 0.1 mg/ml bovine serum albumin (BSA; NEB). Ligation of adapters was done in a total volume of 40 µl by adding 4 µl 10× ligase buffer, 1 U T4 DNA ligase (Promega), 62.5 nM HindIII adapter, 625 nM MseI adapter (Eurogentec, San Diego, CA), and 0.1 mg/ml BSA to the digest and incubating at 16°C overnight. Pre-selective amplification was carried out in a total volume of 20 µl and PCR mixtures contained 4 µl of restricted and ligated template DNA, 25 nM Hind-0 primer, 125 nM Mse-0 primer (Eurogentec) and 15 µl Amplification Core Mix (Applied Biosystems, Foster City, CA). Pre-selective PCR products were diluted 1:20 in HPLC water and a selective PCR was performed in a 10 µl reaction mixture containing 1.5 µl of diluted template, 50 nM 6-FAM-labelled Hind + G primer, 250 nM Mse + C primer (Eurogentec), and 7.5 µl Amplification Core Mix. All PCR reactions were performed in a Gene Amp 9700 Thermal Cycler (Applied Biosystems) and thermal cycling conditions were as described by Keto-Timonen et al. (2003). Amplified fragments were separated by capillary electrophoresis on an ABI 3130 XL Genetic Analyzer (Applied Biosystems). Loading buffer was prepared by mixing 13.5 µl
deionised formamide and 0.5 µl GS-500 ROX standard (Applied Biosystems). This was mixed with 1 µl of selective amplification product, whereafter samples were heated for 3 min at 90°C and immediately put on ice before loading according to the manufacturer’s guidelines. For data collection during the run, GeneMapper® software (Applied Biosystems) was used. Peak data of both bacterial AFLP profiles and internal molecular mass standards were then imported into the BioNumerics version 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium) for normalization and similarity calculations. Genetic similarity of AFLP fingerprints was calculated based on the Pearson product-moment correlation coefficient. Only bands between 35 and 500 bp were considered for similarity calculations and position tolerance was set at 1.0%. In each executed AFLP run, a control DNA sample of the *S. felis* ATCC 49168^T* strain was co-analyzed as an internal reference. Dendrograms were deduced from similarity matrices using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm.

### 3.3.4. Repeatability of the AFLP procedure

An experiment was conducted to analyze the repeatability of the AFLP protocol. From four different type strains (*S. chromogenes* NCTC 10530^T*, *S. saprophyticus* subsp. *bovis* DSM 18669^T*, *S. warneri* ATCC 27836^T* and *S. xylosus* CCM 2738^T*) DNA was three times independently prepared, each time starting from a fresh culture taken from the frozen stock. All 12 DNA preparations were subjected to three separate AFLP reactions. The nine resulting AFLP fingerprints of each strain were then compared with the BioNumerics software.

### 3.3.5. Data analysis and identification strategy

For construction of a CNS library and calculation of genetic similarities, the ‘Libraries & Identification’ module in the BioNumerics software was used. The core of the AFLP identification method is the reference library, which is a collection of library units, which in turn is a selection of AFLP fingerprints in the database that represent a certain species. To create an initial library, all 54 type and reference strains (Table 3-1) were analyzed and fingerprints were stored in separate library units per species. Next, the 247 field isolates were subjected to AFLP and the data set was randomly divided into
two subsets, i.e. a training set and a validation set. Fingerprints of the training set (n = 123) were first identified based on this initial AFLP library. For identification of a given isolate, the software performs pairwise similarity calculations against all fingerprints available in the library. The cut-off value to identify an isolate as a certain CNS species was preliminary set at a minimum of 50% similarity to a type or reference strain (Taponen et al., 2006; Taponen et al., 2007). Only identification on the species level was validated, since subspecies of most bovine isolates were unknown.

Irrespective of the obtained AFLP identification results, fingerprints of the training set were then stored in the corresponding library unit with species identity as determined by the reference method. Subsequently, identification of the validation set (n = 124) was done by means of this extended library, after which the final performance of the AFLP identification method was evaluated. For identification of the validation set, maximum similarity (MS) was used as the statistical algorithm. With MS, isolates are assigned to the library unit (species) to which its most similar library strain belongs. This maximum similarity is used as an identification score and the same cut-off value was applied as for the training set (MS to a library unit ≥50%). To evaluate the performance of the AFLP identification system, typeability and accuracy were determined for both subsets, i.e. before and after the library extension. All isolates for which a species name was generated were considered typeable. Accuracy was defined as the correspondence of AFLP identification with species identification determined by sequencing of housekeeping genes (CLSI, 2007a; Zadoks and Watts, 2009).

For visual examination of all AFLP fingerprints and analysis of genetic distances between strains, dendrograms were generated using the UPGMA clustering algorithm. This algorithm uses mean averages of pairwise similarities, comparing all isolates included in clustering, while the library method compares each isolate separately against all fingerprints in the library. When Staphylococcus type strains are included in the dendrogram, UPGMA clustering provides an alternative approach for species identification of CNS field isolates.
3.4. Results

3.4.1. General features and repeatability of AFLP fingerprints

Each isolate analyzed by AFLP produced a unique genomic fingerprint of 30 to 70 bands that could be used for bacterial identification. All fingerprints of the control DNA sample used as internal reference showed between 91.2 and 98.2% similarity, which indicated that fingerprints between runs were comparable. Repeatability of the AFLP procedure, including DNA preparation, was confirmed in a separate experiment. For each of the four tested staphylococcal type strains, the similarity between the nine fingerprints ranged from 90.6 to 97.2%, indicating a good repeatability (Figure 3-1).

**Figure 3-1.** AFLP fingerprints obtained for the repeatability test conducted on four CNS type strains indicated high repeatability of the AFLP protocol. DNA was prepared for each strain on three time points starting from fresh cultures (t1, t2, and t3) and AFLP was performed three times on each DNA preparation (aflp1, aflp2, and aflp3). Clustering of the fingerprints was obtained by the unweighted pair group method with arithmetic
averages (UPGMA) of Pearson product-moment correlation coefficients. The similarity (%) of the nine resulting AFLP fingerprints per strain is given at the nodes of the corresponding clusters (♦). The vertical dotted line indicates the 90% similarity cut-off.

3.4.2. Validation of library-based species identification

In Table 3-2, the species distribution and an overview of the AFLP identification results of the training set (n = 123) are given. All *S. auricularis*, *S. epidermidis*, *S. fleurettii*, *S. hominis*, *S. hyicus*, *S. saprophyticus*, and *S. simulans* isolates were correctly identified using the initial library and 50% similarity as a cut-off. The majority of isolates belonging to the species *S. chromogenes*, *S. equorum*, *S. haemolyticus*, *S. sciuri*, and *S. xylosus* were readily identified, with percentages of correctly identified isolates between 66.7 and 94.6%. In total, 24 isolates of the training set, distributed over eight different species, remained unidentified. The most troublesome species to identify by AFLP were *S. cohnii*, *S. warneri*, and *M. caseolyticus* with respectively 55.6, 88.9, and 100.0% of isolates which could not be identified using only type and reference strains. Five isolates of the training set were misidentified. One *S. chromogenes* isolate was misidentified as *S. hyicus*, two *S. cohnii* isolates as *S. caprae*, one *S. cohnii* isolate as *S. pasteuri*, and one *S. haemolyticus* isolate as *S. hyicus*. To summarize, based on the initial AFLP reference library, typeability was 80.5%, i.e. 99 of 123 isolates of the training set could be assigned to a species name. Accuracy, being the correctness of all identifications given, was 95.0%.

After addition of all fingerprints of the training set to the correct library unit, the 124 isolates of the validation set were identified using this extended library. AFLP identification results of this set are reported in Table 3-3. The AFLP method now identified 121 isolates correctly (97.6%), of which 97 had an identification score of ≥80%. Two isolates, one *M. caseolyticus* and one *S. haemolyticus*, could not be identified because their AFLP fingerprints did not match with any of the library strains. Only one of the 124 isolates was misidentified, being an atypical *S. hyicus* isolate that had a pattern most similar to a bovine *S. sciuri* isolate of the training set. The library extension ultimately resulted in a typeability of 98.4% and an accuracy of 99.2% for AFLP identification.
### Table 3-2. Species identification results of the training set using amplification fragment length polymorphism (AFLP) analysis and the initial AFLP library.

<table>
<thead>
<tr>
<th>Species identified by sequencing&lt;sup&gt;1&lt;/sup&gt;</th>
<th>No. of representative field strains in training set</th>
<th>No. of strains (% of strains) showing most similarity with corresponding type or reference strain and an identification score&lt;sup&gt;2&lt;/sup&gt; of</th>
<th>No. of strains (% of strains) misidentified&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;50% (no ID&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>&gt;50% (correct ID)</td>
</tr>
<tr>
<td><strong>S. auricularis</strong></td>
<td>2</td>
<td>2 (100.0)</td>
<td>35 (94.6)</td>
</tr>
<tr>
<td><strong>S. chromogenes</strong></td>
<td>37</td>
<td>1 (2.7)</td>
<td>35 (94.6)</td>
</tr>
<tr>
<td><strong>S. cohnii</strong></td>
<td>9</td>
<td>5 (55.6)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td><strong>S. epidermidis</strong></td>
<td>9</td>
<td>1 (10.0)</td>
<td>9 (90.0)</td>
</tr>
<tr>
<td><strong>S. equorum</strong></td>
<td>10</td>
<td>3 (20.0)</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td><strong>S. fleuretii</strong></td>
<td>3</td>
<td>1 (100.0)</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td><strong>S. haemolyticus</strong></td>
<td>15</td>
<td>3 (20.0)</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td><strong>S. hominis</strong></td>
<td>1</td>
<td>1 (100.0)</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td><strong>S. hycus</strong></td>
<td>5</td>
<td>1 (100.0)</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td><strong>S. saprophyticus</strong></td>
<td>1</td>
<td>2 (33.3)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td><strong>S. sciuri</strong></td>
<td>6</td>
<td>2 (33.3)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td><strong>S. simulans</strong></td>
<td>5</td>
<td>2 (22.2)</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td><strong>S. warneri</strong></td>
<td>9</td>
<td>8 (88.9)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td><strong>S. xylosus</strong></td>
<td>9</td>
<td>2 (22.2)</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td><strong>M. caseolyticus</strong></td>
<td>2</td>
<td>2 (100.0)</td>
<td>94 (76.4)</td>
</tr>
</tbody>
</table>

<sup>1</sup>All field isolates were previously identified to species level by sequencing of housekeeping genes (*rpoB*, and if necessary additional genes were sequenced in the following order: *hsp60*, 16S rRNA gene, and *tuf*); 2The identification score for the training set is the percentage similarity to the best matching type or reference strain in the initial library; 3ID = Identification result using the initial library; 4Strains showing >50% similarity to a non-corresponding type or reference strain.
Table 3-3. Species identification results of the validation set using amplification fragment length polymorphism (AFLP) analysis and the extended AFLP library.

<table>
<thead>
<tr>
<th>Species identified by sequencing&lt;sup&gt;1&lt;/sup&gt;</th>
<th>No. of representative field strains in validation set</th>
<th>No. of strains (% of strains) showing highest similarity with corresponding library unit and an identification score&lt;sup&gt;2&lt;/sup&gt; of</th>
<th>No. of strains (% of strains) misidentified&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;50% (no ID&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>50-80% (correct ID)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. capitis</td>
<td>2</td>
<td>1 (50.0)</td>
<td>45 (97.8)</td>
</tr>
<tr>
<td>S. chromogenes</td>
<td>46</td>
<td>1 (2.2)</td>
<td>45 (97.8)</td>
</tr>
<tr>
<td>S. cohnii</td>
<td>2</td>
<td>2 (100.0)</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>12</td>
<td>1 (8.3)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>S. equorum</td>
<td>9</td>
<td>3 (33.3)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>S. fleuretti</td>
<td>4</td>
<td>1 (25)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>8</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>S. hyicus</td>
<td>8</td>
<td>7 (87.5)</td>
<td></td>
</tr>
<tr>
<td>S. neapolensis</td>
<td>1</td>
<td>1 (100.0)</td>
<td></td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>1</td>
<td></td>
<td>1 (100.0)</td>
</tr>
<tr>
<td>S. sciuri</td>
<td>9</td>
<td>2 (22.2)</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>S. simulans</td>
<td>4</td>
<td>1 (25.0)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>S. succinicus</td>
<td>3</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>S. warneri</td>
<td>3</td>
<td></td>
<td>3 (100.0)</td>
</tr>
<tr>
<td>S. xylosus</td>
<td>8</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>M. caseolyticus</td>
<td>4</td>
<td>1 (25.0)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>Total no. of strains</td>
<td>124</td>
<td>2 (1.6)</td>
<td>24 (19.4)</td>
</tr>
</tbody>
</table>

<sup>1</sup>All field isolates were previously identified to species level by sequencing of housekeeping genes (rpoB, and if necessary additional genes were sequenced in the following order: hsp60, 16S rRNA gene, and tuf); <sup>2</sup>The identification score for the validation set was the maximum similarity (%) to the best matching library unit of the extended library; <sup>3</sup>ID = Identification result using the extended library; <sup>4</sup>Correct ID with an intermediate identification score; <sup>5</sup>Correct ID with a good identification score; <sup>6</sup>Strains showing >50% maximum similarity to a non-corresponding library unit.
3.4.3. Cluster analysis of all CNS strains

Species determination based on clustering was less objective and user-friendly than pairwise similarity calculations against the AFLP reference library, due to anomalous clustering of atypical strains. Nevertheless, it is advisable to use both methods complementary, especially for fingerprints that result in low identification scores. Therefore, all identification results obtained with the library method were verified by evaluating clustering of all field isolates and type and reference strains. Fingerprints of isolates of 14 out of 18 analyzed Staphylococcus species showed relatively high homogeneity, which made their identification straightforward. By contrast, the species S. hyicus, S. cohnii, S. warneri, and M. caseolyticus showed more heterogeneous fingerprints and their representative strains clustered somewhat dispersed. In Figure 3-2, UPGMA clustering of a sample of the training set and part of the type strains is illustrated. The bovine S. hyicus isolates appeared to be divided into two separate clusters, of which one included the S. hyicus type strain (CCM 2368T) and the other the two reference strains originating from bovine mastitis (LMG 19100 and LMG 19101) (Figure 3-2). Similarity between the type strain and these two reference strains was very low (20.7%), so it seems that at least two S. hyicus AFLP types exist. The majority of S. cohnii (data not shown) and S. warneri isolates were mutually very similar, but had little similarity with their respective type strains (36.4 and 34.1%, respectively). The species M. caseolyticus on the other hand (data not shown), displayed five different AFLP types among nine analyzed strains in total and was consequently difficult to identify. However, it appeared that the resulting identification problems for these species were partially overcome by extending the library with bovine field isolates (Table 3-3). At the right hand side of Figure 3-2, library-based identification results of four isolates in the cluster, obtained using the initial library, are given as an example, i.e. two isolates of the training set that were correctly identified using only type and reference strains (S. xylosus KS-OS 2.17 and S. chromogenes KS-SP 53), one isolate that was reported as unidentified because similarity with the type strain was less than 50% (S. equorum KS-OS 2.30), and one isolate that was misidentified as S. hyicus (S. chromogenes KS-OS 3.22) and for which AFLP disagreed with gene sequencing.
Figure 3-2. Cluster analysis of AFLP fingerprints of a sample of CNS field isolates from the training set and part of the *Staphylococcus* type and reference strains. Clustering was obtained by the unweighted pair group method with arithmetic averages (UPGMA) of Pearson product-moment correlation coefficients. At the bottom of the figure, the molecular mass standard is depicted with corresponding fragment sizes (expressed as numbers of base pairs). The cluster cut-off value of 50% is indicated by the vertical dotted line and is considered to delineate relevant clusters. At the roots of clusters including a type or reference strain (♦), the percentage of similarity is given. As an example, identification results obtained with the library method are shown for four field isolates on the right hand side of the figure. \(^1\)Identification results using the initial library (identification scores expressed as percentage similarity to a type or reference strain); \(T\): type strain; \(R\): reference strain.


3.5. Discussion

CNS are a taxonomically diverse group of Gram-positive, mainly facultative anaerobes that have great adaptive nature and are able to persist and multiply in a variety of environments. As opportunistic pathogens, they cause a wide spectrum of diseases in both humans and animals (Piette and Verschraegen, 2009). Because of the increasing interest in the clinical significance of CNS in bovine mastitis, accurate identification of veterinary important CNS is desirable. CNS are recovered from milk and teat apices of cows of all ages, but the species responsible for intramammary infection in these age groups seem to partially differ (Taponen et al., 2006). Despite variations between herds and regions, *S. chromogenes*, *S. simulans*, *S. hyicus*, and *S. epidermidis* appear to be the most frequently isolated CNS species from mammary secretions (Luthje and Schwarz, 2006; Taponen et al., 2006; Gillespie et al., 2009; Sampimon et al., 2009b). However, variations in species prevalence exist between studies and these may be partly due to the identification methods used (Bes et al., 2000; Sampimon et al., 2009c). The superiority of genotypic methods over phenotypic methods for staphylococcal identification has been clearly demonstrated (Heikens et al., 2005; Capurro et al., 2009; Sampimon et al., 2009c). Genotypic identification methods have higher typeability and accuracy and enable construction of reference databases that can be updated with information on new species and atypical isolates, which is a lot more troublesome for conventional phenotypic methods (Zadoks and Watts, 2009).

In the current study, AFLP genotyping was used to develop an accurate identification method for bovine associated *Staphylococcus* species. Previous reports have shown the ability of AFLP to delineate genomic groups in a way comparative to DNA-DNA hybridization, which is currently the “gold standard” for species identification. For example, a strong correlation has been found between DNA-DNA hybridization results and similarity levels of AFLP fingerprints for *Xanthomonas* spp. (Rademaker et al., 2000). Recently, AFLP genotyping has been used for identification of CNS isolates from bovine milk in two related studies (Taponen et al., 2006; Taponen et al., 2007). In these studies, AFLP clustering-based identification has been compared with phenotypic API Staph ID 32 testing. The genotypic and phenotypic methods correspond for 66.0% of identified isolates in the first study and 71.9% in the second study, but
AFLP clustering of a number of isolates clearly does not correlate with the API Staph ID 32 species identification. However, this discrepancy has not been further investigated and real validation of AFLP as an identification tool for CNS species is still lacking.

During the present study AFLP was compared to sequencing of housekeeping genes, which is considered a warranted reference methodology (CLSI, 2007a; Zadoks and Watts, 2009), to validate the presented library-based AFLP identification method. All analyzed species were differentiated by their AFLP fingerprints, even species that are closely related according to hsp60, partial tuf, and 16S rRNA gene sequences, e.g. S. haemolyticus/S. hominis and S. capitis/S. epidermidis (Takahashi et al., 1999; Martineau et al., 2001; Kwok and Chow, 2003; Zadoks and Watts, 2009). It was demonstrated that the proposed criteria for library-based identification were appropriate and sufficiently accurate for species level identification of CNS isolates. While Taponen et al. (2006; 2007) were able to identify isolates belonging to nine different CNS species, the applicability of AFLP identification was extended to at least 18 different CNS species (including M. caseolyticus), which are considered relevant in CNS mastitis research. When the extended library was used, typeability of the AFLP method (98.4%) was higher than that of sequencing of only one housekeeping gene (93.6% for sequencing of tuf alone, 88.2% for rpoB alone) (Capurro et al., 2009; Supré et al., 2009), but was comparable with that of successive sequencing of multiple genes. Accuracy of AFLP identification with the extended library was 99.2%, which is practically as high as the accuracy of the reference method. In contrast, studies using phenotypic identification systems on CNS isolates show considerable misidentification, with error rates up to 28% (Capurro et al., 2009; Sampimon et al., 2009c).

AFLP analysis of the 124 isolates of the validation set showed that the discriminating power of this identification method increased considerably after adding bovine CNS isolates to the initial AFLP reference library. The same was observed when bovine CNS strains were added to the tRNA-intergenic spacer PCR fingerprint library in an analogous technical study by Supré et al. (2009). The high typeability and accuracy of the AFLP method could only be established by this library extension. For example, most bovine S. warneri isolates had AFLP fingerprints that differed considerably from that of the human S. warneri type strain, which initially resulted in identification problems for this species. However, after addition of bovine S. warneri isolates to the library, their correct identification was no longer impeded. Analogously, all except one of the 13
analyzed bovine *S. hyicus* isolates had AFLP fingerprints dissimilar to the porcine *S. hyicus* type strain. Similar results have been obtained with hybridization experiments using labeled staphylococcal *hsp60* gene sequences for *S. hyicus* species identification (Goh *et al.*, 1997). When pairwise hybridizations were performed with *hsp60* gene sequences of five *S. hyicus* isolates and the same *S. hyicus* type strain, the four bovine isolates appeared to cross-hybridize with each other, but not with the type strain and one other porcine isolate, which also cross-hybridized. Zadoks and Watts (2009) reported that the *rpoB* sequences of bovine *S. hyicus* isolates show little homology with the *rpoB* sequences of *S. hyicus* available in GenBank (<97%), though sequencing of additional genes (16S rRNA and *hsp60*) indicated that they are *S. hyicus* isolates with more than 99% sequence similarity. These observations with bovine *S. warneri* and *S. hyicus* isolates support the statement that intra-species diversity can exist in genetic profiles of isolates originating from various hosts (Zadoks and Watts, 2009). Furthermore, for the species *S. hyicus*, it was already stated that division into subspecies might be necessary, which is now re-enforced by the AFLP results. Screening of more *S. hyicus* isolates originating from various sources should further clarify the taxonomic classification of this species.

Generally, AFLP detects a large number of DNA polymorphisms and generates more bands than most other molecular techniques. For example, tRNA- and 16S-23S rRNA-intergenic spacer length analysis of staphylococci generate about 8 to 11 DNA fragments, as does ribotyping (Maes *et al.*, 1997; Mendoza *et al.*, 1998; Taponen *et al.*, 2008), while the AFLP fingerprints contained about 30 to 70 bands. These other techniques result in more simple fingerprints that are highly species-specific, but intra-species variation detected by these methods is limited. For sequencing of most of the housekeeping genes, discriminating power below the species level is also relatively low (Heikens *et al.*, 2005). Consequently, these methods are particularly useful for identification on the species level, but less suitable for sub-typing. On the other hand, with AFLP marked strain differences within species could be observed, although fingerprints were on the whole species-specific. This means that, to a certain extent, species identification and sub-typing can be done concurrently. However, to check the validity of subtype or subspecies differentiation by AFLP, a greater number of strains per species should be analyzed.
To clarify the role of individual CNS species in udder health, it is important to identify them accurately. Identification of CNS field isolates on a large scale is important in monitoring species distribution in herds, detecting possible reservoirs involved in bovine mastitis and understanding host-pathogen relationships. AFLP genotyping is a promising technique that enables construction of elaborate databases for identification and future reference. If additional sequencing of housekeeping genes is needed for confirmation of AFLP identification, AFLP clustering enables elimination of redundancy. Gene sequencing, the current reference method for CNS identification, depends in the first place on availability and quality of sequences in GenBank. The lack of high quality of deposited sequences and limited discriminating power for phylogenetically closely related species, *e.g.* for the 16S rRNA gene, can sometimes cause identification problems (Heikens *et al.*, 2005). Also, sequencing sometimes has to deal with failing amplification reactions, so that additional optimization is needed. AFLP has the advantage that a genomic fingerprint for numerical comparison and bacterial identification is always generated. The approach of using a library containing bovine strains next to type and reference strains can probably also be applied for other species than the 18 represented in the analyzed field collection, but this should also be validated. When enough relevant reference strains are included in the fingerprint library, simultaneous differentiation of all known *Staphylococcus* species should be possible.

### 3.6. Conclusions

It is concluded that the presented AFLP library-based method is accurate and reproducible for identification of bovine CNS species and provides a useful identification tool for future field studies on the subject of CNS.

**Acknowledgements**

This research was funded by the agency for Innovation by Science and Technology in Flanders (IWT-Vlaanderen, grant no. 60714). The authors would like to thank Ann Vanhee for her excellent laboratory support.
CHAPTER 4

DISTRIBUTION OF COAGULASE-NEGATIVE
STAPHYLOCOCCUS SPECIES FROM COWS’
MILK AND ENVIRONMENT DIFFERS
BETWEEN HERDS

V. Piessens¹, E. Van Coillie¹, B. Verbist¹, K. Supré², G. Braem³, A. Van
Nuffel¹, L. De Vuyst³, M. Heyndrickx¹, and S. De Vliegher²

¹Institute for Agricultural and Fisheries Research (ILVO), Technology and Food
Science Unit, Melle, Belgium; ²Department of Reproduction, Obstetrics, and Herd Health,
Faculty of Veterinary Medicine, Ghent University, Belgium; ³Research Group of
Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering
Sciences, Vrije Universiteit Brussel, Brussels, Belgium.

4.1. Abstract

In many parts of the world, coagulase-negative staphylococci (CNS) are the predominating pathogens causing intramammary infections (IMI) in dairy cows. The cows' environment is thought to be a possible source for CNS IMI and this was investigated in the present paper.

A longitudinal field study was carried out in six well-managed dairy herds to determine the distribution and epidemiology of various CNS species isolated from milk, causing IMI, and living freely in the cows' environment, respectively. On each herd, quarter milk samples from a cohort of ten lactating cows and environmental samples from stall air, slatted floor, sawdust from cubicles, and sawdust stock were collected monthly (n = 13). CNS isolates from quarter milk samples (n = 134) and the environment (n = 637) were identified to species level using amplified fragment length polymorphism (AFLP) genotyping.

Staphylococcus chromogenes, S. haemolyticus, S. epidermidis, and S. simulans accounted for 81.3% of all CNS milk isolates. Quarters were considered infected with CNS (positive IMI status) only when two out of three consecutive milk samples yielded the same CNS AFLP type. The species causing IMI were S. chromogenes (n = 35 samples with positive IMI status), S. haemolyticus (n = 29), S. simulans (n = 14), and S. epidermidis (n = 6). The observed persistent IMI cases (n = 17) had a mean duration of 149.4 days (range 63.0 to 329.8 days). The CNS species predominating in the environment were S. equorum, S. sciuri, S. haemolyticus, and S. fleurettii. Herd-to-herd differences in distribution of CNS species were observed both in milk and the environment, suggesting herd-level factors are involved in the establishment of particular species in a dairy herd.

Primary reservoirs of the species causing IMI varied. Staphylococcus chromogenes and S. epidermidis were rarely found in the environment, indicating that other reservoirs were more important in their epidemiology. For S. haemolyticus and S. simulans the environment was found as a reservoir, suggesting IMI with these species were possibly environmental in origin.
4.2. Introduction

The last twenty years, mastitis prevention programs (National Mastitis Council, 2009) have resulted in a better control of transmission of contagious mastitis pathogens in lactating dairy cows. In many well-managed dairy herds, the obtained decrease in prevalence of intramammary infections (IMI) caused by contagious major pathogens is, however, associated with a relatively higher proportion of subclinical or mild clinical infections caused by coagulase-negative staphylococci (CNS) (Tenhagen et al., 2006; Bradley et al., 2007; Piepers et al., 2007). Besides, CNS are also the major cause of IMI in heifers, the future milk producers of every dairy herd (Fox, 2009; Piepers et al., 2010). The change in distribution of mastitis pathogens suggests that the current mastitis control measures are less effective in reducing CNS IMI.

In general, CNS induce only a mild inflammatory reaction in infected quarters, as indicated by the modest increase in the milk somatic cell count (SCC) (Schukken et al., 2009). However, they can be a cause of (mild) clinical mastitis (Waage et al., 1999; Taponen et al., 2006) and quarters infected by CNS have been reported to be at greater risk for infection by major mastitis pathogens (Hogan et al., 1988; Lam et al., 1997). On the other hand, in some studies CNS IMI protect quarters against new infections by major mastitis pathogens (Rainard and Poutrel, 1988; Matthews et al., 1991). It has also been demonstrated that teat apex colonization with CNS protects heifers against high SCC and new IMI by major pathogens in early lactation (De Vliegher et al., 2003; Piepers et al., in press). Impact on quarter SCC is species-specific (Supré et al., 2011) and some CNS species are able to persist in the udder for long periods (Chaffer et al., 1999; Gillespie et al., 2009), prolonging their influence on the mammary gland. Whether this influence is harmful or advantageous remains unresolved and subject for debate.

A potential drawback of many studies in the past is the classification of CNS as one homogeneous group (Hogan et al., 1988; Nickerson and Boddie, 1994), which can partly explain the aforementioned contradictory findings on the pathogenic or protective role of CNS. Actually, CNS originating from cows include a variety of species with differences in antimicrobial susceptibility (Luthje and Schwarz, 2006; Sawant et al., 2009) and virulence factors (Park et al., 2011b). Therefore, evaluating the epidemiology of individual CNS species is of great importance to understand their respective
significance. Based on phenotypic characteristics, species identification of CNS isolates from cows is difficult and often inaccurate (Capurro et al., 2009; Sampimon et al., 2009c). Therefore, the last few years several genotypic methods have been developed allowing for more accurate identification of CNS species (Supré et al., 2009; Chapter 2; Braem et al., 2011).

High prevalence of prepartum CNS infections in heifers (Fox, 2009), which have not yet been exposed to the milking process, suggests that other sources than the milking machine exist. As CNS are abundantly free-living in dairy herds (Rendos et al., 1975; Matos et al., 1991), it can be hypothesized that the cows’ environment is a possible source for CNS causing IMI. The aims of the present study were to determine the species distribution of CNS originating from milk and living freely in the cows’ environment, evaluate the ability of the different CNS species to cause IMI, and identify their main reservoirs.

### 4.3. Materials and methods

#### 4.3.1. Herds and cows

During the period May 2008 to May 2009, a longitudinal field study was conducted in six Flemish dairy herds, referred to as herds A to F. Farms were well-managed and had comparable characteristics reflecting the general situation on Flemish dairy farms. An overview of herd and management characteristics of each farm is given in Table 4-1. The mean herd size was 70 Holstein cows (range 49 to 87) with an average production of 9,368 kg of milk per cow per year (range 7,147 to 11,665 kg) and an average bulk milk SCC of 137,725 cells/ml milk (range 85,615 to 202,154 cells/ml). On all six farms, cows were housed in free-stalls with slatted floors and sawdust-bedded cubicles. Sawdust bedding was removed two to three times a day and replaced by fresh sawdust from an indoor storage. Post-milking teat disinfection and dry-cow therapy were practiced in all herds.
Table 4-1. Herd and management characteristics if six Flemisch dairy farms involved in a longitudinal field study of 13 months (May 2008 to May 2009)

<table>
<thead>
<tr>
<th>Herd and management characteristics</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average herd size(^1)</td>
<td>76</td>
<td>87</td>
<td>62</td>
<td>63</td>
<td>49</td>
<td>83</td>
</tr>
<tr>
<td>Average production per cow per year (kg)</td>
<td>8,559</td>
<td>9,955</td>
<td>8,507</td>
<td>10,372</td>
<td>11,665</td>
<td>7,147</td>
</tr>
<tr>
<td>Average bulk milk SCC (cells/ml milk)</td>
<td>195,462</td>
<td>202,154</td>
<td>85,615</td>
<td>175,000</td>
<td>120,846</td>
<td>185,000</td>
</tr>
<tr>
<td>Incidence rate of clinical mastitis cases(^2)</td>
<td>0.180</td>
<td>0.236</td>
<td>0.347</td>
<td>0.485</td>
<td>0.290</td>
<td>0.149</td>
</tr>
<tr>
<td>Milking parlor</td>
<td>2×4</td>
<td>2×6</td>
<td>2×5</td>
<td>2×5</td>
<td>2×4</td>
<td>2×8</td>
</tr>
<tr>
<td>Bedding material</td>
<td>sawdust</td>
<td>sawdust</td>
<td>sawdust</td>
<td>sawdust</td>
<td>sawdust</td>
<td>sawdust or none</td>
</tr>
<tr>
<td>Use of pre-dip (yes/no)</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Post-milking dip/spray</td>
<td>spray</td>
<td>dip</td>
<td>dip</td>
<td>dip</td>
<td>spray</td>
<td>spray</td>
</tr>
<tr>
<td>Post-dip main component</td>
<td>iodine</td>
<td>iodine</td>
<td>chlorhexidine</td>
<td>iodine</td>
<td>lactic/caprylic/capric acid + lauricidin</td>
<td>lactic/salicylic acid or iodine(^3)</td>
</tr>
</tbody>
</table>

\(^1\)Average herd size during the 13 months of the study; \(^2\)Calculated as the number of quarter cases per 365 cow-days at risk; \(^3\)The farmers of herds E and F switched to an iodine post-dip product in the seventh month of the study.
At the beginning of the study, a cohort of ten clinically healthy cows was randomly selected in each herd within parity blocks (four heifers, three cows of second parity, and three cows of third or higher parity). Before the end of the study, 20 out of the 60 cohort cows were culled (ranging from 0 to 5 per herd) for a diversity of reasons, including problems with lameness, udder health, fertility, or milk production. All cohort cows culled before the 12th month of the study (n = 14) were replaced by randomly selected herd mates of the same parity. The cohort cows were by average 11.2 months under study.

4.3.2. Sample collection

Quarter milk samples were collected aseptically with monthly intervals (n = 13) from the cohort cows according to standard procedures (Hogan et al., 1999). Samples were frozen and transported to the laboratory of the Milk Control Centre Flanders (MCC, Lier, Belgium) for bacteriological examination. In addition to the monthly milk samples taken from the cohort cows, extra samples were taken when the farmer noticed clinical signs in a quarter (any visual abnormality of milk or the udder). Clinical milk samples were taken both from cows of the cohort and from cows outside the cohort. The average overall incidence rate of clinical mastitis (IRCM) (all pathogens) was calculated as by Barkema et al. (1998).

Environmental samples were taken once a month (n = 13) in each farm for isolation of CNS. Four different sample types were chosen in close proximity of the cows, i.e. the air in the free-stall, slatted floors, used sawdust bedding from cubicles, and unused sawdust from the storage. For isolation of staphylococci from the air in the free-stall, blank air strips (Biotest, Dreieich, Germany) were filled aseptically with mannitol salt agar (MSA, BD Biosciences, San Jose, CA), and kept at 4 °C until further use. At the time of sampling, an MSA-filled strip was removed from the sterile package, placed in the RCS Standard air sampler (Biotest) and exposed for 1 min, which was equal to sampling of 40 liters of air. The slatted floor was sampled by walking over a whole corridor, wearing polypropylene Sekuroka® overshoes (Fiers, Kuurne, Belgium), after which both overshoes were transferred to a sterile stomacher bag (180 x 300 mm, Medical Lab Service, Menen, Belgium). Used sawdust samples were collected from the
back one-third of 10% of the cubicles. Samples of the sawdust storage were collected at five random places in front of the pile using a sample bore (5.6 cm Ø - 50 cm long). Per herd and per sample type, collected sawdust was commingled and a subsample was transferred to a sterile stomacher bag. In one herd (herd F), no sawdust samples were collected during the first five months of the study, as the farmer did not use any bedding material in the cubicles at that time. All environmental samples were stored at 4 °C and transported the same day to the laboratory for processing.

4.3.3. Sample processing and isolation of CNS

4.3.3.1. Quarter milk samples

Bacteriological culture of quarter milk samples and bacterial identification was done in the MCC as recommended by the NMC (Hogan et al., 1999). Briefly, 0.01 ml of each quarter milk sample was spread on a quadrant of a blood-esculin agar plate and incubated aerobically at 37 °C ± 1 °C for 36 h ± 12 h. A quarter was considered culture-positive when growth of ≥1 colony was detected. A sample was considered contaminated when three or more dissimilar colony types were observed. Phenotypic differentiation of bacterial species was done as described by Piepers et al. (2007). Staphylococci were identified presumptively based on colony morphology, Gram-stain and positive catalase test. Staphylococcus aureus was differentiated from other Staphylococcus spp. based on morphology, pigmentation, haemolysis, and DNAse activity. All non-Staphylococcus aureus staphylococci were a priori considered as CNS. For milk samples yielding at least three CNS colonies (≥ 300 cfu/ml), two colonies were picked up and transferred to trypton soy agar (TSA, Oxoid Ltd., Basingstoke, Hampshire, UK) for further identification. When more than one type of CNS colonies was present, more colonies were picked up. TSA plates were incubated at 37 °C for 18 h.

4.3.3.2. Environmental samples

The same day of sample collection, exposed air strips were incubated at 37 °C for 24 to 48 h. Of each sawdust sample, 25 g was weighed and put in a new sterile stomacher bag. Overshoes were transferred to separate sterile stomacher bags. To each sawdust sample and overshoe, 225 ml of brain heart infusion broth (BHI, Oxoid Ltd.) +
Chapter 4  
Distribution of CNS species from cows’ milk and environment

7.5% NaCl was added. Sawdust samples were homogenized for 1 min in a stomacher and overshoes were massaged by hand for 1 min for equal distribution of the medium. Samples were then incubated for 20 h ± 4 h at 37 °C for selective enrichment of staphylococci. The next day, dilution series of the enrichment media were made in Ringers solution (Oxoid Ltd.), 100 µl of dilutions 10^{-2} to 10^{-5} were spread by use of sterile glass beads (2.5-3.5 mm, VWR International, West Chester, PA) on MSA plates, which were incubated for 20 h ± 4 h at 37 °C. After incubation, *Staphylococcus*-like colonies were picked up from MSA plates and strips and were streaked on TSA. Choice of colonies was based on dissimilar colony morphology and pigmentation. More colonies were picked up from agar plates/strips with more diverse bacterial growth. TSA plates were further incubated for 18 h at 37°C and checked for purity the next day.

4.3.4. Identification of CNS isolates

4.3.4.1. Confirmation of CNS identity

A crude DNA preparation was made for all isolates from milk and for a random selection of four isolates per environmental sample with dissimilar colony morphology and pigmentation. A few fresh colonies were suspended in 45 µl sterile HPLC water + 5 µl lysostaphin (1 mg/ml; Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C for 10 min. Next, 150 µl of Tris-HCl (0.1 M, pH 8.0) + 5 µl proteinase K (2.5 mg/ml; Promega Corporation, Madison, WI) was added and the suspensions were incubated at 60 °C for 10 min followed by 90 °C for 5 min. Cell lysates were vortexed for 1 min, centrifuged at 14000 g for 1 min and kept at -20 °C until further use. To confirm the isolates as *Staphylococcus* species and to rule out *S. aureus* identity, a duplex PCR was performed. Two primer pairs previously described by Mason et al. (2001) were used, the first pair targeting the genus *Staphylococcus*-specific 16S rRNA gene (fragment of 791 bp) and the second primer pair targeting the *S. aureus*-specific *clf*A gene (fragment of 638 bp). PCR was performed in a 25-µl reaction mixture containing 1 µl of cell lysate, 50 pmol of each primer (Eurogentec, San Diego, CA), 1× PCR buffer II (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂, 0.1 mM of each dNTP, and 1 U of AmpliTaq Polymerase (Applied Biosystems). Thermal cycling conditions were 1 min at 95 °C, 30 cycles of 15 s at 95 °C, 15 s annealing at 60 °C and 30 s elongation at 72 °C, and a final elongation step at 72 °C for 8 min. Fragments were analyzed by electrophoresis on 1.5% (w/v) agarose gels. In
Chapter 4  Distribution of CNS species from cows’ milk and environment

each executed PCR run, a positive *S. aureus* control (*S. aureus* subsp. *aureus* DSM 20231ᵀ), a positive CNS control (*S. auricularis* ATCC 33753ᵀ), and a negative control (water) were co-analyzed.

4.3.4.2. Selection of isolates for AFLP genotyping

All isolates considered as CNS by PCR were subsequently analyzed by a rapid fingerprinting technique to avoid redundancy in the CNS collection. Random amplification of polymorphic DNA-PCR (RAPD-PCR) was done on 1 µl of cell lysate using the primer D11344 and PCR conditions as described by Fitzgerald et al. (1997). RAPD-PCR fragments were separated on 2% (w/v) Seakem LE (Lonza, Basel, Switzerland) agarose gels at 100 V for 75 min and RAPD fingerprints of isolates originating from the same milk sample or the same environmental sample were compared visually. When identical RAPD fingerprints were observed, only one isolate per sample was retained in the final CNS collection. Selected CNS isolates were sub-cultured on TSA for another 18 h at 37 °C and stored as frozen stocks in BHI broth (Oxoid) with 15% (w/v) glycerol at –80 °C for further species identification.

4.3.4.3. AFLP genotyping and identification of CNS species

DNA was prepared and AFLP genotyping was done on the final CNS collection as described previously (Chapter 3). The BioNumerics software version 6.01 (Applied Maths, Sint-Martens-Latem, Belgium) was used for normalization of fingerprints and the library and identification module was used for calculation of genetic similarities and identification of the field isolates. Species identification was done based on similarity of AFLP fingerprints to entries in the staphylococcal AFLP library, which contained fingerprints of 54 CNS type and reference strains, representing 49 different CNS species and subspecies, and 247 well identified bovine CNS isolates belonging to 18 CNS species common in cattle (Chapter 3). Similarities were calculated based on the Pearson product-moment correlation coefficient and 50% similarity to a library entry was used as a cut-off for species identification. When an isolate showed less than 50% similarity to all library entries in the numerical analysis, its fingerprint was visually compared with library strains in a dendrogram constructed with the Unweighted Pair Group
Method with Arithmetic Mean (UPGMA) clustering algorithm. Due to different intensities of fingerprints, similarity can be <50%, although fingerprints visually match. When clearly resembling AFLP fingerprints were found in the library, the unknown field isolate was assigned to the corresponding CNS species.

### 4.3.4.4. **Sequencing of the *rpoB* gene**

Isolates showing AFLP fingerprints that could not be assigned to any CNS species by numerical analysis or visual comparison, were further analyzed by sequencing of the *rpoB* gene. Per unknown cluster in the AFLP dendrogram, identification of at least one isolate was done based on *rpoB* gene sequencing as described elsewhere (Supré et al., 2009).

### 4.3.5. **Definition of CNS intramammary infection**

When at least two out of three consecutive quarter milk samples were found culture-positive for the same CNS species with ≥300 cfu/ml, and when consecutive isolates had a similar AFLP type, quarters were considered to have a CNS IMI at the middle sampling. In all other cases, a quarter was considered to be non-infected or of unknown IMI status when no sample was taken in one of the adjacent months (*e.g.* first sampling, dry period, culling). A quarter was considered as having a transient IMI when it had an IMI that was absent at the previous and the next sampling. Cases were considered “persistent” when IMI status remained positive for at least two consecutive samplings. Duration of persistent IMI cases was calculated by assuming infections started at the midpoint between the first detection of IMI and the previous sampling, and ended at the midpoint between the last detection of IMI and the next sampling, respectively. When a cow entered or left the study infected, the extra period of time was calculated as the average number of days between two samplings for that herd (n = 13), divided by 2.
4.4. Results

4.4.1. CNS in milk

Based on bacteriological examination, 154 out of 2,580 cohort quarter milk samples (6.0%) were culture-positive for CNS (≥100 cfu/ml). Only CNS isolates from milk samples yielding ≥300 cfu/ml were further considered for identification. After duplex PCR (confirmation of CNS identity) and RAPD analyses (exclusion of duplicate CNS isolates per sample), 134 CNS isolates originating from 59 different quarters of 37 cows were retained. Six quarter milk samples showed mixed growth of two CNS species. All CNS isolates from milk were identifiable to species level by numerical comparison to the AFLP library (n = 126), by visual comparison of clusters in an AFLP dendrogram (n = 2), or by AFLP and rpoB gene sequencing combined (n = 6). In total, 13 species were differentiated, ranging from five to seven different species per herd (Table 4-2). The predominating CNS species in milk with ≥300 cfu/ml were *S. chromogenes* (30.6% of all isolates), *S. haemolyticus* (27.6%), *S. epidermidis* (11.9%), and *S. simulans* (11.2%). *S. chromogenes* (isolated from 12 different cows) and *S. haemolyticus* (13 cows) were the only species isolated in all herds. *S. epidermidis* was isolated in four herds (9 cows). Remarkably, *S. epidermidis* was isolated at least once from six different cows in herd E. *S. simulans* was isolated in two herds, but originated mostly from one persistently infected cow in herd E. The more rarely isolated CNS species were restricted to one or a few herds.
### Table 4-2. Species distribution of coagulase-negative *Staphylococcus* (CNS) isolates from quarter milk samples taken each month (n = 13) from ten randomly selected cohort cows per herd in six herds

<table>
<thead>
<tr>
<th>CNS species</th>
<th>Total no. of isolates (%)</th>
<th>No. of isolates per herd (no. of cows)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>41 (30.6)</td>
<td>9(2)</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>37 (27.6)</td>
<td>2(2)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>16 (11.9)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>15 (11.2)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>5 (3.7)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>4 (3.0)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>4 (3.0)</td>
<td>3(2)</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>3 (2.2)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>3 (2.2)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. devriesei</em></td>
<td>2 (1.5)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>2 (1.5)</td>
<td>1</td>
</tr>
<tr>
<td><em>M. caseolyticus</em></td>
<td>1 (0.7)</td>
<td>1</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>1 (0.7)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>134 (100.0)</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>

1Total number of isolates per species originating from quarter milk samples with ≥ 300 cfu/ml, and respective proportion (%) of all isolates (n = 134); 2Number of cows per herd from which the isolates originated is given between brackets when >1 isolate was found; 3*Staphylococcus caseolyticus* has been reclassified as *Macrococcus caseolyticus* and is sporadically isolated from cattle.

#### 4.4.2. CNS intramammary infections

In the course of the study, CNS IMI was detected 84 times distributed over 18 quarters of 14 cohort cows (range 0 to 4 cows per herd) (Table 4-3). They were caused by *S. chromogenes* (n = 35 IMI), *S. haemolyticus* (n = 29), *S. simulans* (n = 14), and *S. epidermidis* (n = 6). In total, 17 cases of persistent IMI in 16 quarters were detected for which the same causative CNS AFLP type was repeatedly isolated, and 4 IMI cases in 3 quarters were transient in nature. The mean duration of persistent cases was 149.4 days (range 63.0 to 329.8 days). The longest persistent CNS infection (329.8 days) was caused by an *S. chromogenes* AFLP type that was isolated 11 consecutive times from the same quarter [Fig. 4-1, herd E, cow 6, left hind quarter (LH)]. Another cow was persistently infected with *S. chromogenes* in a quarter in first lactation (134.2 days) and got infected with another *S. chromogenes* AFLP type (102.5 days) in its second lactation in the same quarter (Fig. 4-1, herd A, cow 1, LH). Five other quarters of 5 cows with persistent *S.
chromogenes IMI were detected with a duration ranging from 63.2 to 290.5 days. *Staphylococcus haemolyticus* was the only CNS species found to cause both transient and persistent infections. In herd D, a heifer was infected with *S. haemolyticus* in 3 quarters [Fig. 4-1, herd D, cow 1, left front (LF), right front (RF), and right hind quarter (RH)], experiencing two persistent (74.4 days in LF, 100.4 days in RH) and three transient IMI (one in RH, two in RF). The *S. haemolyticus* isolates from the LF quarter showed an AFLP type diverging from the *S. haemolyticus* library strains and the isolates from the two other infected quarters (Fig. 4-1). However, they were confirmed as *S. haemolyticus* by rpoB gene sequencing. A multiparous cow of the same herd was persistently infected with *S. haemolyticus* in two quarters for 183.0 and 103.5 days with another AFLP type (Fig. 4-1, herd D, cow 8, LH, RH). One other transient *S. haemolyticus* infection was observed in a multiparous cow of herd F and one persistent in a heifer of herd B that lasted for 222.1 days (data not shown). The two quarters with persistent *S. simulans* IMI belonged to the same heifer and were both infected for 214.8 days with the same AFLP type (Fig. 4-1, herd E, cow 3, LH). Persistent *S. epidermidis* IMI were found twice, one in herd B lasting for 117.6 days (Fig. 4-1, herd B, cow 9, RF) and one in herd E for 63.0 days (Fig. 4-1, herd E, cow 5, LF).
Table 4-3. Number of detected intramammary infections (IMI) caused by coagulase-negative *Staphylococcus* (CNS) species in monthly sampled cows in six herds (10 cows/herd)

<table>
<thead>
<tr>
<th>Causative CNS species</th>
<th>Positive IMI status&lt;sup&gt;1&lt;/sup&gt;</th>
<th>No. of IMI cases&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Mean duration (range) of persistent IMI cases (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transient</td>
<td>Persistent</td>
<td>cows</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>35</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>4</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>14</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>6</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>80</td>
<td>21</td>
</tr>
</tbody>
</table>

<sup>1</sup>Number of quarter milk samples with positive CNS IMI status, assigned when at least 2 out of 3 consecutive quarter milk samples were culture-positive for the same CNS species (≥ 300 cfu/ml), a single positive IMI status was classified as a transient IMI, recurrent positive IMI status was considered as persistent IMI; <sup>2</sup>Cases of transient (n = 4) or persistent (n = 17) IMI established by the same CNS AFLP type in a single quarter.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Month (1-13)</th>
<th>Herd</th>
<th>Cow</th>
<th>Quarter</th>
<th>CNS Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>mas0816</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mas0934</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mas0662</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mas0694</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mas1087</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mas0592</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>LH</td>
</tr>
<tr>
<td>mas0524</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>S. chromogenes</td>
</tr>
<tr>
<td>mas0938</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>mas1102</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>mas1221</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>mas1111</td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>mas0452</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mas0585</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>mas0526</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>LH</td>
</tr>
<tr>
<td>mas0397</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>S. chromogenes</td>
</tr>
<tr>
<td>mas0937</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>mas1085</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>mas1112</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>mas0591</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>mas0449</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mas0393</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mas0523</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>mas0661</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>mas0689</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>mas0813</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>mas1006</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>mas1090</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>mas1097</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>mas1110</td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>mas0693</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>mas0815</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>mas0388</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mas0445</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mas0389</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mas0446</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mas0448</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mas0532</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>mas0534</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>mas0519</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>mas0538</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>mas0936</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>mas0670</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>mas0686</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>mas0685</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>mas0669</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>mas0819</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. haemolyticus</td>
</tr>
</tbody>
</table>

Species: S. chromogenes, S. simulans, S. epidermidis, S. haemolyticus.
**Figure 4-1.** Dendrogram of amplified fragment length polymorphism (AFLP) fingerprints of a selection of the *S. epidermidis, S. haemolyticus, S. chromogenes,* and *S. simulans* isolates from quarter milk samples of monthly sampled cows. AFLP types establishing IMI within the same cow or quarter are indicated by boxes. Isolates were identified to species level by comparison of AFLP fingerprints to a staphylococcal AFLP library or by *rpoB* gene sequencing. Clustering was obtained by the unweighted pair group method with arithmetic averages (UPGMA) of Pearson product-moment correlation coefficients, optimization of 1.0%, and curve smoothing of 0.5%. ¹Month of isolation (May 2008 to May 2009); ²Designation of the quarters: left front (LF), left hind (LH), right front (RF) and right hind (RH).
4.4.3. Clinical cases

During the study period, 26 quarters with clinical signs were detected from cohort cows and 83 quarters from non-cohort herd-mates. The average IRCM (all cases) in the herds was 0.281 quarter cases per 365 cow-days at risk (ranging from 0.149 to 0.485 between herds (Table 4-1). In total, 7.5% of clinical quarter milk samples (7 out of 94 sampled quarters) were positive for CNS growth. Three samples yielded CNS exclusively, the other four showed mixed growth with esculine-positive cocci \( (n = 3) \) or Corynebacterium bovis \( (n = 1) \). Species distribution of CNS isolates from clinical samples was two \( S. \) chromogenes, one \( S. \) epidermidis, one \( S. \) equorum, and three unknown (no isolate was preserved). Both \( S. \) chromogenes isolates came from clinical cases yielding CNS exclusively.

4.4.4. CNS in the cows’ environment

CNS were isolated from 75 out of 78 (96.2%) floor samples, from 77 out of 78 (98.7%) air samples, from 65 out of 73 (89.0%) used bedding samples, and from 50 out of 73 (68.5%) sawdust storage samples. After PCR and RAPD analyses, 637 CNS isolates were preserved for further identification. In total, 612 (96.1%) isolates were identified to species level and 25 isolates (3.9%) remained unidentified. Identification results and species distribution over herds are given in Table 4-4. The majority of environmental isolates was readily identifiable by numerical comparison to the AFLP library, namely 531 out of 637 isolates (83.4%). Another 81 isolates could be identified by visual comparison of AFLP fingerprints in a clustering \( (n = 40) \) or by combination of \( rpoB \) gene sequencing and AFLP clustering \( (n = 41) \). The CNS species predominating in the cows’ environment over all herds were \( S. \) equorum (19.0% of the isolates), \( S. \) sciuri (17.9%), and \( S. \) haemolyticus (16.6%). The species \( S. \) cohnii (5.7%), \( S. \) simulans (5.3%), \( S. \) xylosus (3.1%), \( S. \) devriesei (2.8%), and \( S. \) arlettae (2.5%) were also isolated in the environment of each herd, but less frequently. Some notable herd-to-herd differences were observed as it relates to species distribution. Several CNS species were chiefly isolated in one or two herds. \( S. \) fleurettii was isolated predominantly in the environment of herd E, \( S. \) cohnii in herds A and F, \( S. \) simulans in herds C and E, and \( S. \) saprophyticus in herd A. In herd E, \( S. \) sciuri (3.7% of environmental CNS isolates) was less common compared to the other
herds (11.0 to 28.3%). *S. haemolyticus* was less common in herds C (6.9%) and E (8.3%) compared to the other herds (15.7 to 26.4%).

Table 4-4. Species distribution of coagulase-negative *Staphylococcus* (CNS) isolates from environmental samples (stall air, slatted floors, sawdust from cubicles, and sawdust stock) taken monthly (n = 13) on six herds

<table>
<thead>
<tr>
<th>CNS species</th>
<th>Total no. of isolates (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. equorum</em></td>
<td>121 (19.0)</td>
<td>18</td>
<td>21</td>
<td>29</td>
<td>12</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td><em>S. sciuiri</em></td>
<td>114 (17.9)</td>
<td>28</td>
<td>10</td>
<td>28</td>
<td>32</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>106 (16.6)</td>
<td>21</td>
<td>24</td>
<td>7</td>
<td>28</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td><em>S. fleurettii</em></td>
<td>47 (7.4)</td>
<td></td>
<td>5</td>
<td></td>
<td>41</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>36 (5.7)</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>34 (5.3)</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>4</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>20 (3.1)</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>S. devriesei</em></td>
<td>18 (2.8)</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><em>S. arlettae</em></td>
<td>16 (2.5)</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>15 (2.4)</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>12 (1.9)</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>11 (1.7)</td>
<td>1</td>
<td>3</td>
<td></td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>11 (1.7)</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>S. succinus</em></td>
<td>10 (1.6)</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>8 (1.3)</td>
<td></td>
<td>3</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>7 (1.1)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>7 (1.1)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. vitulinus/S. pulvereri</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6 (0.9)</td>
<td></td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>5 (0.8)</td>
<td>1</td>
<td>1</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>4 (0.6)</td>
<td>2</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. caseolyticus</em>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2 (0.3)</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td>1 (0.2)</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. nepalensis</em></td>
<td>1 (0.2)</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>25 (3.9)</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>637 (100.0)</td>
<td>114</td>
<td>91</td>
<td>102</td>
<td>113</td>
<td>109</td>
<td>108</td>
</tr>
</tbody>
</table>

<sup>1</sup>Total number of isolates per species and respective proportion (%) of all isolates (n = 637); <sup>2</sup>*S. vitulinus* and *S. pulvereri* are synonyms for the same species; <sup>3</sup>*Staphylococcus caseolyticus* has been reclassified as *Macroccoccus caseolyticus* and is sporadically isolated from cattle.
4.4.5. Distribution of CNS species over the different niches

When distribution of CNS species over the different niches is compared, certain CNS species tended to favor particular niches (Table 4-5). *Staphylococcus chromogenes* and *S. epidermidis* were predominantly isolated from milk (78.8 and 57.1% of all isolates, respectively) and less from environmental samples. *Staphylococcus haemolyticus* and *S. simulans* were regularly isolated both from milk and the environment (74.1 and 69.4% isolates from environment, respectively). Other CNS species were sporadically isolated from milk, but not causing IMI according to our definition. Common species with a mainly environmental origin (90.0 to 100.0% of all isolates) were *S. equorum, S. sciuri, S. fleurettii, S. cohnii, S. devriesi, S. xylosus, S. arlettae*, and *S. succinus*. CNS species in the environment distributed differently over the sampled locations. *Staphylococcus haemolyticus* and *S. equorum* were isolated mostly from stall air (40.6 and 49.6% of all isolates, respectively), whereas *S. sciuri* and *S. simulans* were isolated on a regular basis from slatted floors and sawdust of the cubicles (72.2% and 67.3%, respectively). *Staphylococcus xylosus* and *S. succinus* were isolated mostly from sawdust stock (55.0 and 80.0%, respectively).
Table 4-5. Distribution of the individual coagulase-negative *Staphylococcus* species isolated from monthly sampled (n = 13) niches on six dairy farms (high occurrence of individual species in a particular niche is indicated in bold)

<table>
<thead>
<tr>
<th>CNS species</th>
<th>Milk²</th>
<th>No. of isolates per niche (% within species)¹</th>
<th>Air</th>
<th>Slatted floors</th>
<th>Sawdust cubicles</th>
<th>Sawdust stock</th>
<th>Total no. (%)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. haemolyticus</td>
<td>37 (25.9)</td>
<td>58 (40.6)</td>
<td>23 (16.1)</td>
<td>9 (6.3)</td>
<td>16 (11.2)</td>
<td>143 (18.6)</td>
<td></td>
</tr>
<tr>
<td>S. equorum</td>
<td>2 (1.6)</td>
<td>61 (49.6)</td>
<td>28 (22.8)</td>
<td>22 (17.9)</td>
<td>10 (8.1)</td>
<td>123 (16.0)</td>
<td></td>
</tr>
<tr>
<td>S. sciuri</td>
<td>1 (0.9)</td>
<td>14 (12.2)</td>
<td>43 (37.4)</td>
<td>40 (34.8)</td>
<td>17 (14.8)</td>
<td>115 (14.9)</td>
<td></td>
</tr>
<tr>
<td>S. chromogenes</td>
<td>41 (78.8)</td>
<td>3 (5.8)</td>
<td>5 (9.6)</td>
<td>2 (3.8)</td>
<td>1 (1.9)</td>
<td>52 (6.7)</td>
<td></td>
</tr>
<tr>
<td>S. simulans</td>
<td>15 (30.6)</td>
<td>1 (2.0)</td>
<td>23 (46.9)</td>
<td>10 (20.4)</td>
<td>49 (6.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. fleurettii</td>
<td>8 (17.0)</td>
<td>20 (42.6)</td>
<td>15 (31.9)</td>
<td>4 (8.5)</td>
<td>47 (6.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cohnii</td>
<td>4 (10.0)</td>
<td>11 (27.5)</td>
<td>6 (15.0)</td>
<td>9 (22.5)</td>
<td>10 (25.0)</td>
<td>40 (5.2)</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>16 (57.1)</td>
<td>1 (3.6)</td>
<td>4 (14.3)</td>
<td>5 (17.9)</td>
<td>2 (7.1)</td>
<td>28 (3.6)</td>
<td></td>
</tr>
<tr>
<td>S. devriesii</td>
<td>2 (10.0)</td>
<td>7 (35.0)</td>
<td>4 (20.0)</td>
<td>3 (15.0)</td>
<td>4 (20.0)</td>
<td>20 (2.6)</td>
<td></td>
</tr>
<tr>
<td>S. xylosus</td>
<td>5 (25.0)</td>
<td>4 (20.0)</td>
<td>11 (55.0)</td>
<td>20 (2.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>4 (21.1)</td>
<td>3 (15.8)</td>
<td>1 (5.3)</td>
<td>4 (21.1)</td>
<td>7 (36.8)</td>
<td>19 (2.5)</td>
<td></td>
</tr>
<tr>
<td>S. arlettae</td>
<td>8 (50.0)</td>
<td>1 (6.3)</td>
<td>1 (6.3)</td>
<td>4 (25.0)</td>
<td>3 (18.8)</td>
<td>16 (2.1)</td>
<td></td>
</tr>
<tr>
<td>S. hominis</td>
<td>5 (31.3)</td>
<td>3 (18.8)</td>
<td>1 (6.3)</td>
<td>6 (37.5)</td>
<td>1 (6.3)</td>
<td>16 (2.1)</td>
<td></td>
</tr>
<tr>
<td>S. auricularis</td>
<td>3 (30.0)</td>
<td>7 (70.0)</td>
<td>8 (80.0)</td>
<td>10 (1.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. succinus</td>
<td>2 (20.0)</td>
<td>1 (12.5)</td>
<td>3 (37.5)</td>
<td>2 (25.0)</td>
<td>2 (25.0)</td>
<td>8 (1.0)</td>
<td></td>
</tr>
<tr>
<td>S. lentus</td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
<td>2 (28.6)</td>
<td>1 (14.3)</td>
<td>7 (0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. capitis</td>
<td>3 (42.9)</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
<td>7 (0.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. warneri</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>3 (0.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. capitis</td>
<td>1 (100.0)</td>
<td>1 (100.0)</td>
<td>1 (100.0)</td>
<td>1 (0.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. nemalensis</td>
<td>1 (100.0)</td>
<td>1 (100.0)</td>
<td>1 (100.0)</td>
<td>1 (0.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>10 (40.0)</td>
<td>12 (48.0)</td>
<td>1 (4.0)</td>
<td>2 (8.0)</td>
<td>25 (3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>134 (17.4)</td>
<td>209 (27.1)</td>
<td>189 (24.5)</td>
<td>135 (17.5)</td>
<td>104 (13.5)</td>
<td>771 (100.0)</td>
<td></td>
</tr>
</tbody>
</table>

¹Distribution of isolates of individual CNS species over the sampled niches given in numbers and proportion (%) of total number of isolates within the respective species; ²Isolates from quarter milk samples of monthly sampled cows in the 6 herds (10 cows/herd) yielding ≥300 cfu/mL CNS; ³Total number of isolates per species and respective proportion (%) of all isolates (n = 771)


4.5. Discussion

In this longitudinal field study, distribution of CNS species isolated from quarter milk samples and the cows’ environment was determined in six herds. CNS isolates were genotyped and identified to species level by means of AFLP, a well validated and reproducible whole-genome based method (Chapter 3). This is in contrast with the majority of previous field studies on CNS in which for species differentiation phenotypic methods are generally used (Matos et al., 1991; Thorberg et al., 2009). In a number of studies, however, it has been demonstrated that the performance of phenotypic methods is insufficient for identification of CNS isolates from the bovine (Bes et al., 2000; Capurro et al., 2009; Sampimon et al., 2009c). Currently, genotyping is preferred to gain accurate information on CNS species level (Zadoks and Watts, 2009). In the past, CNS from cows’ environment have only been studied with phenotypic methods (Matos et al., 1991) and as far as we know, their genotypes have never been compared with those of CNS causing IMI. Amplified fragment length polymorphism genotyping not only enables species identification, but also genotypic comparison of CNS isolates and tracking of the spread of specific CNS genotypes, which provides valuable information on epidemiology of these udder pathogens. Because different control practices are needed when either contagious or environmental pathogens cause a significant proportion of all IMI in a herd, determining the epidemiology of individual CNS species is useful for the dairy industry to prevent infections with particular more pathogenic CNS species.

According to the postulated IMI definition of the present study, only the CNS species S. chromogenes, S. haemolyticus, S. simulans, and S. epidermidis were causing IMI in the studied cows. This is in line with other studies, where these species have also been isolated from IMI (Aarestrup and Jensen, 1997; Chaffer et al., 1999; Taponen et al., 2007), although other CNS species, e.g. S. xylosus (Thorberg et al., 2009) and S. hyicus (Gillespie et al., 2009), have also been reported. The majority of detected CNS IMI in the present study was persistent in nature, although this was largely due to the rather stringent definition for CNS IMI based on three consecutive monthly samples. There are no standard criteria for diagnosis of CNS IMI in the bovine udder, although attempts to define a general IMI definition have recently been published (Dohoo et al., 2011).
recent consensus is, however, based on weekly samplings, whereas the current study used monthly data. Because of the relatively large time interval between samplings and the drawbacks of bacterial culturing, the real number of IMI in the present study is most likely underestimated, especially for IMI of short duration. Nevertheless, the impact of these transient CNS IMI on general udder health is probably minor, whereas long duration of undetected subclinical infections increases impact on SCC and the possibility of spread of the causative bacteria. Although all CNS species isolated from milk in the present study were evaluated with the same definition, striking differences were found among CNS species and only four were further considered of particular interest. Persistence of S. chromogenes, S. haemolyticus, S. epidermidis, and S. simulans has been diagnosed in previous studies by repeated isolation of the same species from the same quarter (Aarestrup and Jensen, 1997; Chaffer et al., 1999; Thorberg et al., 2009). The observations in the current study further confirm that particular CNS strains within these species, as evidenced by the repeated isolation of the same AFLP type, are able to survive and persist in the udder.

Comparing prevalence of different CNS species between studies should be done with caution, as there are often differences in identification methods used, definitions for CNS IMI, or sampling schedule. However, it can be assumed that distribution of CNS species infecting the udder varies between herds (Thorberg et al., 2009; this study; Gillespie et al., 2009). Part of this variation could be explained by factors such as type of germicide used for teat dipping (Hogan et al., 1987) or age of the cows (Matthews et al., 1992; Taponen et al., 2006). Between herds, marked differences were also seen in CNS species distribution in the environment, although farm and management characteristics of the herds included in the present study were similar. This also indicates that as yet unknown herd-level factors or environmental conditions are determining the establishment of staphylococcal species in a dairy herd. In two studies in which extramammary CNS isolates have also been differentiated, it has been demonstrated that type of housing (White et al., 1989) and type of bedding (Matos et al., 1991) influences the distribution of CNS species found on body sites of heifers and in bedding samples, respectively. Although no attempt was made to quantify individual CNS species in the environment, it can be concluded that each dairy farm probably harbors its own CNS microbiota. To explain this remarkable variation in occurrence of particular CNS
species, further study on the influence of different herd and environmental factors on CNS prevalence and distribution is required.

The findings of the present study suggest that CNS species are abundantly present in the studied type of dairy herds, but that their primary reservoirs differ according to species. For *S. chromogenes* and *S. epidermidis*, the udder was found as a main reservoir. These two species were rarely isolated from the environment and IMI probably originated from other sources, *e.g.* cow’s skin, milkers’ hands, or other cows. *Staphylococcus chromogenes* caused the most and longest persistent subclinical IMI and was found as the most likely cause of two clinical cases. As *S. chromogenes* is a commonly isolated mastitis pathogen (Aarestrup and Jensen, 1997; Gillespie et al., 2009; Sawant et al., 2009) able to induce an elevation in SCC as high as for infections with the major pathogen *S. aureus* (Supré et al., 2011), its role in udder health might not be as minor as previously thought. *Staphylococcus chromogenes* has been isolated from udder skin, teat apices and other body sites in previous studies (White et al., 1989; De Vliegher et al., 2003; Taponen et al., 2008). According to Taponen et al. (2008), this species is a typical skin opportunist. In contrast with *S. chromogenes*, *S. epidermidis* is uncommon in normal bovine skin microbiota. Alternatively, *S. epidermidis* is one of the most prevalent staphylococcal species on human skin and may be transmitted from milkers to cows (Thorberg et al., 2006).

Two CNS species were found, being *S. haemolyticus* and *S. simulans*, that caused IMI and also survived well in environmental conditions. Especially *S. haemolyticus* caused a large proportion of all IMI and appeared to have a considerable environmental reservoir. In several studies, this species has not or rarely been isolated from IMI (Matthews et al., 1992; Gillespie et al., 2009), whereas in others it has been found frequently (Chaffer et al., 1999; Thorberg et al., 2009). The apparent differences in *S. haemolyticus* prevalence could be due to either true variation in its occurrence or the difficulty to identify this species. Among the *S. haemolyticus* isolates, two very distinct AFLP types were differentiated of which one could only be identified by *rpoB* gene sequencing. Possibly, the latter type could lead to identification problems with other methods. Within herds, similar AFLP types of *S. haemolyticus* were observed among isolates originating from environment and IMI, strongly indicating potential environmental sources for IMI with this species. *Staphylococcus haemolyticus* was also commonly isolated from the teat apices of the cows under study (Braem G., Verbist B.,
Piessens V., De Vliegher S., De Vuyst L., unpublished results), indicating its adaptability to various niches in the dairy herd. *Staphylococcus simulans* on the other hand is uncommon on cow skin, and the source of IMI with this species is still unclear. Environmental *S. simulans* isolates mostly originated from slatted floors and used bedding, which might imply contamination of the environment by infected cows. *Staphylococcus simulans* tends to cause more severe mastitis and predominates in studies conducted on clinical mastitis (Myllys, 1995; Waage et al., 1999). In a study by Aarestrup et al. (1999), a variety of ribotypes of *S. simulans* have been recovered from IMI in the same herds and within different quarters of the same cows. The observed genetic diversity of infectious *S. simulans* ribotypes in that study might imply multiple environmental sources, although this has not yet been demonstrated.

The origin of all other CNS species isolated was primarily environmental. Besides, 25 CNS were isolated from the environment that could not be identified by the AFLP method. These isolates could represent previously unknown CNS species; however, they were not studied further because of their overall low prevalence. In two other studies using molecular techniques for CNS differentiation, unidentifiable genetic profiles were also generated for a number of presumptive CNS isolates with transfer DNA-PCR (Supré et al., 2009) and AFLP (Taponen et al., 2006). Further study of these atypical isolates led to the characterization and description of two new CNS species, being *S. devriesei* (Supré et al., 2010) and *S. agnetis* (Taponen et al., 2011), respectively. Surprisingly, some species that were mainly environmental in the present study have been found as significant causes of IMI in others, e.g. *S. xylosus* (Thorberg et al., 2009), *S. sciuri* (Davidson et al., 1992), and *S. cohnii* (Supré et al., 2011). It can be speculated that when the infection pressure is high or when the immunity of cows is compromised, these CNS species may possibly act as environmental opportunistic pathogens. Also, it could be that some strains within these species are better adapted to cause infection. Despite prevention measures such as teat disinfection and dry-cow therapy, prevalence of CNS infections in some herds is remarkably high for unknown reasons (Piepers et al., 2007; Schukken et al., 2009). In the past, only few studies have been set up to identify possible reservoirs of CNS by comparing strains originating from mastitis with strains originating from other sources (Thorberg et al., 2006; Taponen et al., 2008). Knowledge on primary reservoirs of particular CNS species in herds, e.g. *S. xylosus* in sawdust stock, could be useful in prevention of CNS infections contracted from the environment. The present
study was an attempt to identify potential environmental sources of CNS IMI. However, before possible transmission routes of individual CNS species can be demonstrated or reservoirs of strains causing IMI can be identified, clonal diversity within CNS species observed in the AFLP fingerprints should be further confirmed by (an)other molecular subtyping technique(s).

4.6. Conclusions

Distribution of CNS species from cows’ milk and environment differed among herds. Epidemiology of CNS infecting the udder varied among species. *Staphylococcus chromogenes* and *S. epidermidis* seemed to act more as host-adapted pathogens specialized in surviving in the udder and the environment was not found as a likely source of IMI. *Staphylococcus haemolyticus* and *S. simulans* on the other hand had a considerable reservoir in the environment and could potentially act as environmental opportunists.

Acknowledgments

This research was funded by the agency for Innovation by Science and Technology in Flanders (IWT-Vlaanderen, grant no. 60714). The authors would like to thank Ann Vanhee for her excellent laboratory support.
CHAPTER 5

INTRA-SPECIES DIVERSITY AND EPIDEMIOLOGY VARIES AMONG DIFFERENT COAGULASE-NEGATIVE STAPHYLOCOCCUS SPECIES CAUSING BOVINE INTRAMAMMARY INFECTIONS

V. Piessens¹, S. De Vliegher², B. Verbist¹, K. Supré², G. Braem³, A. Van Nuffel¹, L. De Vuyst³, M. Heyndrickx¹, and E. Van Coillie¹

¹Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium; ²Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Belgium; ³Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium.

5.1. Abstract

Although many studies report coagulase-negative staphylococci (CNS) as the predominant cause of subclinical bovine mastitis, their epidemiology is poorly understood.

In the current study, the genetic diversity within four CNS species frequently associated with bovine intramammary infections, *Staphylococcus haemolyticus*, *S. simulans*, *S. chromogenes*, and *S. epidermidis*, was determined. For epidemiological purposes, CNS genotypes recovered from bovine milk collected on six Flemish dairy farms were compared with those from the farm environment, and their distribution within the farms was investigated. Genetic diversity was assessed by two molecular typing techniques, amplification fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD) analysis.

Subtyping revealed the highest genetic heterogeneity among *S. haemolyticus* isolates. A large variety of genotypes was found among environmental isolates, of which several could be linked with intramammary infection, indicating that the environment could act as a potential source for infection. For *S. simulans*, various genotypes were found in the environment, but a link with IMI was less obvious. For *S. epidermidis* and *S. chromogenes*, genetic heterogeneity was limited and the sporadic isolates from environment displayed largely the same genotypes as those from milk. The higher clonality of the *S. epidermidis* and *S. chromogenes* isolates from milk suggests that specific genotypes probably disseminate within herds and are more udder-adapted.

Environmental sources and cow-to-cow transmission both seem to be involved in the epidemiology of CNS, although their relative importance might substantially vary between species.
5.2. Introduction

Coagulase-negative staphylococci (CNS) have become the most commonly isolated microorganisms from bovine milk in many countries and are regarded as emerging mastitis pathogens (Aarestrup and Jensen, 1997; 2007; Bradley et al., 2007; Pyörälä and Taponen, 2009). As CNS causing intramammary infections (IMI) increase the somatic cell count (SCC) in the milk, they are considered harmful for milk quality and yield, but particularly need attention in herds with low bulk milk SCC (Schukken et al., 2009). Besides infected mammary glands, CNS have reservoirs on various bovine body sites, including the skin, mucosa and teat canals, and in the dairy farm environment (White et al., 1989; Matos et al., 1991; Piessens et al., 2011). Although CNS are generally considered as opportunistic pathogens originating from bovine skin, studies on risk factors for CNS IMI have indicated that heifers and cows likely get infected from environmental sources (Sampimon et al., 2009b; Piepers et al., 2011). Hence, it is conjectured that IMI-causing CNS originate from multiple sources and can use different routes of transmission. In a previous study conducted in six Flemish dairy herds, the IMI-causing CNS species were identified as *S. haemolyticus*, *S. simulans*, *S. chromogenes*, and *S. epidermidis* by amplified fragment length polymorphism (AFLP) analysis (Piessens et al., 2011). *Staphylococcus haemolyticus* and *S. simulans* are isolated regularly both from IMI and environmental samples, whereas *S. epidermidis* and *S. chromogenes* do not seem to have a significant reservoir in the environment.

The aims of the current study were to assess the genetic diversity within isolates of the four above mentioned CNS species causing IMI in dairy cows, to compare genotypes from milk and the environment, and to gather data on the dissemination of individual genotypes within farms. A combination of two molecular typing methods, AFLP and random amplified polymorphic DNA (RAPD) analysis, were used for intra-species comparison.
5.3. Materials and methods

5.3.1. Bacterial isolates

This study included 213 CNS isolates originating from dairy cows’ milk (n = 54) and the dairy farm environment (n = 159) (Table 5-1). The isolates were collected in the course of a longitudinal field study conducted between May 2008 and May 2009 on six Flemish dairy farms (referred to as A to F) and belong to the four CNS species causing IMI in those herds. Briefly, quarter milk samples of ten randomly selected cows per herd, and samples from different sites in the farm environment (slatted floor alleyways, air in the free stalls, sawdust bedding from the cubicles, and unused sawdust from the storage) were taken at monthly intervals during 13 months for isolation of CNS. Collection of samples, isolation and identification of CNS, and determination of IMI status of consecutively sampled quarters have been described in detail previously (Chapter 4). Of the 54 CNS isolates from milk, 17 originated from persisting IMI cases (Table 5-1). Cases were considered “persistent” when quarters were diagnosed to have a CNS IMI for at least two consecutive samplings (i.e. out of three consecutive samplings, the middle sampling was considered to have an IMI when identical AFLP fingerprints were isolated at least twice; see Chapter 4). Per quarter diagnosed with persistent IMI, only one of the consecutive isolates was included for further study. The other 37 isolates from milk were recovered once (not causing IMI) or twice in three consecutive milk samples (so called transient IMI) and were considered “non-persistent” for the current study. Two S. chromogenes isolates and one S. epidermidis were isolated from quarters showing clinical signs. Environmental CNS isolates originated from different sites in the dairy farm and were isolated at different time points throughout the aforementioned longitudinal study.
Table 5-1. Species distribution and origin of 213 coagulase-negative *Staphylococcus* isolates collected in six Flemish dairy farms (A to F) during a longitudinal study from May 2008 to May 2009

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin of isolates</th>
<th>Herd of origin</th>
<th>No. of isolates per origin over all herds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A  B  C  D  E  F</td>
<td></td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>Milk</td>
<td>non-persistent&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2  3  5  2  3  15</td>
</tr>
<tr>
<td>(n = 124)</td>
<td></td>
<td>persistent&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1  4  5  5  23</td>
</tr>
<tr>
<td></td>
<td>Environment</td>
<td>alleyway</td>
<td>2  8  2  5  1  5  23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stall air</td>
<td>9  11 4  15 8  10  57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bedding</td>
<td>2  1  4  7  7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sawdust storage</td>
<td>8  3  1  4  1  17</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>Milk</td>
<td>non-persistent</td>
<td>1  2  1  2  3</td>
</tr>
<tr>
<td>(n = 37)</td>
<td></td>
<td>persistent</td>
<td>1  1  2  2</td>
</tr>
<tr>
<td></td>
<td>Environment</td>
<td>alleyway</td>
<td>1  3  5  4  5  3  21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bedding</td>
<td>1  1  4  5  11</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>Milk</td>
<td>non-persistent*</td>
<td>4  1  1  1  2*  9</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td>persistent</td>
<td>3  2  1  1  1  8</td>
</tr>
<tr>
<td></td>
<td>Environment</td>
<td>alleyway</td>
<td>3  2  2  5  5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stall air</td>
<td>1  2  3  3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bedding</td>
<td>2  2  2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sawdust storage</td>
<td>1  1  1</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Milk</td>
<td>non-persistent*</td>
<td>1  1*  7  1  10</td>
</tr>
<tr>
<td>(n = 24)</td>
<td></td>
<td>persistent</td>
<td>1  1  2  2</td>
</tr>
<tr>
<td></td>
<td>Environment</td>
<td>alleyway</td>
<td>2  1  1  4  4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stall air</td>
<td>1  1  1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bedding</td>
<td>2  3  5  5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sawdust storage</td>
<td>1  1  2</td>
</tr>
</tbody>
</table>

<sup>1</sup>Isolated once from milk (not causing intramammary infection (IMI)), or the same AFLP type isolated twice from three consecutive samples (so called transient IMI) (see Chapter 4); <sup>2</sup>Infections were considered persistent when a CNS IMI was diagnosed for at least two consecutive samples (see Chapter 4); *One isolate was cultured from a clinical mastitis case.
5.3.2. AFLP analysis

Isolates were recultured on trypton soy agar overnight at 37°C from the -80°C stocks in brain heart infusion broth with 15% (v/v) glycerol. Genomic DNA extraction, DNA restriction with Msel and HindIII, generation of AFLP fingerprints, and subsequent library-based species identification of all isolates was done as previously described (Chapter 3). In the current study, AFLP analysis was used for the assessment of intra-species diversity (subtyping). Isolates of each species were first subdivided into genetically similar AFLP-clusters with the BioNumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium), using the unweighted pair group method with arithmetic averages (UPGMA) clustering of Pearson product-moment correlation coefficients (optimization, 1.0%; curve smoothing, 0.5%). To improve the discriminatory power at the intra-species level, species-specific fragments in the high-molecular-mass range with high intensity and present in all isolates of a species were left out of the numerical analysis. When fingerprints within clusters were visually homogeneous, clusters were defined as an individual AFLP type. When fingerprints within clusters showed heterogeneity, i.e. absence or presence of one or more clear bands in multiple isolates, the cluster was further subdivided visually into multiple AFLP types based on these marked band differences. A single band difference in a single fingerprint was not considered a different AFLP type. Each AFLP type was given an arbitrary number.

5.3.3. RAPD analysis

For complementary subtyping, isolates were analyzed by random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR). RAPD-PCR was done on 1 μl of DNA (50 ng/μl) using the primer D11344 and PCR conditions as described by Fitzgerald et al. (1997). Generated fragments were separated on 1.5% (w/v) Seakem LE agarose (Lonza, Basel, Switzerland) gels in 1× Tris-Borate-EDTA buffer (Invitrogen Ltd., Paisly, UK) at 120 V for 4 h. All isolates of the same CNS species were analyzed in the same PCR run, except for the S. haemolyticus isolates, which were analyzed in two runs. A control DNA sample (S. auricularis ATCC 33753) was co-analyzed in each executed RAPD run as an internal reference. RAPD patterns were analyzed with the BioNumerics
software (UPGMA clustering of Pearson product-moment correlation coefficients; optimization, 1.0%; curve smoothing, 0.5%), and RAPD types were assigned based on clustering and visual assessment of band differences. Isolates showing the same number and size of bands were considered the same RAPD type, designated by an arbitrary lower case letter.

5.3.4. Delineation of genotypes

In the context of this study, a CNS genotype was defined as a unique combination of a specific AFLP and RAPD type. Genotypes were indicated by the number and letter combination representing the AFLP and RAPD types, respectively. Discriminatory power of the combination of both techniques was determined for each species by calculating the Simpson's diversity index (D) with 95% confidence intervals using the PAST software (Hammer et al., 2001), to assess the heterogeneity in a population, and to statistically compare the diversity among species. Corrections for multiple comparisons were not employed.

5.4. Results

5.4.1. Genetic relatedness and genotypes within CNS species according to AFLP and RAPD

AFLP and RAPD fingerprints were generated for all S. haemolyticus, S. simulans, S. chromogenes, and S. epidermidis isolates from milk and environment (Table 5-1). Per species, fingerprints generated by both methods were analyzed separately by construction of dendrograms and their visual examination, and each isolate was independently assigned an AFLP and RAPD type to determine a final CNS genotype.

AFLP subdivided the 124 S. haemolyticus isolates into three main clusters showing distinct AFLP profiles (19.6% internal similarity) (Figure 5-1A). Within the largest AFLP-cluster (number of isolates = 105; 53.0% internal similarity), clear band differences were found and isolates were grouped into 13 homogeneous AFLP types (1 to 13), containing one to 18 isolates per type. The two smaller AFLP clusters were closer related to each other (49.8% similarity) than to the large cluster and were represented
by 12 (76.9% internal similarity) and 7 isolates (64.2% internal similarity), respectively. Both clusters could be subdivided further into two AFLP types each, 14 (n = 9) and 15 (n = 3), and 16 (n = 4) and 17 (n = 3), respectively. With RAPD, two main clusters displaying very distinct RAPD profiles (20.8% similarity) were generated with the 124 S. haemolyticus isolates (data not shown); one large cluster represented by the same 105 isolates (46.8% internal similarity) as for the largest AFLP cluster, and the other cluster represented by the 19 isolates (76.1% internal similarity) which were found in the two smaller AFLP clusters. The clusters were subdivided into ten (a to j) and four (k to n) RAPD types, respectively. Concordant typing results were often obtained with AFLP and RAPD, and isolates within an AFLP type mostly showed the same RAPD type (Figure 5-1A). However, not all isolates within AFLP types were identified as the same RAPD type, and vice versa. In particular, isolates within the most common AFLP types 1 and 2 could show heterogeneous RAPD types. Likewise, within each of the RAPD types a, b and d, multiple AFLP types could be differentiated.

Overall similarity among AFLP fingerprints of the 37 S. simulans isolates was 69.7% (Figure 5-1B). Isolates were subdivided into two clusters and four singletons not clustering with other isolates (AFLP types 5 to 8). The largest cluster (n = 22; 90.6% internal similarity) showed heterogeneous fingerprints and could be subdivided further into three AFLP types, 1 (n = 10), 2 (n = 7), and 3 (n = 5). The smallest cluster (n = 11; 87.5% internal similarity) had homogeneous fingerprints and was totally assigned to AFLP type 4. With RAPD, the S. simulans isolates were differentiated into eight distinct RAPD types (overall similarity of 26.0%): three RAPD types a (n = 10), b (n = 11), and c (n = 11) were represented by clusters of isolates (data not shown), and five RAPD types were represented by one isolate each (d to h). In general, isolates within an AFLP type showed one main RAPD type, although there were some exceptions.

The AFLP fingerprints of the 28 S. chromogenes isolates were very homogeneous with an overall similarity of 81.4% (Figure 5-1C). Few clear band differences were seen and only three AFLP types were differentiated, 1 (n = 16), 2 (n = 11), and 3 (n = 1). The S. chromogenes RAPD fingerprints were also visually alike and only minor differences were seen. Three closely resembling RAPD types (59.9% similarity, data not shown) were differentiated, RAPD type a (n = 22), b (n = 5), and c (n = 1).
Figure 5-1A.
**Figure 5-1 (continued).** Dendrograms generated with the amplified fragment length polymorphism (AFLP) fingerprints of the *Staphylococcus haemolyticus* (A), *S. simulans* (B), *S. chromogenes* (C), and *S. epidermidis* (D) isolates from milk and farm environment (stall air, alleyways, used sawdust bedding, and unused sawdust storage).

For each isolate the corresponding random amplification of polymorphism DNA (RAPD) profiles are represented, but were not used in the clustering. Genotypes were defined as

[97]
the combination of the assigned AFLP (number) and RAPD type (letter). Clustering settings were: unweighted pair group method with arithmetic averages (UPGMA) of Pearson product-moment correlation coefficients (optimization, 1.0; curve smoothing, 0.5%).

AFLP subdivided the 24 S. epidermidis isolates into two main clusters diverging at 56.1% similarity (Figure 5-1D). The largest cluster (n = 19; 83.9% internal similarity) was subdivided into three AFLP types, 1 (n = 11), 2 (n = 7), and 3 (n = 1). The smaller cluster (n = 5; 86.3% internal similarity) was subdivided into AFLP types 4 (n = 4), and 5 (n = 1). Similar as for AFLP, RAPD subdivided the S. epidermidis isolates into two main clusters (50.1% similarity, data not shown), and five RAPD types were differentiated. The largest cluster contained RAPD types a (n = 11), b (n = 7), and c (n = 1); the smaller cluster consisted of RAPD types d (n = 4) and e (n = 1). Subtyping of S. epidermidis isolates by RAPD fully corresponded with the results obtained by AFLP.

The number of genotypes found within each species and source is given in Table 5.2. The combination of AFLP and RAPD revealed 27 genotypes among 124 S. haemolyticus isolates, 13 genotypes among 37 S. simulans isolates, 5 genotypes among 28 S. chromogenes isolates, and 5 genotypes among 24 S. epidermidis isolates. The Simpson’s diversity index was significantly higher for S. haemolyticus (94.4%) compared to the other three species (p < 0.001), indicating a higher genetic heterogeneity within S. haemolyticus. In its turn, S. simulans (D = 83.9%) was more diverse than S. epidermidis (D = 67.4%; p < 0.02) and S. chromogenes (D = 63.3%; p < 0.01). The genetic diversity within the S. epidermidis and S. chromogenes isolates did not significantly differ.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of genotypes/number of isolates</th>
<th>Simpson’s Diversity Index (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. haemolyticus</td>
<td>12/20 milk 26/104 farm environment 27/124 total</td>
<td>94.4 (92.6 - 94.5)</td>
</tr>
<tr>
<td>S. simulans</td>
<td>3/5 milk 12/32 farm environment 13/37 total</td>
<td>83.9 (75.2 - 86.8)</td>
</tr>
<tr>
<td>S. chromogenes</td>
<td>4/17 milk 4/11 farm environment 5/28 total</td>
<td>63.3 (45.2 - 71.7)</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>3/12 milk 5/12 farm environment 5/24 total</td>
<td>67.4 (50.4 - 73.6)</td>
</tr>
</tbody>
</table>
5.4.2. Comparison of genotypes from milk and environment

Within CNS species no clear associations were found between source and genotype. In general, isolates from milk could not be discriminated from isolates from the environment, and only few genotypes were isolated from milk exclusively (Figure 5-1). In contrast, 15 *S. haemolyticus* and 10 *S. simulans* genotypes were isolated from environmental samples exclusively.

5.4.3. Distribution of genotypes between and within herds

The distribution of the genotypes over the different sources within herds is given in Table 5-3. For *S. haemolyticus*, considerable genotypic heterogeneity was found within and between herds, although identical genotypes were found in multiple herds. Per herd, one to six different genotypes were identified among isolates from milk, and five to twelve among isolates from the environment. Among the 20 isolates from milk from the six herds, twelve genotypes were differentiated, and milk isolates from different cows within the same herd mostly displayed different genotypes. Among the 104 environmental isolates from the six herds, 26 genotypes were differentiated, of which 11 were shared by isolates from milk and the environment. Fourteen out of 20 (70.0%) *S. haemolyticus* isolates from milk belonged to genotypes which were also recovered from the environment of the same herd. These genotypes in the environment linked with IMI were mostly retrieved from alleyway samples (herds A, B, C, D, and F) and the air in the free-stalls (herds A, D, and E). In herd D where the highest prevalence of *S. haemolyticus* in milk samples was found, common genotypes were also retrieved from used bedding samples or from the sawdust storage, including two associated with persistent IMI (Table 5-3; Figure 5-1A).

Among *S. simulans* isolates, genotypic heterogeneity was found within and between herds, although certain genotypes were isolated in multiple herds (Table 5-3; Figure 5-1B). Twelve different *S. simulans* genotypes were isolated from environmental samples from the six herds, with two to four different genotypes per herd. Most genotypes were isolated once or sporadically from the environment in a single herd, except for genotype 1b that was isolated on multiple occasions from alleyways and used bedding samples in herd E. Two out of 5 isolates from milk from the six herds belonged
Table 5-3. Distribution of *S. haemolyticus, S. simulans, S. chromogenes*, and *S. epidermidis* genotypes recovered from milk and the environment in six dairy herds as determined by combination of AFLP and RADP analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>Herd</th>
<th>Isolates from milk</th>
<th>Isolates from farm environment</th>
<th>Environmental sources of genotypes shared by isolates from milk and the environment within a herd (genotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Distribution of genotypes</td>
<td>n</td>
<td>Distribution of genotypes</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>A</td>
<td>2</td>
<td>10h (1); <strong>11b</strong> (1)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1</td>
<td><strong>2b</strong> (1)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3</td>
<td>1a (1); 4b (1); <strong>13i</strong> (1)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>9</td>
<td>2b (2); 3a (1); <strong>8e</strong> (1); <strong>10h</strong> (1)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3c (1); <strong>8e</strong> (2); <strong>16l</strong> (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2</td>
<td><strong>2d</strong> (2)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3</td>
<td><strong>1a</strong> (1); 1b (1); <strong>2b</strong> (1)</td>
<td>16</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>A</td>
<td>NI</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>NI</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td><strong>4a</strong> (1)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>2</td>
<td><strong>2c</strong> (1); 2f (1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2</td>
<td><strong>4a</strong> (2)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>NI</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>A</td>
<td>3</td>
<td><strong>1a</strong> (1); 2a (1); <strong>2b</strong> (1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6</td>
<td><strong>1a</strong> (1); 2a (1); 3a (1); <strong>2b</strong> (1)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td><strong>1a</strong> (1)</td>
<td>1a (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>2</td>
<td><strong>1a</strong> (1)</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2</td>
<td><strong>1a</strong> (1)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3</td>
<td><strong>1a</strong> (2)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td></td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----</td>
<td>--------</td>
<td>----</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4d (1)</td>
<td>2</td>
<td>1a (1)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1a (4)</td>
<td>4</td>
<td>1a (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = number of isolates; NI: not isolated; Bold: genotypes recovered both from quarter milk samples and environmental samples within the same herd; ¹Genotypes were determined by combination of amplified fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD) analysis, with numbers indicating the AFLP type and letters the RAPD type. The number of isolates per genotype is given between brackets. Isolates from milk were distributed into persistent and non-persistent strains based on the definitions mentioned in the materials and methods section. Unless stated otherwise, isolates of the same genotype were isolated from different cows within the herd; ²Two isolates with the same genotype originated from different quarters of the same cow.
to genotypes which were also recovered from the environment in the same herd. In herd C, genotype 4a was found both in a milk sample and in alleyway and bedding samples. In herd D, genotype 2c was found both in milk and in alleyway samples.

Limited genotypic heterogeneity was found among *S. chromogenes* isolates between and within herds (Table 5-3; Figure 5-1C). Per herd, one to four different genotypes were identified among isolates from milk, and one to three among isolates from the environment. Three out of five differentiated genotypes were shared by isolates from milk and the environment. Among the 17 isolates from milk from the six herds, four genotypes were differentiated with 1a as the predominating genotype (11/17 isolates). Genotype 1a was isolated from multiple cows within herds and was involved in 5 persistent IMI cases. In herds B, E and F, genotype 1a was also recovered from alleyway samples, used bedding, and the air, respectively. Likewise, genotypes 2b (herds A and B) and 2a (herd F) were also linked with IMI and were recovered from the environment within the same herd (sawdust storage, alleyways, or air).

Heterogeneity among *S. epidermidis* isolates was limited, although there was some between-herd heterogeneity (Table 5-3; Figure 5-1D). Only five genotypes were differentiated, of which three were shared by isolates from milk and the environment. Among the 12 isolates from milk from the six herds, three genotypes were differentiated, and milk isolates from different cows within the same herd mostly displayed the same genotypes. Genotype 2b predominated in herd E and was isolated from milk from four different cows, of which two were affected in two quarters at different time points. In the same herd, genotype 4d was recovered from two cows. Recovery of *S. epidermidis* genotypes from the environment was mostly sporadic, except for genotype 1a that was isolated on multiple occasions in herds A and B from bedding and alleyways. In herd B, this genotype was also isolated from milk of two different cows, including one persistent case.
5.5. Discussion

*Staphylococcus chromogenes*, *S. simulans*, *S. haemolyticus*, and *S. epidermidis* have been isolated from bovine IMI in several studies (Aarestrup and Jensen, 1997; Taponen *et al.*, 2007; Rajala-Schultz *et al.*, 2009; Thorberg *et al.*, 2009). Moreover, these four species were identified as the only CNS species to be causing IMI in six Flemish dairy herds when using a stringent IMI definition based on multiple samplings (Chapter 4). In only a few recent studies, the genetic diversity within CNS species causing IMI (*S. simulans*, *S. epidermidis*, *S. hyicus*, and *S. chromogenes*) has been investigated with ribotyping or pulsed field gel electrophoresis (PFGE) (Aarestrup *et al.*, 1999; Thorberg *et al.*, 2006; Rajala-Schultz *et al.*, 2009; Gillespie *et al.*, 2009; Sawant *et al.*, 2009; Jaglic *et al.*, 2010). In two studies, CNS isolates from milk have been compared with those from other sources within the dairy farm (milker’s hands, bovine skin, and body sites) using molecular methods (Thorberg *et al.*, 2006; Taponen *et al.*, 2008). Large populations of non-*S. aureus* staphylococci in used bedding samples have been reported (Rendos *et al.*, 1975; Matos *et al.*, 1991). Nonetheless, the transmission of CNS via environmental sources in the dairy farm has not been studied previously.

In the present study, distribution of CNS genotypes from milk and the environment in six dairy herds were investigated using two molecular methods. PFGE is considered the gold standard for subtyping in terms of reproducibility and discriminatory power, but it is also time-consuming and laborious. AFLP and especially RAPD represent more rapid techniques and were used complementary in this study to increase resolution. Depending on the species analyzed and the molecular method used, varying degrees of intra-species polymorphism were found. For *S. haemolyticus* and *S. simulans*, a higher discriminatory power was obtained by combining both techniques. In contrast, within *S. chromogenes* little genotypic variation was seen with either method, which could reflect high genetic conservation within the species. For the *S. epidermidis* isolates, both methods gave fully concordant results and no extra information was gained by application of a second technique. Because the number of *S. haemolyticus* isolates was much higher than for the other three species, it is not surprising that the number of genotypes that could be defined was also higher. On the other hand, overall similarity
levels of the *S. haemolyticus* AFLP fingerprints were also considerably lower, indicating considerable genetic divergence within this species.

The within-herd genetic diversity found among *S. haemolyticus* isolates from IMI suggested the existence of multiple sources rather than a contagious nature of the pathogen. A multitude of *S. haemolyticus* genotypes was found on different sites of the dairy farm, including the sawdust storage that had not been in direct contact with the cows. Genotypes linked with IMI were recovered regularly from alleyways and the air in the free-stall, indicating a possible means by which this species is transmitted. Other reservoirs exist for *S. haemolyticus* besides infected udders and the environment. In a parallel study conducted on the same cows as in this study (Braem *et al.*, unpublished results), as well as in other studies (Devriese and Dekeyser, 1980; Baba *et al.*, 1980; Supré *et al.* unpublished results), *S. haemolyticus* has been isolated regularly from teat apices, suggesting a commensal lifestyle. Alternatively, teat apices can get colonized with bacteria originating from the environment. The abundant occurrence in milk, on teats, and in the environment, and the ability of *S. haemolyticus* to cause persistent IMI (Chapter 4; Chaffer *et al.*, 1999), indicate that this species is a highly versatile opportunist that can adapt to intramammary as well as to skin and environmental conditions. Moreover, the *S. haemolyticus* genotypes involved in persisting IMI were all found free-living in the environment as well, suggesting individual genotypes can be highly adaptable to various conditions. Although *S. haemolyticus* has been described as a cause of IMI before (Chaffer *et al.*, 1999; Thorberg *et al.*, 2009), no other reports could be found on the genetic diversity among bovine isolates for comparison with the present findings. However, *S. haemolyticus* is also part of the human skin microbiota, and in human isolates extreme genome flexibility has been demonstrated (Takeuchi *et al.*, 2005). A common trait within the species is that many insertion sequences are present in the genome, promoting frequent genomic rearrangements. Similar mechanisms could be involved in the diversification of bovine *S. haemolyticus* isolates, possibly explaining the high genetic diversity among isolates from bovine milk and the farm environment.

The set of *S. simulans* isolates from milk in the present study was too small to draw final conclusions on genetic heterogeneity or preference of specific genotypes for the udder. In two other studies determining genetic diversity among *S. simulans* isolates from IMI, it has been demonstrated that usually one or two genotypes predominate within herds, suggesting that specific *S. simulans* genotypes are more effective than
others in causing IMI and disseminate within herds (Aarestrup et al., 1999; Taponen et al., 2008). *Staphylococcus simulans* has been rarely isolated from extramammary samples and seems to be well adapted to the udder (Taponen et al., 2008, Braem et al., Supré et al., unpublished results). Nonetheless, a variety of *S. simulans* genotypes were found in the environment in proximity of the cows, namely on alleyways and in bedding from the cubicles. Though not conclusive, this finding indicates that particular *S. simulans* genotypes might be able to survive in the environment. Animals infected with *S. simulans* could contaminate their own environment and establish a potential source of infection. However, further study on the survival and multiplication of this species in the environment is required to clarify if the environment is a reservoir of importance for transmission of *S. simulans* to the udder. In any case, identical *S. simulans* genotypes were found sporadically in milk samples and the environment within herds.

Heterogeneity in the AFLP and RAPD fingerprints of the *S. chromogenes* isolates was limited. These observations are in agreement with the findings of Shimizu et al. (1997) who analyzed 21 *S. chromogenes* from cows and pigs with PFGE and also found highly conserved patterns. In contrast, others have reported more diversity among PFGE fingerprints of *S. chromogenes* isolates: Taponen et al. (2008) identified 10 pulsotypes among 46 isolates from milk and extramammary sites in a single herd, Gillespie et al. (2009) found 33 pulsotypes among 66 isolates from milk in 3 herds, and Rajala-Schultz et al. (2009) found 8 pulsotypes among 27 isolates from milk in two herds. However, composition of the analyzed sets of isolates and the interpretation of PFGE fingerprints differs among studies, making comparison of typing results difficult. Overall, different *S. chromogenes* pulsotypes seem to occur within herds, although the relatively high similarity levels between defined pulsotypes indicates close genetic relatedness (Gillespie et al., 2009; Rajala-Schultz et al., 2009). For identification of specific *S. chromogenes* strains, a higher discriminatory power might be required than feasible with the methods used in the present study. However, comparison with the other species in this study is justified, as they were analyzed accordingly. The seemingly higher conservation of *S. chromogenes* suggests that specific genotypes are well adapted to the udder, which is typical for a true udder-adapted pathogen. Consequently, cow-to-cow transmission might play a more important role for this CNS species. The sporadic *S. chromogenes* genotypes found in the environment were mostly similar to those from milk, indicating that they originated from shedding cows, and probably did not
constitute an important source of infection. Furthermore, *S. chromogenes* has been commonly isolated from bovine teat apices and skin samples (White *et al.*, 1989; De Vliegher *et al.*, 2003). Surprisingly, in a parallel study determining the CNS biota on the teat apices of the cows in the present study, *S. chromogenes* was rarely isolated (Braem *et al.*, data not shown). Likewise, in the study by Taponen *et al.* (2008) *S. chromogenes* does not belong to the predominating species isolated from skin samples, although a number of shared pulsotypes could be identified among isolates from milk and different bovine body sites, indicating the possibility of a skin source. Alternatively, no association has been found between colonization of teat apices of heifers with *S. chromogenes* prior to calving and IMI in early lactation with the same bacterium (De Vliegher *et al.*, 2003). Although *S. chromogenes* can reside on the bovine skin, the importance of this source should be studied further, as this CNS species is a frequent cause of subclinical mastitis (Aarestrup and Jensen, 1997; Taponen *et al.*, 2006).

In the one herd that had a high prevalence of *S. epidermidis* in milk, one predominating type was found affecting multiple cows, indicating clonal distribution within the farm. These observations were similar to those of other studies, in which particular *S. epidermidis* pulsotypes are shown to disseminate within herds, although many other strains have also been differentiated (Thorberg *et al.*, 2006; Sawant *et al.*, 2009; Jaglic *et al.*, 2010). As for *S. chromogenes*, the environment is probably not important as a reservoir for *S. epidermidis* (Chapter 4; Supré *et al.*, unpublished results). *Staphylococcus epidermidis* is relatively uncommon on bovine skin (Baba *et al.*, 1980; White *et al.*, 1989). Rather, it is hypothesized that *S. epidermidis* is a mainly human commensal which might be introduced in a dairy herd via human sources, in particular the milkers’ hands (Thorberg *et al.*, 2006; Jaglic *et al.*, 2010). However, in a recent study conducted in three herds, only two out of 37 CNS isolates from milkers’ skin are identified as *S. epidermidis*. Possibly, the low level of colonization of the milkers’ skin in these herds could explain the low prevalence of *S. epidermidis* IMI (Supré *et al.*, unpublished results). On the other hand, cow-to-cow transmission from infected udders cannot be ruled out as a means of spread, as specific *S. epidermidis* strains have been shown to disseminate within herds (Thorberg *et al.*, 2006; Sawant *et al.*, 2009).

Associations made in the current study between intra-species diversity and epidemiological behaviour of different CNS species are as yet speculative, and are based on a limited number of isolates and herds. However, some of these findings are
consolidated by other reports that have characterized CNS species on the subspecies level with other techniques, including PFGE. It is possible that the observations are not representative for other herds, and further validation is needed through the study of more epidemiologically related CNS in more herds. The data of this work provide novel insights into the population structure and dissemination of CNS species involved in bovine IMI. When needed, appropriate infection control measures could be suggested in the future for management of CNS IMI by more harmful species or strains.

5.6. Conclusions

Genomic DNA fingerprinting revealed many different *S. haemolyticus* genotypes in the cows’ environment, of which part could be linked with IMI. Reservoirs in the environment likely played a role in the epidemiology of this opportunistic and versatile CNS species. A variety of genotypes was also found among *S. simulans* isolates from the cows’ environment. Given the few shared genotypes with isolates from milk, their association with IMI was less pronounced, but there is a possibility that opportunistic infections with *S. simulans* originate from the environment. In contrast, a higher clonality was found among *S. chromogenes* and *S. epidermidis* isolates. The predominance of a limited number of genotypes causing IMI suggests a more udder-adapted and a possibly contagious nature for these species.

Acknowledgments

This research was funded by the the agency for Innovation by Science and Technology in Flanders (IWT-Vlaanderen, grant no. 60714). The authors would like to thank Ann Vanhee and Katrien Verheyen (Institute for Agricultural and Fisheries Research, Melle, Belgium) for their excellent laboratory support.
CHAPTER 6

CHARACTERIZATION OF COAGULASE-NEGATIVE *STAPHYLOCOCCUS* SPECIES FROM COWS’ MILK AND ENVIRONMENT BASED ON THE PRESENCE OF *bap*, *icaA*, AND *mecA* GENES AND PHENOTYPIC SUSCEPTIBILITY TO ANTIMICROBIALS AND TEAT DIP PRODUCTS

V. Piessens¹, S. De Vliegher², B. Verbist¹, G. Braem³, A. Van Nuffel¹, L. De Vuyst³, M. Heyndrickx¹, and E. Van Coillie¹

¹Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium; ²Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Belgium; ³Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium.

In progress.

[109]
6.1. Abstract

The aim of this study was to compare characteristics of coagulase-negative staphylococci (CNS) from different niches in the dairy farm to find possible associations with the ability to cause intramammary infections (IMI).

CNS isolates belonging to 22 different CNS species, either associated with IMI or the farm environment, were analysed by PCR for the presence of the biofilm associated genes \textit{bap} and \textit{icaA}, and the methicillin resistance gene \textit{mecA}. In addition, phenotypic susceptibility to five antibiotics and two teat dip formulations of 82 selected CNS isolates belonging to CNS species commonly causing IMI (\textit{S. chromogenes}, \textit{S. epidermidis}, \textit{S. haemolyticus}, and \textit{S. simulans}) or living freely in the environment (\textit{S. sciuri} and \textit{S. equorum}), was tested and compared between both species groups. Antimicrobial susceptibility was determined by Etest, and a microdilution method was optimized to determine minimal biocidal concentrations for teat dips.

Carriage of \textit{bap}, \textit{icaA} and \textit{mecA} genes was detected significantly more in CNS originating from cows’ environment than in the IMI-causing CNS species. Antimicrobial resistance was mainly to erythromycin (23%) or oxacillin (16%), and was also detected more often in environmental than in IMI-causing CNS species. The isolates least susceptible to teat dips belonged to the IMI-causing species \textit{S. chromogenes} and \textit{S. simulans}.

It was concluded that carriage of biofilm associated genes and antimicrobial resistance do not play a major role in CNS pathogenicity, as these characteristics were more common in environmental than in IMI-causing CNS species. Contrasting, increased tolerance to biocides may be favourable for some CNS species to establish bovine IMI.
6.2. Introduction

In many countries, coagulase-negative staphylococci (CNS) are the most commonly isolated microorganisms from intramammary infections (IMI) in dairy cattle (Pitkalä et al., 2004; Tenhagen et al., 2006; Piepers et al., 2007). CNS are a heterogeneous group of bacterial species that commonly colonize the bovine skin and mucosa, but also occur free-living in the stall environment. Hence, it is assumed that CNS IMI originate from skin or environmental sources, although sources of infection might differ according to the species (Chapter 4; Taponen et al., 2008). The cow's environment harbours a variety of CNS species that rarely cause IMI such as Staphylococcus equorum and S. sciuri (so-called environmental CNS) (Chapter 4; Matos et al., 1991). Two species, S. haemolyticus and S. simulans, are both recovered regularly from the environment and from IMI within herds, acting as so-called environmental opportunistic pathogens. By contrast, two other IMI-causing species, S. chromogenes and S. epidermidis, are rarely isolated from the environment and are put forward as udder-adapted species (Chapter 4; Supré et al., unpublished results).

Although CNS IMI are rarely clinical, certain CNS species induce a stronger inflammatory reaction (Supré et al., 2011), or seem better able to persist in the udder than others (Chapter 4; Thorberg et al., 2009). Biofilm production might represent a colonization advantage in the establishment of (persistent) IMI in cattle (Cucarella et al., 2004; Melchior et al., 2006), but its role in the pathogenesis of CNS mastitis is largely unknown. Two major surface components have been identified that contribute significantly to the formation of staphylococcal biofilms: the polysaccharide intercellular adhesin (PIA) (Cramton et al., 1999), and the biofilm-associated protein (Bap) (Cucarella et al., 2001). PIA is a matrix molecule synthesized by the proteins encoded by the icaADBC gene cluster, which is distributed among a variety of CNS species from nosocomial human infections (Cramton et al., 1999) and food processing environments (Moretro et al., 2003). Carriage of the ica operon has been well established as an important virulence factor of S. epidermidis associated with nosocomial CNS infections in humans (Frebourg et al., 2000; de Silva et al., 2002). Bap is a surface protein first identified in strong biofilm-forming S. aureus isolates from bovine mastitis and is associated with the persistence of infections (Cucarella et al., 2001). Also in CNS isolates
from chronic mastitis cases, homologues of the \textit{bap} gene and production of the encoded protein have been demonstrated (Tormo \textit{et al.}, 2005).

The widespread use of antimicrobials in dairy cattle poses a risk for the emergence of resistant bacteria, but as of now there is no evidence for an increasing trend in antimicrobial resistance in mastitis pathogens (Erskine \textit{et al.}, 2002; Pitkala \textit{et al.}, 2004). Nonetheless, antimicrobial resistance can develop in mastitis pathogens as well as in commensal and environmental microbiota, and plays a major role in the dissemination of pathogens (Catry \textit{et al.}, 2003). Of particular interest is the emergence of methicillin-resistant staphylococci (MRS) in food-producing animals (Lee, 2003; Zhang \textit{et al.}, 2009; Huber \textit{et al.}, 2011), which are resistant to all classes of \(\beta\)-lactam antibiotics due to the expression of the \textit{mecA} gene encoding a penicillin-binding protein with low \(\beta\)-lactam affinity. Furthermore, MRS tend to be more resistant to antibiotics of other classes as well (van Duijkeren \textit{et al.}, 2004; Moon \textit{et al.}, 2007).

Also, post-milking teat disinfection is a widely used practice. As is true for antimicrobials, frequent exposure to sub-inhibitory concentrations of teat dip components could give rise to bacterial populations with increased tolerance for these biocides (Smith \textit{et al.}, 2008). Genes mediating biocide resistance have been found in various CNS species isolated from bulk milk and quarter milk samples, and individual biocide-resistant CNS strains have been found disseminating and persisting within herds (Bjorland \textit{et al.}, 2005; Bjorland \textit{et al.}, 2006).

It can be hypothesized that the predominance of particular CNS species involved in bovine IMI is explained by possible colonization advantages, such as biofilm production, or reduced susceptibility to antimicrobials and/or biocides. The aim of the present study was to compare specific characteristics of the so-called udder-adapted and opportunistic CNS species on the one hand (typically causing IMI) and the so-called environmental species (only causing IMI sporadically) on the other hand. The investigated markers were (i) the presence of the \textit{bap} and \textit{icaA} loci involved in biofilm formation, (ii) the presence of the \textit{mecA} gene encoding methicillin resistance, (iii) the phenotypic susceptibility to various antimicrobials, and (iv) the phenotypic susceptibility to teat dip products as determined through an adapted microdilution assay.
6.3. Materials and methods

6.3.1. Bacterial isolates

A total of 366 CNS isolates belonging to 22 different CNS species were used in this study (Table 6-1). Isolates originated from dairy cows’ milk (n = 77), teat apices (n = 7), and the dairy farm environment (n = 282), and were obtained during a longitudinal study of six herds in Flanders (Belgium) as described previously (Chapter 4). Isolates were genotyped and identified to species level by amplified fragment length polymorphism (AFLP) analysis. According to a strict IMI definition based on both culturing and AFLP typing of isolates from consecutive monthly quarter milk samples, only four CNS species were found to cause IMI in the studied herds: S. chromogenes, S. epidermidis, S. simulans, and S. haemolyticus (Chapter 4). Throughout the current study, these four species are further referred to as “IMI-causing species”, encompassing species that were put forward before as udder-adapted and opportunistic species, respectively. Isolates of these species cultured from the environment were also included. The other 18 CNS species originated primarily from the dairy farm environment (slatted floor alleyways, air in the free-stalls, used sawdust bedding, and fresh sawdust), and were only sporadically isolated from milk samples (Table 6-1). These species were not considered to cause IMI and are referred to as “environmental species” throughout this work. Over the six herds, S. equorum and S. sciuri were the most frequently isolated environmental CNS species. All 366 isolates were analysed for the presence of the bap, icaA, and mecA genes. For phenotypic susceptibility testing, a subset of 82 isolates was selected based on origin (milk or environment) and distinct AFLP fingerprints (S. haemolyticus, n = 32; S. chromogenes, n = 10; S. simulans, n = 10; S. epidermidis, n = 10; S. equorum, n = 10, and S. sciuri, n = 10).

6.3.2. Bap, icaA, and mecA PCR assays

DNA extraction of all isolates was done as previously described (Chapter 3). Genomic DNA was analyzed by specific PCR assays for detection of the biofilm-associated gene bap with primers sasp-6m and sasp-7c (Tormo et al., 2005), the intracellular adhesion protein gene icaA with primers ica4f and ica2r (Moretro et al., 2005), and the methicillin resistance gene mecA with primers mecA1 and mecA2 (Loutit et al., 1992).
2003), and the methicillin resistance gene mecA with primers mecA1 and mecA2 (Murakami et al., 1991). The PCR assay was performed in a 25-µl reaction mixture containing 0.25 µl of each primer (100 µM; Eurogentec, San Diego, CA), 1× PCR buffer II (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂ (Applied Biosystems), 0.1 mM of each dNTP, 1 U of AmpliTaq Polymerase (Applied Biosystems), and 1 µl of bacterial DNA. Thermal cycling conditions were 5 min of denaturation at 95°C; 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 58°C (bap and icaA) or 52°C (mecA), and 30 s of elongation at 72°C; and a final elongation step at 72°C for 8 min. Fragments were analyzed by electrophoresis on 2% (w/v) agarose gels. In each PCR run, a negative (water) and positive control were co-analyzed. The strains used as positive controls were S. xylosus CCM 2738ᵀ (bap), S. capitis subsp. capitis LMG 13353ᵀ (icaA), and S. aureus MRSA ST 398 strain MB 4393 (mecA). The expected amplicon sizes were 971 bp, 568 bp, and 533 bp, respectively.

6.3.3. MIC determination by Etest

Minimal inhibitory concentrations (MIC) of five antimicrobial agents representing different antibiotic classes were determined for a selection of isolates (n = 82) by an Etest according to the manufacturer’s guidelines (AB bioMérieux, Marcy l’Etoile, France). The antimicrobials were cephalothin, enrofloxacin, erythromycin, gentamicin, and oxacillin, representing the cephalosporins, fluoroquinolones, macrolides, aminoglycosides, and penicillins, respectively. Staphylococcus aureus ATCC 29213, S. aureus ATCC 43300, and Enterococcus faecalis ATCC 29213 were used for quality control as recommended by the manufacturer. MIC results were evaluated based on the CLSI document M31-A3 (CLSI, 2007b), with resistance breakpoints of 32 µg/ml for cephalothin, 4 µg/ml for enrofloxacin, 8 µg/ml for erythromycin, 8 µg/ml for gentamicin, and 0.5 µg/ml for oxacillin. Isolates with an MIC in the intermediate range were also categorized as resistant, and half-log MIC values were rounded up to the next upper log value of standard two-fold dilution series (Schwarz et al., 2010).
6.3.4. MBC determination by a microdilution test

6.3.4.1. Optimization of a teat dip susceptibility test

Susceptibility tests were developed for two commercial ready-to-use teat dip products: one containing 0.15% (wt/v) of available iodine and 2% (v/v) emollient (pH 4.8-5.0) (further referred to as “iodine teat dip”), and one containing 0.42% (wt/v) chlorhexidine and 10.5% (v/v) emollient (pH 7.0) (further referred to as “chlorhexidine teat dip”). A microdilution plate assay was optimized for determination of the minimal biocidal concentrations (MBC) of both products for two contact times, and recommendations described in the European standard suspension test (EN 1656, 2000) were followed for dilution/neutralization of the biocides. The neutralizer consisted of 3% polysorbate 80 (v/v), 0.3% lecithin (wt/v), 0.5% sodium thiosulfate (wt/v), 0.1% L-histidine (wt/v), 0.1% peptone (wt/v), and 0.3% sodium chloride (wt/v) (Merck KgaA, Darmstadt, Germany), and was prepared according to the EN 1656 standard. During preliminary tests, the toxicity and efficiency of the neutralizer were tested for both products with the *S. aureus* control strain ATCC 6538 and six CNS test strains (one each of *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. sciuri*, and *S. equorum*) using trypton soy agar (TSA, Oxoid Ltd., Cambridge, UK) as the recovery medium for the determination of viable bacteria (EN 1656, 2000). The iodine product was effectively neutralized for all seven strains, but neutralization of the chlorhexidine teat dip was insufficient, leading to carry-over of the product after exposure. As alternative, Dey-Engley (D/E) neutralizing agar (BD Biosciences, Franklin Lakes, NJ) was tested as the recovery medium. The components in the D/E neutralizing agar effectively quenched residual activity of chlorhexidine and a successful recovery of viable bacteria was obtained. The absence of lethal effects of the experimental conditions was also verified for both products and the seven test strains (one *S. aureus* ATCC 6538 and six CNS). D/E neutralizing broth (BD Biosciences) was used in the final microdilution plate assay as the recovery medium (for both products) to avoid carry-over of chlorhexidine, and to facilitate reading of the MBC (bacterial growth induces a color change of the medium from purple to yellow).
6.3.4.2. MBC determination

Isolates were grown on TSA at 37°C for 24 h, and were transferred at least two times before testing. Bacterial suspensions were made in diluent (EN 1656, 2000) and adjusted to ~ 3.10^8 colony forming units (cfu)/ml (McFarland standard 2). For each tested isolate, appropriate dilutions were spread on TSA for determination of bacterial counts in the inoculum. Two-fold dilutions of both products were freshly made in sterile distilled water and distributed into microtiter plates (160 µl/well), which were inoculated within 2 h after preparation. Subsequently, bacterial suspensions of the 10^-2 dilution (~ 3.10^6 cfu/ml) were diluted two-fold in the designated interfering substance (10% skimmed milk, Oxoid Ltd.), and 40 µl of this mixture was used to inoculate the challenge plates. Each isolate was challenged in duplicate against five concentrations of the iodine dip (1,200 – 75 ppm) and six concentrations of the chlorhexidine dip (3,360 – 105 ppm) at 30°C for two contact times (5 and 30 min). One column of the challenge plates was filled with water as a control for test conditions and neutralizer toxicity. After the contact time, 20 µl of the test suspensions was transferred to plates containing 180 µl of neutralizer per well. After 5 min of neutralization at room temperature, 20 µl was transferred to plates containing D/E Neutralizing Broth (200 µl/well) for recovery of viable bacteria. Recovery plates were read after 48 h of incubation at 37°C, and the MBC was defined as the lowest concentration for which no growth was visible (no color change). In this assay, the MBC represented the biocide concentration that resulted in a 2 to 3 log reduction in viable counts for the respective contact time. A deviation of one dilution step between duplicates was accepted and the MBC values of repeated tests were averaged. Repeatability of the microdilution assay was tested for four CNS strains (one each of *S. chromogenes*, *S. epidermidis*, *S. simulans*, and *S. haemolyticus*) by repeating the test on another day starting from a fresh culture. The MBC values among repeats differed no more than one dilution, indicating repeatability of the protocol.

6.3.5. Sequence analysis

A selection of mecA amplicons (n = 10) was sequenced by a commercial sequencing facility (Macrogen, Seoul, Korea). The same primers as for the PCR were
used for the sequencing reaction and sequence comparisons were done using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.gov/BLAST/).

6.3.6. Statistical analysis

All statistical analyses were done with the Statistica software version 9 (StatSoft, Tulsa, OK). The associations between groups (CNS species causing IMI versus environmental CNS species) and PCR results (positive versus negative) were analysed using two-by-two tables and Chi-square or Fisher exact tests as appropriate. To compare antimicrobial and biocide susceptibility between IMI-causing and environmental species, survival of both groups was plotted using a Kaplan-Meier survival analysis, with the MIC or MBC values defined as the time-to-event (Sampimon et al., 2011). When isolates were killed at the lowest test concentration or were still growing at the highest test concentration, observations were coded as left or right censored, respectively. The Logrank test was used to search for significant differences in survival between both IMI-causing and environmental species. Significance was set at P<0.05.

6.4. Results

6.4.1. Presence of bap, icaA, and mecA genes

In total, bap was found in 11.2% (41/366) of CNS isolates and icaA in 5.2% (19/366) (Table 6-1). Environmental CNS species were significantly more bap-positive (28.1%) or icaA-positive (12.3%) than isolates belonging to the IMI-causing species (0.0% and 0.5%, respectively; P < 0.001). Bap-positive isolates belonged to two CNS species, whereas icaA-positive isolates were distributed over six CNS species. The presence of bap was common among S. equorum isolates (97.1%, 33/34). Of the S. xylosus isolates, 5/9 were positive for bap solely, and 3/9 both for bap and icaA. The icaA gene was found in 29.0% (9/31) of the S. sciuri isolates. Furthermore, icaA was found in 1/24 S. epidermidis, 4/4 S. capitis, 1/5 S. cohnii, and 1/7 S. saprophyticus isolates.
### Table 6-1. PCR results for detection of the *bap*, *icaA*, and *mecA* genes in 366 bovine isolates belonging to 22 coagulase-negative *Staphylococcus* species.

<table>
<thead>
<tr>
<th>CNS species group</th>
<th>N¹</th>
<th>Origin</th>
<th>N (%) of PCR positive isolates</th>
<th>milk</th>
<th>environment</th>
<th><em>bap</em></th>
<th><em>icaA</em></th>
<th><em>mecA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IMI-causing²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>28</td>
<td>Milk</td>
<td>11</td>
<td></td>
<td></td>
<td>1 (4.2)</td>
<td>8 (33.3)</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>24</td>
<td>Milk</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>131</td>
<td>Environment</td>
<td>111³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>37</td>
<td>Milk</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>220</td>
<td>Milk</td>
<td>166</td>
<td>0 (0.0)</td>
<td>1 (0.5)</td>
<td>8 (3.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Environmental</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>34</td>
<td>Milk</td>
<td>29</td>
<td>33</td>
<td>97.1</td>
<td>9 (29.0)</td>
<td>29 (93.5)</td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>31</td>
<td>Milk</td>
<td>30</td>
<td>8 (88.9)</td>
<td>3 (33.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>9</td>
<td>Milk</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. devriesii</em></td>
<td>8</td>
<td>Milk</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>7</td>
<td>Milk</td>
<td>4</td>
<td>1</td>
<td>14.3</td>
<td>1 (20.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>7</td>
<td>Milk</td>
<td>4</td>
<td>1</td>
<td>14.3</td>
<td>1 (20.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>7</td>
<td>Milk</td>
<td>4</td>
<td>1</td>
<td>14.3</td>
<td>1 (20.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. fleurettii</em></td>
<td>6</td>
<td>Milk</td>
<td>6</td>
<td>6</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. arlettae</em></td>
<td>6</td>
<td>Milk</td>
<td>6</td>
<td>6</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>5</td>
<td>Milk</td>
<td>4</td>
<td>1</td>
<td>20.0</td>
<td>1 (20.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>5</td>
<td>Milk</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. succinus</em></td>
<td>5</td>
<td>Milk</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>4</td>
<td>Milk</td>
<td>4</td>
<td>4</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>4</td>
<td>Milk</td>
<td>4</td>
<td>4</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. vitulinus/pulvereri</em></td>
<td>3</td>
<td>Milk</td>
<td>3</td>
<td>3</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. caseolyticus</em></td>
<td>2</td>
<td>Milk</td>
<td>1</td>
<td>1</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>2</td>
<td>Milk</td>
<td>1</td>
<td>1</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. nepalensis</em></td>
<td>1</td>
<td>Milk</td>
<td>1</td>
<td>1</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>146</td>
<td>Milk</td>
<td>123</td>
<td>41 (28.1)</td>
<td>18 (12.3)</td>
<td>35 (24.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>366</td>
<td>Milk</td>
<td>289</td>
<td>41 (11.2)</td>
<td>19 (5.2)</td>
<td>43 (11.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹N: number of isolates; ²Intramammary infection (IMI) was defined as the isolation of the same CNS strains, based on AFLP fingerprinting, at least twice in three consecutive monthly milk samples from the same quarter; ³Including seven isolates originating from teat apices

MecA was detected in 11.7% (43/366) of CNS isolates, and *mecA*-positive isolates were distributed over three species (Table 6-1). Environmental CNS were significantly more *mecA*-positive (24.0%) than the IMI-causing species (3.6%) (P < 0.001). MecA was detected in 33.3% (8/24) of *S. epidermidis* isolates, and was common in *S. sciuri* and *S.
Chapter 6  Characterization of antimicrobial susceptibility and biofilm genes in CNS

fleurettii [93.5% (29/31) and 100% (6/6), respectively]. In 22.6% (7/31) of the S. sciuri isolates, mecA was found concomitantly with icaA.

For all S. chromogenes isolates (24/24) an mecA amplicon with a slightly higher position than the control was obtained. Therefore, the mecA amplicons of a selection of PCR-positive S. chromogenes (n = 4), S. epidermidis (n = 2), S. fleurettii (n = 2), and S. sciuri (n = 2) isolates were sequenced. For the S. chromogenes amplicons no homology with mecA was found in a BLAST search, indicating non-specific amplification. The sequences obtained from the other species were confirmed to be mecA (2 S. chromogenes, 2 S. fleurettii, and 1 S. sciuri), or an mecA homologue (1 S. sciuri).

6.4.2. Antimicrobial susceptibility

All 82 CNS isolates tested were susceptible to cephalothin and gentamicin according to an Etest, and only one isolate was found intermediate resistant to enrofloxacin (Table 6-2). Nineteen isolates (23.2%) were classified as (intermediate) resistant to erythromycin, and 13 isolates (15.9%) to oxacillin. No significant difference was found in survival between IMI-causing and environmental CNS species against increasing erythromycin concentrations (Logrank test; P > 0.05), but the environmental CNS species survived significantly higher oxacillin concentrations than the IMI-causing species (Figure 6-1) (Logrank test; P < 0.001).

Species distribution and resistance patterns of the 82 isolates are given in Table 6-3. In total, 51 isolates (62.2%) were susceptible to all five tested antimicrobials, and the species with the highest proportion of pansusceptible isolates were S. haemolyticus (90.6%) and S. simulans (90.0%). Twenty-nine isolates showed resistance to a single compound, either erythromycin (22.0%) or oxacillin (13.4%), and one S. sciuri isolate was resistant to both. Another S. sciuri isolate was resistant to oxacillin and intermediate resistant to enrofloxacin. Erythromycin-resistant isolates were found among all six CNS species, with the highest proportion in S. equorum (90.0%) and S. epidermidis (30.0%). The oxacillin-resistant isolates belonged to three species: S. sciuri (80.0%), S. epidermidis (30.0%), and S. chromogenes (20.0%). Carriage of mecA was confirmed by PCR in ten (three S. epidermidis and seven S. sciuri) out of 13 oxacillin-resistant isolates. One S. sciuri isolate and two S. chromogenes isolates were phenotypically oxacillin-resistant (MIC 0.5-1.0 µg/ml), but mecA was not found by PCR. There was a significant difference
between mecA-positive and mecA-negative CNS isolates in survival against increasing oxacillin concentrations (Figure 6-2) (Logrank test; $P < 0.001$).

Table 6-2. Minimal inhibitory concentrations (MIC) of five antimicrobial agents as determined by an Etest for 82 CNS isolates from milk ($n = 33$), teat apices ($n = 7$), and the dairy farm environment ($n = 42$)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Number of isolates with MIC (µg/ml)</th>
<th>Number (%) of (intermediate) resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>35</td>
<td>26</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>41</td>
<td>9</td>
</tr>
</tbody>
</table>

Italic: intermediate resistant; grey shade: resistant.
Table 6-3. Species distribution and resistance patterns of 82 bovine CNS isolates tested for their susceptibility to cephalothin, enrofloxacin (EF), erythromycin (EM), gentamicin, and oxacillin (OX) by an Etest.

<table>
<thead>
<tr>
<th>CNS species group</th>
<th>N</th>
<th>Origin</th>
<th>Phenotypic resistance profile</th>
<th>N (%)</th>
<th>pansusceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Milk</td>
<td>EM</td>
<td>OX</td>
<td>OX-EM</td>
</tr>
<tr>
<td>IMI-causing^2</td>
<td></td>
<td>Environment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>32</td>
<td>12</td>
<td>20^3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Subtotal</td>
<td>62</td>
<td>29</td>
<td>33</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Environmental</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>10</td>
<td>1</td>
<td>9</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Subtotal</td>
<td>20</td>
<td>4</td>
<td>16</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>33</td>
<td>49</td>
<td>18</td>
<td>11</td>
</tr>
</tbody>
</table>

^1Number (%) of intermediate resistant or resistant isolates per species or group; ^2Intramammary infection (IMI) was defined as the isolation of the same CNS, based on AFLP fingerprinting, at least twice in three consecutive monthly milk samples from the same quarter; ^3Including seven isolates originating from teat apices
Figure 6-1. Percentage survival of 82 CNS isolates with increasing concentrations of oxacillin. Dotted line: CNS belonging to IMI-causing species (n = 62), full line: CNS belonging to environmental species (n = 20), +: left censored (Log-rank test: P < 0.001).

Figure 6-2. Percentage survival of 82 CNS isolates with increasing concentrations of oxacillin. Dotted line: mecA-positive CNS isolates (n = 13), full line: mecA-negative CNS isolates (n = 69), +: censored (Log-rank test: P < 0.001).
6.4.3. Biocide susceptibility

The MBC of one *S. haemolyticus* isolate could not be determined, because no recovery of the unexposed control was obtained, indicating possible toxicity of the neutralizer or the experimental conditions for this strain. The MBC of the 81 CNS isolates for two contact times is given in Table 6-4 (iodine teat dip) and Table 6-5 (chlorhexidine teat dip). No significant differences were found between the IMI-causing and environmental species in survival against increasing biocide concentration (data not shown). Isolates of *S. chromogenes*, *S. simulans*, and *S. sciuri* were generally less susceptible to the iodine dip (Table 6-4), and isolates of *S. chromogenes* and *S. simulans* were generally less susceptible to the chlorhexidine dip (Table 6-5). The most tolerant isolates belonged to the species *S. simulans* (for both iodine and chlorhexidine) and *S. chromogenes* (for chlorhexidine).

The bactericidal activity of the individual products against the two isolates least susceptible in the microdilution assay was also determined according to the EN 1656 standard, except that D/E neutralizing agar was used for recovery and counting of viable bacteria. As a reference, the bactericidal activity of both products against the *S. aureus* ATCC 6538 control strain was tested. After 30 min of exposure to the respective undiluted products, more than a 5 log reduction in bacterial counts was obtained in all tests, indicating adequate bactericidal activity of both products against the *S. aureus* ATCC 6538 control strain and all tested CNS strains.
Table 6-4. *In vitro* susceptibility of 81 CNS isolates exposed for 5 and 30 min to two-fold dilutions of the iodine teat dip (range 75 – 1200 ppm)

<table>
<thead>
<tr>
<th>MBC of iodine (ppm)</th>
<th>IMI-causing species</th>
<th>Environmental species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. chromogenes</td>
<td>S. epidermidis</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>75 5 min</td>
<td>1 (10.0)</td>
<td>6 (60.0)</td>
</tr>
<tr>
<td>75 30 min</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>112.5 5 min</td>
<td>1 (10.0)</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>112.5 30 min</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>150 5 min</td>
<td>3 (20.0)</td>
<td>2 (20.0)</td>
</tr>
<tr>
<td>150 30 min</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>150 125.5 5 min</td>
<td>1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>150 125.5 30 min</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>150 150 5 min</td>
<td>28</td>
<td>9 (90.0)</td>
</tr>
<tr>
<td>150 150 30 min</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>300 5 min</td>
<td>1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>300 30 min</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>450 5 min</td>
<td>1 (10.0)</td>
<td>-</td>
</tr>
<tr>
<td>450 30 min</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

MBC: minimal biocidal concentration; ppm: parts per million;  
1Intramammary infection (IMI) was defined as the isolation of the same CNS, based on AFLP fingerprinting, at least twice in three consecutive monthly milk samples from the same quarter; 2Bactericidal activity of the respective teat dip was tested against the two least susceptible isolates (EN 1656); 30 min exposure to the undiluted product resulted in >5 log reduction in viable bacteria.
<table>
<thead>
<tr>
<th>MBC of chlorhexidine (ppm) after exposure for</th>
<th>IMI-causing species</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>30 min</td>
<td>N</td>
</tr>
<tr>
<td>105</td>
<td>105</td>
<td>6</td>
</tr>
<tr>
<td>157.5</td>
<td>105</td>
<td>3</td>
</tr>
<tr>
<td>210</td>
<td>210</td>
<td>7</td>
</tr>
<tr>
<td>210</td>
<td>210</td>
<td>32</td>
</tr>
<tr>
<td>315</td>
<td>210</td>
<td>9</td>
</tr>
<tr>
<td>315</td>
<td>315</td>
<td>1</td>
</tr>
<tr>
<td>420</td>
<td>210</td>
<td>20</td>
</tr>
<tr>
<td>420</td>
<td>315</td>
<td>1</td>
</tr>
<tr>
<td>420</td>
<td>420</td>
<td>2</td>
</tr>
</tbody>
</table>

MBC: minimal biocidal concentration; ppm: parts per million; 1 Intramammary infection (IMI) was defined as the isolation of the same CNS, based on AFLP fingerprinting, at least twice in three consecutive monthly milk samples from the same quarter; Bactericidal activity of the respective teat dip was tested against the two least susceptible isolates (EN 1656); 30 min exposure to the undiluted product resulted in >5 log reduction in viable bacteria.
6.5. Discussion

6.5.1. Biofilm genes

This study was conducted to compare characteristics between CNS species causing IMI (S. chromogenes, S. epidermidis, S. simulans, and S. haemolyticus) and those that are primarily free-living in the environment (S. equorum, S. sciuri, and other CNS species). The biofilm mode of growth has been associated with persistence of IMI, as it protects bacteria against the lethal effects of the host defence system and antimicrobial treatment (Melchior et al., 2006). Both Bap and the product of the ica operon (PIA) promote adhesion, cell-to-cell interactions, and bacterial accumulation of staphylococci, and especially the presence of Bap has been associated with persistence of IMI (Cucarella et al., 2004; Tormo et al., 2005). Therefore, it was investigated whether CNS species causing bovine IMI possibly harbour these genes more often than other CNS species primarily colonizing extramammary niches. However, no association was found between the presence of these loci and an increased ability to establish IMI. On the contrary, bap and icaA genes were detected almost exclusively in CNS from the farm environment. Biofilm formation might increase the ability of CNS to persist in the dairy farm environment, where bacteria experience considerable stress caused by changing conditions (e.g. humidity and ambient temperature), mechanical shear, or disinfection. Strong biofilm-forming CNS have also been recovered from food-processing environments, suggesting that biofilm production might promote CNS survival in the environment (Moretro et al., 2003).

The biofilm-forming process of CNS is complex and can be mediated by other factors than Bap or PIA (Cucarella et al., 2004; Cerca et al., 2005b; Fredheim et al., 2009). Therefore, the failure to detect bap or icaA genes does not necessarily imply inability to form a biofilm. Furthermore, the presence of bap or ica loci showing low homology with the currently available sequences cannot be ruled out. Nonetheless, the primers used to amplify the bap gene have been designed from the S. aureus bap gene, from which the sequence has been determined to be more than 97% similar to the bap homologues in S. epidermidis, S. simulans, S. chromogenes, and S. hyicus mastitis isolates, but only 74% similar to the bap gene of S. xylosus (Tormo et al., 2005). The used icaA primers have been designed from conserved regions in icaA genes of S. aureus, S. epidermidis, and S.
caprae (Moretro et al., 2003). Whereas the icaA genes of clinical S. haemolyticus and S. epidermidis strains have been described as closely related (Fredheim et al., 2009), the icaA genes of 38 strains belonging to nine food-related CNS species were less conserved, with sequence similarities of 55 to 82% and considerable sequence diversity between strains within species (Moretro et al., 2003). Given the considerable similarity in bap and ica genes across staphylococci, and the fact that these genes were detected in a variety of species in this study, it is concluded that bap- and ica-mediated biofilm formation likely does not play a role in the pathogenicity of CNS species causing bovine IMI. To consolidate the findings obtained by PCR, the in vitro biofilm production of the different species should be additionally tested by phenotypic methods.

6.5.2. Antimicrobial resistance

Although the importance of CNS in mastitis has not been univocally established, their tendency toward increased antimicrobial resistance is a matter of concern (Myllys et al., 1998; Gentilini et al., 2002; Moon et al., 2007; Botrel et al., 2010). Because of their commensal lifestyle, CNS could serve as a reservoir of transferable resistance determinants for other mastitis pathogens. In the present study, the mecA gene was found in only three out of 22 analysed CNS species. Carriage of mecA in the IMI-causing CNS species was low, but was found in one-third of the S. epidermidis isolates. Occurrence of mecA-positive S. epidermidis in bovine milk samples has been reported previously (Sawant et al., 2009; Fessler et al., 2010; Sampimon et al., 2011), and clonal dissemination of multidrug-resistant S. epidermidis strains carrying mecA within herds has been observed (Sawant et al., 2009; Fessler et al., 2010). Occurrence of methicillin-resistant S. epidermidis (MRSE) in cattle might require more attention, and some researchers even recommend culling of animals infected with methicillin-resistant CNS (MR-CNS) (Gentilini et al., 2002).

Among the CNS species prevailing in the environment, mecA was found commonly in isolates of the species S. sciuri and S. fleurettii. In a recent Swiss study, S. sciuri and S. fleurettii constituted the largest proportion of MR-CNS isolated from bulk tank milk samples (Huber et al., 2011). Although these CNS species are rarely associated with IMI, both S. fleurettii and S. sciuri frequently occur in the dairy farm environment (Piessens et
al., 2011; Supré, unpublished results; Huber et al., 2011), thus representing a considerable reservoir of the mecA gene. However, it is known that S. sciuri and S. fleurettii inherently harbour a mecA homologue that is closely related to the mecA gene of MRSA, but is not located on a mobile genetic element and not necessarily confers phenotypic methicillin resistance (Wu et al., 1996; Tsubakishita et al., 2010). Interpretation of the results should be prudent, as the carriage of a non-functional mecA homologue might be of minor importance.

A selection of 82 CNS isolates belonging to six CNS species was additionally tested phenotypically for their susceptibility to antimicrobial agents belonging to five different antibiotic classes. The MIC values for enrofloxacin, gentamicin, and cephalothin were well below the recommended breakpoints (CLSI, 2007b). The observed effectiveness of these antimicrobials against CNS is in accordance with other studies (Owens and Watts, 1988; Trinidad et al., 1990c; Gentilini et al., 2002; Sampimon et al., 2011). Erythromycin-resistant isolates were found in each of the six CNS species tested, but the highest proportion was found in S. equorum. This is consistent with a previous study in which erythromycin resistance was significantly more common in S. equorum than in other CNS species (Sampimon et al., 2011). Oxacillin-resistant isolates were found among S. chromogenes, S. epidermidis, and S. sciuri isolates. It has been proposed that CNS from animals with oxacillin MICs of 0.5-2.0 µg/ml should be confirmed by mecA-PCR before they are reported as methicillin-resistant (Fessler et al., 2010). Consequently, the borderline resistance observed in two S. chromogenes and one S. sciuri mecA-negative isolates cannot be considered methicillin resistance, and is likely mediated by another mechanism, e.g. hyperexpression of β-lactamases (Chambers, 1997). By contrast, the mecA-positive S. sciuri and S. epidermidis isolates were significantly less susceptible to oxacillin than the mecA-negative CNS, although oxacillin MICs of the mecA-positive isolates were relatively low (0.5-4 µg/ml). Furthermore, no cross-resistance to cephalothin was observed, indicating these mecA-positive staphylococci are methicillin heteroresistant (Chambers, 1997). Lack of cross-resistance to cephalothin has been described before in heteroresistant human S. epidermidis strains (Thornsberry and Mcdougal, 1983). These findings emphasize the difficulties of correct assessment of methicillin resistance in CNS species by phenotypic methods (Chambers, 1997; Fessler et al., 2010).
Overall, a higher proportion of isolates of the environmental (85%) than the IMI-causing CNS species (23%) showed antimicrobial resistance to one or more compounds, notably to oxacillin and erythromycin. In analogy with these findings, others have found higher proportions of resistant isolates among CNS species less likely to colonize the udder compared to CNS species commonly causing IMI (Sampimon et al., 2011; Persson Waller et al., 2011). These results indicate that commensal and environmental CNS might be more important in the development and spread of resistance in dairy farms, and go against the general assumption that antimicrobial use selects for more resistant mastitis pathogens. In contrast to the udder, the environment harbours a mixed and large microbial population, providing a better climate for horizontal transfer than the relatively sterile environment of the udder. The determinants for oxacillin and erythromycin resistance in staphylococci (e.g. mecA, msrA, ermA) are often located on mobile genetic elements favouring their distribution across staphylococcal species (Luthje and Schwarz, 2006; Zhang et al., 2009). Long-term residency in a polybacterial environment (e.g. bovine skin or environment) may have favoured the acquisition of resistance genes carried on mobile genetic elements, especially in an environment where antimicrobials are intensively used (Schwarz and Chaslus-Dancla, 2001; Bjorland et al., 2005).

### 6.5.3. Biocide susceptibility

No standard method is available for testing of the susceptibility of microorganisms to biocides, and several researchers have been making use of end-point microdilution or plating methods (Hogan and Smith, 1989; Bjorland et al., 2005; Smith et al., 2008). However, biocides have no specific targets in the bacterial cell and exert considerable residual activity after exposure. Therefore, neutralization of bactericidal activity after exposure is recommended for standardized determination of the MBC. A microdilution method including a neutralization step was optimized to test isolates against five iodine and six chlorhexidine concentrations in microtiter plates for two contact times. The initial problem regarding the difficulty of neutralizing chlorhexidine has been reported before (Kampf et al., 2005; Mehrgan et al., 2006), but was overcome by using a commercial recovery broth containing neutralizing components. In the assay, both the
iodine and chlorhexidine teat dips were effective against all CNS strains if used at the ready-to-use concentrations (0.15% iodine, 0.42% chlorhexidine). According to the results of this investigation, however, sublethal concentrations of the tested biocides allow some *S. chromogenes* and *S. simulans* strains to survive other CNS species. Possible explanations for these findings might be the presence of efflux pumps, which are known to be widespread among CNS from animals (Bjorland *et al.*, 2005), or differences in the permeability of the cell wall. The ability to survive at higher biocide concentrations could provide a selective advantage for these CNS strains, especially when sublethal concentrations would be used in practice. However, the biological relevance of this finding remains uncertain and requires further study.

### 6.6. Conclusions

Environmental CNS species, *S. sciuri* and *S. fleurettii* in particular, constituted a larger reservoir of *meca* than the CNS species involved in IMI. Except for *S. epidermidis*, CNS species causing IMI did not harbour the *meca* gene. Biofilm genes and antimicrobial resistance were found more commonly in environmental than in IMI-causing CNS species, contradicting the assumption that these characteristics have a major role in CNS pathogenicity. A few *S. chromogenes* and *S. simulans* strains showed higher tolerance to the iodine or chlorhexidine teat dip biocides, which might provide a possible colonization advantage for these species.

### Acknowledgements

This research was funded by the agency for Innovation by Science and Technology in Flanders (IWT-Vlaanderen, grant no. 60714). The authors would like to thank Ann Vanhee and Katrien Verheyen (Institute for Agricultural and Fisheries Research, Melle, Belgium) for their excellent laboratory support.
CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

7.1. Introduction

The scope of this thesis was to improve our understanding of the epidemiology of the coagulase-negative *Staphylococcus* (CNS) species involved in bovine intramammary infections (IMI) and to elucidate the role of CNS present in the dairy farm environment. A molecular method based on amplified fragment length polymorphism (AFLP) analysis was optimized and validated to obtain accurate information on the CNS species level in future work (Chapter 3). The CNS species diversity in the milk and the environment, and persistence of CNS IMI in six dairy herds were investigated by AFLP (Chapter 4). Both milk and environmental isolates of four IMI-causing CNS species were typed by two complementary genotypic methods, AFLP and RAPD, to study dissemination and potential sources within herds (Chapter 5). Furthermore, characteristics associated with biofilm formation, antimicrobial resistance, and biocide tolerance were compared between IMI-causing and environmental CNS species to find possible associations with pathogenicity (Chapter 6). In this final chapter, the results and some new insights into the epidemiology of CNS infections are discussed. The topics that are dealt with are:

- Application of AFLP for species identification and strain typing
- CNS species diversity in milk and the farm environment
- Pathogenic potential and persistence in the mammary gland of CNS
- Epidemiology of individual IMI-causing CNS species
- Characteristics of CNS species causing IMI as compared to environmental CNS species
- Future research topics

[133]
7.2. Application of AFLP genotyping for species identification and strain typing

7.2.1. CNS species identification by AFLP

Gene sequencing is currently widely accepted as the standard method for species identification of *Staphylococcus* species and many other microorganisms (CLSI, 2007a; Zadoks and Watts, 2009). The GenBank database can be consulted worldwide, and species identification relies on a universally meaningful quantitative measure of homology, facilitating interpretation and inter-laboratory comparison of results. Nonetheless, some disadvantages are associated with gene sequencing, which is after all a PCR-based method requiring some level of foreknowledge. Problems with failing amplification reactions, probably due to mismatches in the primer annealing sites, have been reported (Heikens *et al.*, 2005; Supré *et al.*, 2009; Onni *et al.*, 2010). Limitations in discriminatory power, *e.g.* for the 16S rRNA gene, or poor quality of sequences deposited in GenBank may also necessitate additional genes to be sequenced, increasing time and cost (Takahashi *et al.*, 1999; Heikens *et al.*, 2005; Park *et al.*, 2011a). Furthermore, species identification of CNS isolates associated with publicly available gene sequences may be incorrect, leading to misidentification.

The past twenty years there has been a spectacular increase in the number and quality of genotyping methods for the identification and typing of microorganisms (van Belkum *et al.*, 2007). A number of straightforward DNA fingerprinting methods, such as RFLP-PCR (Santos *et al.*, 2008b), tDNA-PCR (Supré *et al.*, 2009), (GTG)$_5$-PCR (Braem *et al.*, 2011), and ITS-PCR (Mendoza *et al.*, 1998) have been employed successfully for the identification of CNS isolates from bovine origin. These methods enable accurate, low cost, high throughput, and rapid CNS species identification, provided a validated reference database is available. Contrasting, AFLP is considerably more expensive, complicated, and takes more time, but has a superior discriminatory power. In a comparative study in which the same 28 bovine associated CNS isolates were identified by three different fingerprinting methods, AFLP showed the highest accuracy compared to tDNA-PCR and (GTG)$_5$-PCR (unpublished results). Furthermore, more-intraspecific
genetic diversity can be observed with AFLP than with (GTG)_5- and tDNA-PCR due to the higher number of visualized polymorphisms (Supré et al., 2009; Braem et al., 2011).

Although the construction and validation of the CNS AFLP reference library required considerable preliminary work (Chapter 3), the resulting accuracy and typeability were highly lucrative in the following field study (Chapter 4). Given the high species-specificity of fingerprints of both CNS type and field strains, misidentification by AFLP was highly unlikely (Chapter 3). Several less common CNS species, e.g. S. lentus, S. vitulinus, S. gallinarum, and S. nepalensis, were recognized using the optimized AFLP library, and only 3.2% of isolates remained unidentified, confirming the good typeability (Chapters 3 & 4). When exploring more heterogeneous bacterial populations, as are found in the environment, a good typeability for identification is crucial, as has been demonstrated in the study by Taponen et al. (2008). In that study, approximately half of the extramammary CNS isolates could not be identified using API Staph because of the poor typeability, making it difficult to estimate the true CNS species diversity. Typeability of the AFLP method was improved by adding a broad range of bovine CNS field strains to the database (Chapter 3), as well as aberrant strains such as for the atypical S. haemolyticus cluster (Chapter 4).

Primarily thanks to the advanced molecular techniques that are more and more applied for microbiological research, new Staphylococcus species from cows, such as S. agnetis and S. devriesei, have been characterized (Supré et al., 2010; Taponen et al., 2011). Commercial phenotypic methods are not able to recognize these new species, and updating their databases could impossibly keep pace with the increasing number of newly described Staphylococcus species from animals. Given the high flexibility and resolution of AFLP fingerprinting, the library could easily be optimized to correctly identify all currently known Staphylococcus species, subspecies, and biotypes within, which is harder to obtain for phenotypic methods and less discriminatory molecular methods.

7.2.2. CNS strain differentiation by AFLP

The key benefit of AFLP genotyping is that species identification and strain typing can be done simultaneously (Savelkoul et al., 1999). AFLP has been previously used for strain typing of S. aureus and S. epidermidis isolates from humans, and performed as well
as MLST and PFGE in discriminating epidemiologically related strains, and was more discriminatory than RAPD (Sloos et al., 2000; Melles et al., 2007). AFLP and MLST require practically the same equipment, i.e. a PCR machine, a sequencer, and specialized software. However, for MLST multiple genes have to be sequenced, whereas AFLP fingerprints are generated in a single sequencing run. Each microorganism can be genotyped by AFLP without any foreknowledge, which is highly convenient when analyzing a heterogeneous bacterial population such as CNS. By contrast, MLST requires optimization and validation of primers and conditions for each individual species, and MLST schemes are currently only available for *S. aureus* and *S. epidermidis* (Enright et al., 2000; Thomas et al., 2007). Like AFLP, PFGE is also a generally applicable and highly reproducible method that performs very well in discriminating CNS strains (Thorberg et al., 2006; Gillespie et al., 2009). However, the labour-intensiveness of PFGE makes it less practical for high-throughput analysis.

AFLP showed good repeatability, enabling the construction of a large database for comparison of CNS isolates over time (Chapter 2). Homology between AFLP fingerprints is expressed as a similarity coefficient, Pearson correlation, which is relatively sensitive to fluctuations in background and band intensities because entire densitometric curves are compared. This can somewhat complicate the automated analysis of AFLP fingerprints and can be a source of ambiguity (Savelkoul et al., 1999; Huys et al., 2000). Therefore, AFLP fingerprints were also examined visually, and RAPD was used to complement AFLP typing. As it is also a whole-genome method, RAPD has good discriminatory power (Savelkoul et al., 1999; van Belkum et al., 2007). RAPD has the major advantage that fingerprints are simple and easy to interpret, and that it is a fast and cheap typing method. Although RAPD is less suited for the construction of large databases due to its limited reproducibility, it is very convenient in case studies and for detailed comparison of smaller sets of isolates, as was done in Chapter 5.

Knowledge of the overall genetic structure within bacterial populations is important for the accurate interpretation of typing results, but limited information concerning clonality of CNS species was available when this project was initiated. According to AFLP and RAPD, genetic diversity clearly differed between four IMI-causing CNS species, *S. haemolyticus*, *S. simulans*, *S. epidermidis*, and *S. chromogenes* (Chapter 5). AFLP seemed to be highly discriminatory for *S. haemolyticus*, *S. simulans*, and *S. epidermidis*, and gave largely concordant typing results as for RAPD. Contrasting,
AFLP and RAPD typing revealed little genetic variation within \textit{S. chromogenes}, indicating this species might be more clonal. Alternatively, genetic variation might have been limited because the \textit{S. chromogenes} isolates in the present study originated from only six herds. In other studies, more diversity has been found among \textit{S. chromogenes} isolates with PFGE, but identical pulsotypes were also recovered from different herds (Gillespie \textit{et al.}, 2009; Rajala-Schultz \textit{et al.}, 2009). A polyphasic approach using highly discriminatory methods and the analysis of more epidemiologically unrelated \textit{S. chromogenes} isolates will be needed to assess the strain diversity within bovine isolates, and to confirm if this species is really clonal. In theory, the AFLP method described in Chapter 3 can easily be adjusted to obtain additional strain information by adding or replacing nucleotides in the selective extensions of the primers used for the final selective amplification in the AFLP protocol. Adding additional selective nucleotides would reduce the number of generated fragments, facilitating the interpretation of the otherwise complex AFLP fingerprints. Replacing selective nucleotides would generate alternative fingerprints visualizing other polymorphisms, providing additional strain information.

In conclusion, the methods presented in this dissertation for strain typing contributed to a better understanding of the species and strain diversity of CNS from cattle. However, it is clear that different typing methods will produce different outcomes, and that a combination of methods is preferred. AFLP can be used to group, deduplicate, and select isolates for further study, and for typing of non-clonal CNS species. However, validation of AFLP as a single typing method for individual CNS species is subject for further study. In addition, RAPD is useful for comparison of smaller numbers of well defined CNS isolates in a limited time frame.

7.3. CNS species diversity in milk and the farm environment

At present, there is a more accurate insight into the CNS species diversity occurring in milk samples than before. Large collections of CNS isolates from milk (without background information) have recently been identified by gene sequencing, and it is clear that only a few CNS species predominate in IMI, in addition to a large
diversity of other CNS species that are more sporadic (Supré et al., 2009; Sampimon et al., 2009c; Park et al., 2011a). In spite of the availability of various accurate DNA-based identification methods for human-associated CNS species since about fifteen years (Goh et al., 1996; Maes et al., 1997; Poyart et al., 2001; Martineau et al., 2001; Edwards et al., 2001; Drancourt and Raoult, 2002a), inadequate phenotypic methods have been employed in nearly all field studies on CNS mastitis conducted in the last twenty-five years. Field studies relying on DNA-based identification including well characterized CNS from IMI and extramammary sources are only recently being carried out (this dissertation; Taponen et al., 2006; Taponen et al., 2007; Taponen et al., 2008; Supré et al., 2011; Braem et al., unpublished results), and are essential for the proper understanding of the dynamics and epidemiology of individual CNS species in dairy farms.

The CNS species most commonly isolated from quarter milk samples were *S. chromogenes* and *S. haemolyticus*, which were recovered in all six study herds, and accounted for over 50% of all milk isolates (Chapter 4). The predominance of *S. chromogenes* as a cause of subclinical mastitis is in agreement with most other recent studies that performed phenotypical or molecular species identification (Taponen et al., 2007; Thorberg et al., 2009; Rajala-Schultz et al., 2009; Gillespie et al., 2009; Sawant et al., 2009; Sampimon et al., 2009b; Supré et al., 2011). By contrast, *S. haemolyticus* has been mentioned as a commonly isolated species in only a limited number of previous studies using phenotypic identification (Chaffer et al., 1999; Thorberg et al., 2009). The identification of bovine *S. haemolyticus* isolates based on phenotype has been shown to be problematic (Langlois et al., 1983; Watts et al., 1984; Bes et al., 2000; Capurro et al., 2009; Sampimon et al., 2009c; Park et al., 2011a). Consequently, this species could have been previously underestimated as a subclinical mastitis pathogen. Likewise, occurrence of *S. haemolyticus* might differ among regions, or might have increased over time. The third and fourth most common CNS species in this work, *S. epidermidis* and *S. simulans*, were found in milk samples of only part of the herds, indicating herd variation in CNS species distribution. Admittedly, only ten cows were sampled repeatedly per herd, but a considerable herd variation in CNS species causing IMI is also indicated by other studies in which more cows per herd have been sampled (Gillespie et al., 2009; Thorberg et al., 2009; Supré et al., 2011).
The most isolated CNS species from environmental samples were *S. equorum*, *S. sciuri*, and *S. haemolyticus* (Chapter 4; Table 7-1). Because selective enrichment was used (except for air samples), and no bacterial counts per species were performed, the CNS species distribution does not provide quantitative information. Nonetheless, the repeated isolation of the same CNS species in various locations in the herds strongly indicated their ubiquitous nature (Table 7-1).

**Table 7-1.** Percentage of environmental samples, taken monthly from four niches on six Flemish dairy farms during 13 months, that were culture-positive for the six most isolated CNS species from the stall environment\(^a\) and/or milk samples\(^b\)

<table>
<thead>
<tr>
<th>Species</th>
<th>Bedding</th>
<th>Alleyways</th>
<th>Air</th>
<th>Sawdust storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. equorum</em>(^a)</td>
<td>26.0</td>
<td>30.8</td>
<td>56.4</td>
<td>11.0</td>
</tr>
<tr>
<td><em>S. sciuri</em>(^a)</td>
<td>39.7</td>
<td>46.2</td>
<td>15.4</td>
<td>19.2</td>
</tr>
<tr>
<td><em>S. haemolyticus</em>(^{a,b})</td>
<td>12.3</td>
<td>35.9</td>
<td>60.3</td>
<td>17.8</td>
</tr>
<tr>
<td><em>S. simulans</em>(^b)</td>
<td>14.0</td>
<td>26.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>S. chromogenes</em>(^b)</td>
<td>2.7</td>
<td>6.4</td>
<td>3.9</td>
<td>1.4</td>
</tr>
<tr>
<td><em>S. epidermidis</em>(^b)</td>
<td>5.5</td>
<td>5.1</td>
<td>1.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Bold: commonly presence in an environmental niche.

In a previous study in a single American dairy farm using phenotype-based identification, high numbers of *S. xylosus*, *S. sciuri*, *S. saprophyticus*, and *S. cohnii* have been isolated from used bedding samples (Matos et al., 1991). These free-living CNS species are designated “environmental staphylococci”, because they are restricted to the environment and are not implicated in mastitis. These findings are partly corroborated in the present work, as most CNS species common in the environment were not or rarely found in milk samples (Figure 7-1). The most notable exceptions were *S. haemolyticus* and *S. simulans*, which were recovered from a considerable proportion of environmental samples and were also a cause of IMI (Table 7-1; Chapter 4). Contrasting, *S. chromogenes* and *S. epidermidis*, the other two IMI-causing species, were recovered only sporadically from environmental samples. Whereas *S. haemolyticus* was regularly recovered from all four sampled environmental niches, *S. simulans* was found exclusively in bedding and alleyway samples (Table 7-1).
Figure 7-1. Distribution of the CNS species isolated at least on ten occasions from milk or the farm environment (total number of isolates per species is given above the bars – data from Chapter 4).

More pronounced as in milk, considerable herd variation was observed in the predominating environmental CNS species. For instance, *S. fleurettii* was isolated in one herd from 69% of bedding samples, 92% of alleyway samples, 46% of air samples, and 23% of the sawdust storage samples, whereas it was sporadically isolated from only two other herds. Possibly, such differences might be attributed to the type or source of bedding (Matos et al., 1991). Despite the ubiquitous presence of *S. fleurettii* in this herd, no IMI caused by this species were detected, indicating the environment harboured strains without pathogenic potential. Even so, *S. fleurettii* has been found as a cause of IMI in other studies (Sampimon *et al.*, 2009c; Supré *et al.*, 2011).

The species most commonly cultured from the teat apices of the cows sampled in this investigation showed higher agreement with those occurring in the environment, than with those from milk (Braem *et al.*, unpublished results). The most cultured CNS species from teat apices over the six herds were *S. haemolyticus, S. equorum, S. cohnii*, and *S. saprophyticus*. Except for *S. haemolyticus*, the species common on the teat apices were cultured primarily from the environment and only sporadically from milk. This observed analogy indicates that the majority of the CNS microbiota present on bovine teats might be the result of environmental contamination. Again, there was a
considerable herd variation in CNS species distribution from teat apices (Braem et al., unpublished results).

In conclusion, the species that seem well adapted to the mammary gland were mainly different from those prevailing in the environment, with the exception of *S. haemolyticus* and *S. simulans*. These findings emphasize the variation in epidemiological behaviour, characteristics, and reservoirs of the different CNS species, which have been falsely considered as a homogeneous group before. The reason why some of the common environmental CNS species caused IMI (*e.g.* *S. haemolyticus*), and others did not (*e.g.* *S. equorum, S. sciuri, S. fleurettii*), is unknown, but suggests that species-specific characteristics play an important role in the ability to establish IMI. Despite that herds overall had similar properties in terms of production, BMSCC, herd size, udder health management (such as implementation of teat dipping), and bedding material, the remarkable herd differences found in the milk, on teat apices, and the cows’ surroundings strongly indicate that herd factors influence the CNS species distribution on dairy farms.

### 7.4. Pathogenic potential and persistence in the mammary gland of CNS species causing IMI

Nearly all studies determining the impact of CNS IMI on SCC or milk yield have regarded CNS as a group, and concluded that they are of minor importance for udder health (Rainard et al., 1990; Schukken et al., 2009; Piepers et al., 2009a; Paradis et al., 2010). The contribution of CNS as a group to the BMSCC is believed to be minimal, and a higher milk yield has even been found for cows and heifers with CNS IMI (Schukken et al., 2009; Piepers et al., 2011). However, some CNS species seem to be more pathogenic than others, as indicated by studies that have differentiated CNS (Waage et al., 1999; Sampimon et al., 2009b; Persson Waller et al., 2011; Supré et al., 2011; Park et al., 2011b), and their harmful impact on udder health might have been extenuated by the nonpathogenic CNS.

To assess the impact of CNS IMI, the definition of what is an CNS IMI is of key importance. A large proportion of CNS IMI seems to be transient (Taponen et al., 2007; Thorberg et al., 2009; Persson Waller et al., 2011; Supré et al., 2011), thus elevating SCC
only for a short period. Furthermore, CNS are common colonizers of teat keratin, and might be cultured from milk samples as contaminants. To avoid misclassification (false-positives), multiple milk samples were considered to define CNS IMI status: two out of three consecutive monthly samples had to be culture-positive (≥300 cfu/ml) for the same CNS AFLP type (Chapter 4). As the time interval of one month between samplings was quite large, the IMI definition was rather strict, and singled out the more persistent IMI, but due to this strictness, a clear distinction could be made between CNS species able to establish IMI, and species being easily removed from the udder (Chapter 4).

Dohoo et al. (2011) proposed that culturing two CNS colonies from a single quarter milk sample (0.01 ml) would suffice to determine an IMI, and that inclusion of a SCC threshold as an additional criterion in the IMI definition would reduce sensitivity, but increase specificity. Although this IMI definition based on a single milk sample is much more sensitive than the one used in the present study, many IMI with insignificant impact would get included. Furthermore, including SCC as a criterion in the IMI definition to increase specificity would exclude the possibility to analyse the impact of CNS IMI on SCC. Thus, despite the low sensitivity, it can be concluded that the IMI definition of the present study is well suited for future study on the impact of CNS IMI, because the most relevant CNS IMI are selected for, and it enables determination of their impact on SCC. For future research, it is also important to unify the definition of what is a transient or persistent CNS IMI to allow comparison of studies, which is now difficult due to the differences in IMI definitions and sampling schedules between studies (Taponen et al., 2007; Thorberg et al., 2009; Persson Waller et al., 2011).

Our longitudinal data allowed for evaluation of persistence of CNS genotypes in the mammary gland. However, it should be kept in mind that persistence does not necessarily imply pathogenicity. Protective effects of naturally occurring *S. chromogenes* IMI against *S. aureus* infections have been demonstrated, suggesting that these types of CNS IMI might be beneficial (Matthews et al., 1990). Contrasting, a recent study showed that *S. chromogenes* IMI can increase SCC to almost the same extent as *S. aureus* (Supré et al., 2011). Whether a CNS IMI turns out to be beneficial, innocuous, or harmful, probably depends on the CNS species involved, although host effects can certainly not be overlooked. Moreover, strain-dependent virulence might also be in play. In Chapter 5, identical CNS genotypes were found to cause both persistent and non-persistent IMI. Analogously, Taponen et al. (2007) found isolates with similar AFLP patterns both in
transient and persistent IMI, indicating that host-pathogen interactions might indeed be crucial in the establishment of (persisting) CNS IMI and the development of clinical symptoms.

As CNS are rarely implicated in clinical mastitis, their overall pathogenic potential is clearly limited. However, since subclinical mastitis accounts for substantial economic loss (Ott, 1999; Halasa et al., 2009), and CNS is the most common cause, further research is warranted. Assessment of the impact and virulence of the most important CNS is needed, and it is crucial that this kind of studies is conducted on the CNS species level, using molecular identification and meaningful IMI definitions to avoid misinterpretation and to allow comparison over studies.

7.5. Epidemiology of individual CNS species: udder-adapted (contagious) versus environmental (opportunistic) species

Despite the general use of dry-cow therapy and teat dipping, the number of CNS IMI remains relatively constant. This observation is probably not a result of the ineffectiveness of these control measures, but could rather be attributed to the epidemiological behaviour of CNS. Contrasting to the typically “contagious” mastitis pathogens, CNS probably have other ways to spread than during the milking process. However, little work has been done on the relationship between the infectious and the commensal/environmental lifestyle of CNS from dairy cattle, making it hard to explain where CNS IMI originate from. Because the CNS include normal residents of the bovine skin, CNS have generally been regarded as skin opportunistic pathogens. Furthermore, there have been several indications that CNS IMI might originate from environmental sources: (i) the prevalence of CNS IMI is highest in heifers that have never been in contact with the milking machine, indicating these young animals might contract IMI from the environment, (ii) CNS have been found ubiquitously in used bedding samples and on teat apices (Braem et al., unpublished results; Rendos et al., 1975; Matos et al., 1991), indicating that teat apex colonization by CNS might be the result of environmental contamination, and (iii) risk factors for CNS IMI were similar to those for
major environmental mastitis pathogens (Sampimon et al., 2009b; Piepers et al., 2011). However, the assumption that CNS IMI originate from skin or environmental sources has been merely speculative, and does not rely on accurate strain or even species differentiation of CNS from IMI and extramammary sources. Moreover, some studies suggested that the CNS species causing IMI differ from those found on bovine skin (Devriese and Dekeyser, 1980) and in the environment (Matos et al., 1991), contradicting the common belief that these are major sources. The only way to elucidate these conflicting findings is to study the epidemiology of CNS species individually, preferably on the strain level.

The results of the current work, in combination with those of the parallel study of CNS found on the teat apices of the same cows (Braem et al., unpublished results), strongly indicated that not all CNS species fit the definition of skin or environmental opportunistic pathogens. For S. chromogenes and S. epidermidis, the existence of a large environmental source was countered (Chapter 4, Table 7-1). Still, 22% and 43% of the recovered isolates belonging to these species, respectively, originated from environmental niches, and genotypes identical to those causing IMI were found (Table 4-5; Table 5-3). In Chapter 5, a possibly contagious nature was proposed for these species, and the common genotypes were explained as originating from shedding cows. However, the possibility of an environmental source of infection cannot be ruled out with certainty, as there is no way to determine the directionality of transmission. However, given the limited proportion of environmental samples from which these species were recovered, the latter scenario may be deemed less likely (Table 7-1). The putative reservoir of S. chromogenes on teat apices, as has been indicated previously (De Vliegher et al., 2003; Taponen et al., 2008), was not confirmed in the studied herds. Neither was S. epidermidis common on teat apices (Braem et al., unpublished results). The main reservoir of these species seems to be the udder, although other unknown sources might exist. The predominance of a limited number of S. chromogenes and S. epidermidis genotypes causing IMI in multiple cows within herds indicated clonal dissemination, or the existence of a common source (Chapter 5). Possibly, these predominating strains dispose of mechanisms to evade the host immune response, favouring colonization of the udder and persistence (udder-adaptation). Staphylococcus chromogenes and S. epidermidis IMI are mostly subclinical and often persistent (Taponen et al., 2007; Thorberg et al., 2009; Persson Waller et al., 2011). As this kind of IMI usually
remains undetected, transmission of the causative agent is likely to occur. When the majority of subclinical IMI in a herd is caused by CNS strains that are possibly more “udder-adapted”, the farmer could benefit from controlling contagious transmission. Especially when MR-CNS are involved, as was observed for a clonally disseminated mecA-positive *S. epidermidis* genotype in herd E (Chapters 5 & 6), controlling the transmission among cows might be recommended, even more so because *S. epidermidis* is known to be causing long-lasting udder health problems in some herds (Thorberg *et al*., 2006).

*Staphylococcus simulans* has been previously regarded as a specific mastitis pathogen because it is rarely isolated extramammary (Taponen *et al*., 2008). Although this finding was supported by the parallel study on CNS species colonizing the teat apices (Braem *et al*., unpublished results), it was not in the current study, as *S. simulans* was regularly recovered from environmental samples in close proximity of the cows (Table 7-1, Chapter 4). It seems that *S. simulans* has an intermediate epidemiology: the udder is likely the original reservoir, but transmission might occur via the environment (alleyways and bedding). Little evidence was found for this hypothesis by genotyping due to the limited number of *S. simulans* strains involved in IMI. However, a great level of diversity was found in genotypes from the environment, indicating that the environment supports the survival of many different *S. simulans* strains. Whether *S. simulans* can also multiply in bedding or on the slatted floor alleyways is unknown, but if so, these niches might be considered potentially important sources.

*Staphylococcus haemolyticus* was clearly well adapted to conditions in the environment, on the skin, and in the udder (Chapter 4; Braem *et al*., unpublished results). The ubiquitous presence of *S. haemolyticus* in the environment (Table 7-1) and on skin indicates that the exerted infection pressure for this species might be high. Indeed, a large diversity of genotypes causing IMI was found, indicating a multiplicity of sources (Chapter 5). *Staphylococcus haemolyticus* was isolated from 60% of the air samples taken in the free-stalls, and identical genotypes were isolated from IMI and the air within herds, indicating the possibility of an airborne transmission (Chapters 4 & 5). Furthermore, *S. haemolyticus* was isolated from 18% of fresh bedding samples, which also suggested this species might be a true environmental opportunist. Contrasting to *S. haemolyticus*, the other two ubiquitous environmental species, *S. equorum* and *S. sciuri*, were not implicated in IMI, which might indicate that *S. haemolyticus* isolates dispose of
characteristics promoting colonization of the udder. In a preliminary study, a selection of 70 *S. haemolyticus* isolates was tested overnight at 37°C on Columbia agar supplemented with 5% sheep blood, and all appeared haemolytic (data not shown). Production of cytotoxins might represent a virulence trait playing a role in CNS pathogenesis (Ebrahimi and Taheri, 2009), but the potential role in the mammary gland of this haemolytic property of *S. haemolyticus* needs further investigation.

*Staphylococcus xylosus* is a species closely related to *S. haemolyticus*, and was found confined to the environment in the current work (Figure 7-1), and that of Matos et al. (1991). However, *S. xylosus* has been found as a common mastitis pathogen in other studies using genotypic identification (Boerlin *et al.*, 2003; Supré *et al.*, 2011), and is the most commonly isolated CNS species from milk samples of cows with elevated SCC in Flanders [Annual report Milk Control Centre Flanders (MCC), 2010]. Admittedly, in the routine diagnostics by MCC API Staph is used for identification, but a good agreement has previously been shown between the API Staph system and rpoB gene sequencing for this species (Sampimon *et al.*, 2009c). Furthermore, persistence of *S. xylosus* in the mammary gland has been demonstrated by tDNA-PCR, as well as an SCC increase comparable to *S. chromogenes, S. simulans*, and *S. aureus* IMI (Supré *et al.*, 2011). In the study of Thorberg et al. (2009), *S. xylosus* and *S. haemolyticus* caused a considerable proportion of IMI in 11 herds, but less than 20% of these IMI persisted. These differences between studies might indicate that IMI with particular “environmental” CNS species are more prevalent in some herds than in others, possibly explaining part of the herd variation, and that particular strains within these species might be better adapted to cause (persistent) infections.

Several studies report a high percentage of CNS IMI at calving, followed by a considerable drop during the first weeks in lactation (Matthews *et al.*, 1992; Aarestrup and Jensen, 1997; Piepers *et al.*, 2010). A possible explanation for this observation might be that many CNS IMI around calving are caused by “environmental” CNS which are quickly removed from the udder, whereas the more udder-adapted CNS are more likely to persist. Prevention of CNS infections by environmental CNS opportunists might be important in periods that cows are more vulnerable, and can be achieved by hygienic measures such as regular disinfection of stalls, replacement of bedding, general cow hygiene, etc.
Means of prevention diverge depending on epidemiology of pathogens, which clearly differed among CNS species, but probably also between strains. Given the considerable diversity within the CNS group and between herds, molecular strain typing is indispensable to further unravel the dynamics of CNS mastitis. Analysis of more epidemiologically related isolates of relevant CNS species would improve our understanding on the origin of CNS infections.

7.6. Characteristics of CNS species causing IMI as compared to environmental CNS species

7.6.1. Antimicrobial and biocide susceptibility

The use of antimicrobial drugs and biocides is known as an important selective force in bacterial ecology (Hogan et al., 1987; Rajala-Schultz et al., 2004; Pol and Ruegg, 2007; Vali et al., 2008), and there is increasing evidence that CNS from bovine mastitis constitute a reservoir of antimicrobial resistance genes (Luthje and Schwarz, 2006; Olsen et al., 2006; Fessler et al., 2010; Sampimon et al., 2011). Besides their epidemiologic behaviour, reduced susceptibility to antimicrobials or teat dips might explain for a part why CNS have become the major bacteria establishing IMI in dairy cattle. The common use of antimicrobials might have promoted the emergence and spread of resistant CNS in dairy cattle, possibly compromising the future effectiveness of therapy against staphylococcal infections. Nonetheless, antimicrobial resistance seemed to be more common in CNS species rarely associated with IMI, with the exception of Staphylococcus epidermidis (Chapter 6). Not only pathogens, but also commensal and environmental bacteria can be affected by subtherapeutic concentrations of antimicrobials intended for therapy (Schwarz and Chaslus-Dancla, 2001; Russell, 2003; Catry et al., 2003). Regarding CNS on dairy farms, free-living CNS species likely constitute a more significant reservoir of resistance determinants than the species commonly associated with IMI.

The only antimicrobial resistance of importance found in bovine associated CNS in this dissertation was for oxacillin (16%) and erythromycin (23%) (Chapter 6). The
former is mediated by the mecA gene, whereas the latter can be conferred by a diversity of mechanisms encoded by different genes (Luthje and Schwarz, 2006). The mecA homologue of S. fleurettii and S. sciuri isolates (Chapter 6) is generally not contained on a mobile genetic element (Tsubakishita et al., 2010), contrasting to methicillin-resistant S. aureus, S. epidermidis, and most other CNS species found so far, of which the mecA gene is part of a transferable staphylococcal chromosomal cassette mec (Zhang et al., 2009; Fessler et al., 2010). With the PCR used in Chapter 6, no distinction can be made between the non-mobile mecA homologue and the real mecA gene, thus the nature of the mecA reservoir in these environmental CNS species needs further study. Contrasting, the genes conferring macrolide resistance in staphylococci mostly have a plasmid location (Luthje and Schwarz, 2006). In CNS isolates from bovine mastitis, ermC and lnuA genes have been found on similar sized and structurally related plasmids among different CNS species, suggesting an interspecies exchange of these mobile resistance determinants (Luthje and Schwarz, 2006). We found macrolide resistance in six different CNS species common in milk or the cows’ surroundings, which might indicate dissemination of the involved resistance genes (Chapter 6). However, more research on the mechanisms, spread and epidemiology of the involved determinants is needed to confirm this.

As there is no standard method to evaluate biocide susceptibility of bacterial isolates, a microdilution method was optimized which allowed testing of multiple isolates, contact times, products and concentrations at the same time. This convenient new method can easily be adjusted for testing of other mastitis pathogens, products, or a broader range of biocide concentrations, as required. Regarding the CNS isolates tested a few S. simulans and S. chromogenes isolates were found to be slightly more tolerant to the chlorhexidine and iodine products compared to S. haemolyticus, S. epidermidis, S. equorum, and S. sciuri (Chapter 6). Possibly, increased tolerance to biocides might favour the continued colonization of teat skin and teat orifices, increasing the chance of establishing IMI, but the biological relevance of our findings is subject for further study. Increased tolerance to disinfectants in staphylococci is mostly conferred by integral drug transporter systems, and efflux mechanisms common in staphylococci are those encoded by the various qac genes (Russell, 1997; Hassan et al., 2007; Smith et al., 2008). These are generally located on plasmids and mediate efflux of hydrophobic drugs, including quaternary ammonium compounds (QAC), intercalating dyes (e.g. ethidium bromide), and cationic biocides (e.g. bisguanides such as chlorhexidine). The
mechanisms that increase tolerance of bovine CNS isolates to biocides requires more study, as these might explain why CNS have become so successful as mastitis pathogens despite the general use of post-milking teat disinfection.

Our knowledge on bovine staphylococci as reservoirs or recipients of resistance determinants is limited, and the potential role of antimicrobial and disinfectant resistance in their spread remains to be determined. The sensible use of antimicrobials should help minimizing the risk for selection and spread of resistance determinants in bovine CNS isolates. Continued surveillance of resistance both in mastitis pathogens and commensals is important to ensure the future efficacy of antimicrobials and disinfectants for use in dairy cattle.

7.6.2. Biofilm formation

Nontetheless, the biofilm associated genes icaA and bap were detected more frequently in environmental than in IMI-causing CNS species, contradicting a potential role for biofilm formation in pathogenicity and establishment of persistent IMI (Chapter 6). In accordance with these findings, no correlation has been found in another study between presence of the bap gene, in vitro biofilm formation, and the ability to cause persistent IMI in CNS from bovine mastitis (Simojoki et al., 2010). Possibly, biofilm formation might be more important in environmental CNS species for survival in dry, nutrient-poor, or otherwise challenging environments. In addition, staphylococcal biofilms in dairy farms might play a role in transmissibility when formed on potential fomites, e.g. on milking liners.

The mechanisms of biofilm formation used by CNS other than S. epidermidis have hardly been studied. Furthermore, little information is available on the presence of bap or ica genes in other CNS species, or their homology with previously described sequences. Although the PCR results did not support the notion that biofilm formation is a virulence factor of bovine CNS isolates (Chapter 6), further study of staphylococcal surface components and biofilm formation would surely be useful. This kind of investigation could contribute to a better understanding of host-pathogen interactions, or the development of new approaches for control, as it is relevant in the light of vaccine development and immunization against staphylococcal mastitis. A combination of
phenotypic and genotypic methods is recommended for the detection of biofilm production by CNS.

**7.7. Future perspectives**

The environmental reservoirs, possible sources, transmission routes, antimicrobial resistance and biofilm formation of individual CNS species causing IMI have been dealt with in this dissertation. Nevertheless, there are still many uncertainties concerning the epidemiology, impact, and pathogenesis of CNS species associated with bovine IMI.

First of all, the impact of the different CNS species on udder health needs to be elucidated further. In many studies the number of infected quarters with one particular species (other than *S. chromogenes*) is often too low to make a valid comparison with other mastitis pathogens. Therefore, a meta-analysis of existing literature on well characterized CNS from dairy cattle could be executed. In addition, new studies including more herds could be performed, or efforts could be undertaken to conduct such a study on an (inter)national level. As multiple herd factors may influence the CNS species distribution, a cross-sectional study on various herds using different management styles (*e.g.* different types of housing, bedding materials, teat dip products, prevalence of other mastitis pathogens, *etc.*) could be conducted to identify possible risk factors for IMI caused by individual CNS species. Alternatively, differences in risk factors between udder-adapted and environmental CNS species could be investigated in order to formulate specific control measures. However, analysing quarter milk samples of all cows of multiple dairy farms by conventional methods for the presence of specific CNS species would take a lot of effort and time. As an alternative, fast, reliable, and sensitive methods, such as real-time PCR, could be optimized to identify a number of relevant CNS species. What is more, real-time PCR assays could be improved to use milk as a template, omitting the need for time-consuming culturing and DNA extractions. Real-time PCR protocols making use of probes targeting species-specific polymorphisms in the 16SrRNA (*Edwards et al.,* 2001) or *tuf* (*Martineau et al.,* 2001) genes have been optimized to rapidly identify a number of clinically significant CNS species in humans. Analogously, a real-time PCR assay for the detection of the most relevant CNS species in bovine milk, *e.g.* *S. chromogenes, S. simulans,* and *S. epidermidis,* could be designed using
probes complementary to species-specific sequences of *e.g.* the *tuf* gene, for rapid (quantitative) diagnosis of CNS IMI in multiple milk samples simultaneously. Once the CNS species with a more harmful impact on udder health have been recognized, methods with less technical requirements might be proposed for routine diagnostics. When no phenotypic criteria are available that can unequivocally distinguish them from other CNS species, faster and sensitive PCR-based assays for identification of staphylococcal isolates could be optimized. In addition, PCR-based identification could be combined in a multiplex PCR assay with the detection of resistance genes (*e.g.* mecA, *blaZ*, *msrC*) to perform species identification and resistance profiling simultaneously.

Bedding materials are primary sources of environmental pathogens in between milkings. A direct correlation between bacterial counts in lactating cow bedding and rates of IMI with environmental major pathogens has been demonstrated (Hogan *et al.*, 1989b), and the same might be true for “environmental” CNS. Survival and growth of pathogens in the environment promotes their persistence and spread within herds, and more knowledge on environmental reservoirs of CNS species that are linked with udder health, such as *S. haemolyticus*, *S. xylosus*, and *S. simulans*, is important to assess the infection pressure exerted by these species. In addition, other extramammary sources, such as foodstuffs, drinking water, manure, humans, other animals, bovine skin, hair coat, *etc.*, might also be worth studying as vectors for opportunistic CNS. Sensitive sampling procedures and quantitative isolation methods could be optimized to study the CNS load in these niches. Alternatively, culture-independent metagenomic analysis could be applied to characterize the mixed bacterial populations of CNS species from the environment or other polybacterial reservoirs.

Potential sources for the more udder-adapted CNS species (*e.g.* *S. chromogenes*) include the air in the milking parlour (spread via aerosols), the milking machine, teat orifices, and teat keratin. The teat canal is the barrier between the mammary gland and the environment, and CNS colonizing the keratin might function as a source of infection. On the other hand, CNS in the teat duct could also act competitively against other invading bacteria and thus provide a defence mechanism. In ewes, such a protective effect has been demonstrated for CNS that are present in large numbers within the teat duct (Fragkou *et al.*, 2007). Indications have been found that the isolation of *S. xylosus* in pure culture from bovine milk samples might represent teat canal colonization rather
than IMI (Thorberg et al., 2009). The exact location in the mammary gland and the subsequent impact on udder health of different CNS is subject for further study.

To assess the role of the aforementioned sources and to understand host-pathogen relationships, strain differentiation of the involved CNS is indispensable. For comprehensive epidemiological studies within a limited time frame, PFGE, RAPD, and AFLP are highly suitable methods with good discriminatory power. For more clonal species, such as *S. chromogenes*, resolution can be increased by combining different methods. Alternatively, an MLST scheme could be optimized to study *S. chromogenes* isolates from cattle. MLST has been previously used on bovine *S. aureus* isolates from different origins and regions, which also have a clonal population structure (Smith et al., 2005a; Smith et al., 2005b). As MLST has a typeability and reproducibility near 100% (Smith et al., 2005a), and is truly portable among laboratories, it would be highly suited to improve our understanding of the epidemiological behaviour of *S. chromogenes*. With this library method, both local and global epidemiology of this common subclinical mastitis pathogen could be studied, as well as the evolution of particular *S. chromogenes* lineages.

Strain-differences among CNS could be associated with differences in virulence traits, such as production of toxins, biofilm formation, invasive capacity and intracellular survival. Strains originating from transient and persistent infections, or from quarters with and without clinical signs, could be compared to give an indication if certain CNS factors are important in the establishment of persisting IMI or the development of clinical symptoms. Subsequent study of the genomic content of these IMI-causing CNS should help to identify the epidemiological markers important in virulence or udder-adaptation.

Finally, the interaction of CNS with other mastitis pathogens and their reservoir function for mobile resistance genes are of interest. Ecological information on potential changes in susceptibility of extramammary CNS is virtually non-existent. Using time-to-event analysis of MIC or MBC values (Chapter 6), potential differences or shifts in susceptibility between CNS populations can be detected. The epidemiology of resistance in its turn can be assessed by studying the involved resistance genes.
REFERENCES


CLSI. 2007a. Interpretive criteria for microorganism identification by DNA target sequencing; proposed guideline. CLSI document MM18-P. Clinical and Laboratory Standards Institute, Wayne, PA.


EN 1656. 2000. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary field - Test method and requirements (phase 2/step 1). European Committee for Standardization.


References


Jaglic, Z., E. Michu, M. Holasova, H. Vlkova, V. Babak, M. Kolar, J. Bardon, and J. Schlegelova. 2010. Epidemiology and characterization of *Staphylococcus*


SUMMARY

In many countries, coagulase-negative staphylococci (CNS) are currently the most common cause of intramammary infection (IMI) in lactating cows. Although CNS usually cause subclinical mastitis, the increased prevalence worldwide of CNS IMI in cows of all ages and especially heifers has raised questions about the assumption that CNS are harmless, nonpathogenic commensals, so-called “minor pathogens”. Mastitis researchers initially regarded CNS as one homogeneous group and did not differentiate CNS to the species level. This approach has worked for years, as research was focused primarily on control of major mastitis pathogens. However, the importance of CNS as a group in bovine mastitis is disputed and mastitis researchers are still undecided on the impact of CNS IMI on udder health. Protective effects of preexisting CNS IMI against mastitis with major pathogens have been demonstrated, suggesting that CNS might be beneficial for udder health. On the other hand, it has been suggested that CNS must be regarded as emerging mastitis pathogens that deserve attention on dairy farms aiming for a low BMSCC.

In Chapter 1, the literature on these different aspects of CNS mastitis and general characteristics of CNS is reviewed. However, data on clinical relevance, epidemiology, pathogenesis, or management of CNS mastitis based on accurate species level identification is lacking, and more CNS species-specific research is required to clarify the contradictory findings on CNS regarding their protective or pathogenic behaviour.

The main objectives of this thesis are outlined in Chapter 2. In brief, these were to develop and validate an accurate CNS species identification method (Chapter 3), to determine the species diversity of CNS causing IMI and living freely in the farm environment (Chapter 4), to investigate the epidemiology of CNS species involved in bovine IMI (Chapter 5), and to evaluate the possible role of certain virulence and resistance traits in the ability of CNS to cause IMI (Chapter 6).

Phenotypic identification of CNS species appears to be unreliable due to the variable expression of traits within species, and the large and still increasing number of known CNS species. Therefore, more accurate, reproducible and robust molecular typing methods are preferred for CNS identification. In Chapter 3, the use of amplified
fragment length polymorphism (AFLP) genotyping was validated for the identification of bovine associated CNS species. An initial reference library was generated with AFLP fingerprints of 52 different CNS type and reference strains. Next, 247 bovine CNS field isolates with known species identity were analyzed by AFLP as well. These field isolates had been previously identified by gene sequencing and were randomly divided into two subsets, i.e. a training set and a validation set. The training set was identified against the initial reference library containing only type and reference strains, which resulted in a typeability of 80.5%. Accuracy of the AFLP identifications, being the correspondence with gene sequencing results, was 95.0%. Fingerprints of the training set were then added to the initial library and identification of the validation set was done by means of this extended library. By adding bovine CNS to the library, performance of the AFLP identification method improved considerably. Final typeability and accuracy were 98.4% and 99.2%, respectively. Numerical analysis of AFLP fingerprints proved to be an accurate genotypic method for identification of CNS from bovine origin, and the constructed AFLP library provided a useful identification tool for field studies.

As a group, CNS have long been regarded as skin opportunistic pathogens. However, the cows’ environment is also thought to be a possible source for CNS IMI and this was investigated in a longitudinal field study described in Chapter 4. On 6 well-managed Flemish dairy farms, the distribution and the main reservoirs of various CNS species isolated from milk, causing IMI, and living freely in the cows’ environment were determined. On each herd, quarter milk samples from a cohort of 10 lactating cows and environmental samples from stall air, slatted floor, sawdust from cubicles, and sawdust stock were collected monthly during 13 months. CNS isolates from quarter milk samples (n = 134) and the environment (n = 637) were identified to species level using the validated AFLP library (Chapter 3). *Staphylococcus chromogenes*, *S. haemolyticus*, *S. epidermidis*, and *S. simulans* accounted for 81.3% of all CNS milk isolates. Quarters were considered infected with CNS (positive IMI status) only when 2 out of 3 consecutive milk samples yielded the same CNS AFLP type. The species causing IMI were *S. chromogenes* (n = 35 samples with positive IMI status), *S. haemolyticus* (n = 29), *S. simulans* (n = 14), and *S. epidermidis* (n = 6). The observed persistent IMI cases (n = 17) had a mean duration of 150 days. The CNS species predominating in the environment were *S. equorum*, *S. sciuri*, *S. haemolyticus*, and *S. fleurettii*. Herd-to-herd differences in distribution of CNS species were observed both in milk and the environment, suggesting
herd-level factors are involved in the establishment of particular species in a dairy farm. The results of this study indicated that primary reservoirs of the species causing IMI varied. *S. chromogenes* and *S. epidermidis* were rarely found in the environment, indicating that other reservoirs were more important in their epidemiology. For *S. haemolyticus* and *S. simulans* the environment was found as a reservoir, suggesting IMI with these species were possibly environmental in origin.

To determine whether CNS species act as environmental opportunists or as udder-adapted, possibly contagious mastitis pathogens, a better understanding of the epidemiology of individual CNS species and genotypes in dairy farms is needed. However, the genetic diversity within CNS species associated with bovine IMI is poorly documented, and CNS occurring in the environment have never been genotypically compared with those of IMI-causing CNS. In Chapter 5, the genetic diversity within four CNS species found associated with bovine IMI, *S. haemolyticus, S. simulans, S. chromogenes,* and *S. epidermidis* (Chapter 4), was determined. For epidemiological purposes, CNS genotypes from milk and the environment were compared, and their distribution within the farms was investigated. Genetic diversity was assessed by two molecular typing techniques, AFLP and random amplification of polymorphic DNA (RAPD) analysis. AFLP- and RAPD- typing revealed the highest genotypic diversity among *S. haemolyticus* isolates. A large variety of genotypes was found among environmental isolates, of which several could be linked with IMI, confirming that the environment could act as a potential source for infection. For *S. simulans*, various genotypes were found in the environment, but a link with IMI was less obvious. For *S. epidermidis* and *S. chromogenes,* genotypic heterogeneity was limited and the sporadic isolates from environment displayed largely the same genotypes as those from milk. The higher clonality of the *S. epidermidis* and *S. chromogenes* isolates from milk suggests that specific genotypes probably disseminate within herds and are more udder-adapted. The findings of this epidemiological study indicated that environmental sources and cow-to-cow transmission both seem to be involved in the epidemiology of CNS, although their relative importance might substantially vary between species.

CNS are significantly less pathogenic than the coagulase-positive species *S. aureus.* Nonetheless, CNS are more often resistant to various antimicrobials and biocides due to carriage of mobile resistance genes. In addition, a number of CNS species have been shown to dispose of virulence factors, such as biofilm formation, which might promote
colonization of the mammary tissue and persistence in the udder. The aim of the study described in Chapter 6 was to assess the potential role of biofilm formation and antimicrobial and biocide resistance in the establishment of bovine CNS IMI, by comparing these characteristics among CNS originating from different niches in the dairy farm. Isolates from bovine milk, teat apices and dairy farm environment (n = 366) belonging to 22 different CNS species were analysed by PCR for the presence of the biofilm associated genes *bap* and *icaA*, and the methicillin resistance gene *meca*. In addition, phenotypic susceptibility to five commonly used antibiotics and two teat dip formulations was tested for a selection of 82 CNS isolates belonging to the most common CNS species causing IMI (*S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, and *S. simulans*) or living freely in the environment (*S. sciuri* and *S. equorum*). Antimicrobial minimal inhibitory concentrations (MIC) were determined by commercial Etest for enrofloxacin, erythromycin, gentamicin, cephalothin, and oxacillin, and a microdilution method was optimized to determine minimal biocidal concentrations (MBC) for two teat dip products (0.15% iodine and 0.42% chlorhexidine). Carriage of *bap*, *icaA* and *meca* genes was detected significantly more often in CNS originating from cows’ environment than in the IMI-causing CNS species. Among the environmental CNS species, *bap* was detected commonly in *S. equorum* (97%) and *S. xylosus* (89%), *icaA* was found primarily in *S. capitis* (100%), *S. xylosus* (33%), and *S. sciuri* (29%), and *meca* was found in *S. fleurettii* (100%) and *S. sciuri* (94%). Among the IMI-causing species, only in *S. epidermidis* *icaA* (4%) or *meca* (33%) were detected. Phenotypic resistance was found mainly to oxacillin (16%) and erythromycin (23%), and was also detected more often in environmental than in IMI-causing CNS species. The CNS isolates least susceptible to the iodine and chlorhexidine teat dips belonged to the IMI-causing species *S. chromogenes* or *S. simulans*. The results of this study indicated that carriage of biofilm associated genes and antimicrobial resistance, including *meca* carriage, were more common in environmental than in IMI-causing CNS species, and that reduced susceptibility to biocides might play a role in the ability of CNS species to establish IMI.

In Chapter 7, the overall results, some new insights on the epidemiology of CNS, and future perspectives of CNS mastitis research are discussed.
SAMENVATTING

De coagulase-negative staphylococci (CNS) zijn tegenwoordig wereldwijd de meest voorkomende oorzaak van intramammaire infecties (IMI) bij melkvee. De laatste jaren is de prevalentie en incidentie van CNS IMI duidelijk gestegen bij koeien van alle leeftijden en in het bijzonder bij vaarzen. Hoewel de symptomen van CNS mastitis meestal subklinisch zijn, worden er tegenwoordig vragen gesteld bij de algemene veronderstelling dat CNS niet-pathogene, zogenaamd “minor” pathogenen zouden zijn. Mastitis onderzoekers beschouwden CNS doorgaans als een homogene groep, en maakten geen onderscheid tussen de verschillende species. Deze benadering was lange tijd geschikt, aangezien mastitis onderzoek voornamelijk was toegespitst op de controle van “major” mastitis pathogenen. Het belang van CNS als groep wordt momenteel echter in vraag gesteld en onderzoekers zijn het niet eens over het feit of CNS nu een belangrijke impact hebben op de uiergezondheid of niet. Er is namelijk aangetoond dat door CNS veroorzaakte IMI bescherming kunnen bieden tegen mastitis veroorzaakt door major pathogenen, wat suggereert dat CNS eerder een voordeel dan een nadeel zouden zijn voor de algehele uiergezondheid. Anderzijds worden CNS ook omschreven als opkomende mastitis pathogenen waar aandacht aan besteed moet worden op bedrijven die een laag celgetal beogen.

In Hoofdstuk 1 wordt een samenvatting gegeven van de beschikbare literatuur over de verschillende aspecten van CNS mastitis en algemene eigenschappen van CNS. De informatie over klinische relevantie, epidemiologie, pathogenese en preventie van CNS mastitis gebaseerd op accurate species identificatie is echter beperkt. Om de tegenstrijdigheden betreffende de beschermende en pathogene eigenschappen van CNS te verklaren, is meer onderzoek nodig op CNS species niveau.

De voornaamste doelstellingen van deze thesis worden kort omschreven in Hoofdstuk 2. Deze omvatten, in het kort, het ontwikkelen en valideren van een nauwkeurige identificatiemethode voor CNS species (hoofdstuk 3), het bepalen van de diversiteit aan CNS species die IMI veroorzaken en die in de stalomgeving voorkomen (hoofdstuk 4), het onderzoeken van de epidemiologie van CNS die IMI veroorzaken (hoofdstuk 5), en het evalueren van de rol van bepaalde virulentie- en resistentiekenmerken in het vermogen van CNS om IMI te veroorzaken (hoofdstuk 6).
Identificatie van CNS species op basis van fenotype is onbetrouwbaar, gezien de variabele expressie van eigenschappen binnen species en het grote, nog steeds toenemende aantal bekende CNS species. Daarom moeten bij voorkeur meer betrouwbare, reproduceerbare en robuuste moleculaire typeringsmethoden aangewend worden voor de identificatie van CNS species. In Hoofdstuk 3 werd “amplified fragment length polymorphism” (AFLP) genotypering gevalideerd als methode voor de identificatie van CNS species geassocieerd met bovienne mastitis. Aanvankelijk werd een referentiebibliotheek samengesteld met de AFLP patronen van 52 verschillende CNS type- en referentiestammen. Vervolgens werden ook 247 CNS veldisolaten afkomstig van speentoppen en melk geanalyseerd met AFLP. Deze veldisolaten waren voordien door middel van gensequenering tot op CNS species niveau geïdentificeerd en werden willekeurig in twee groepen verdeeld: een trainingset en een validatieset. De trainingset werd geïdentificeerd door middel van de bibliotheek met enkel type- en referentiestammen wat resulteerde in een typeerbaarheid van 80,5%. De nauwkeurigheid van de AFLP identificatie, zijnde de overeenkomst met de identificatie op basis van gensequenering, was 95,0%. De AFLP patronen van de trainingset werden toegevoegd aan de referentiebibliotheek en vervolgens werd de validatieset geïdentificeerd op basis van deze uitgebreide referentiebibliotheek. Het toevoegen van de bovienne veldisolaten aan de referentiebibliotheek verbeterde de performantie van de AFLP methode aanzienlijk. De uiteindelijke typeerbaarheid en nauwkeurigheid van de methode waren respectievelijk 98,4% en 99,2%. De resultaten van deze validatie toonden aan dat het numeriek vergelijken van AFLP patronen een nauwkeurige genotypische methode is voor de identificatie van CNS species van bovienne oorsprong. De uitgebreide AFLP referentiebibliotheek biedt een uiterst geschikt identificatiemiddel voor veldstudies.

Als groep werden CNS lange tijd beschouwd als opportunistische pathogenen afkomstig van de huid. Nochtans zou de omgeving ook een mogelijke bron van CNS infecties kunnen zijn en dit werd onderzocht in een longitudinale studie beschreven in Hoofdstuk 4. In deze veldstudie werd op 6 goed gerunde Vlaamse mekveebedrijven de distributie bepaald van CNS species in melk, IMI en de stalomgeving. Op elk bedrijf werden maandelijks kwartiermelksstal genomen van een cohort van 10 lacterende koeien per bedrijf gedurende 13 maanden. Daarnaast werden ook maandelijks omgevingsstalen genomen van de lucht en de roostervloeren in de loopstal, het strooisel
in de ligbedden en het strooisel van de voorraad. CNS isolaten uit de melk (n = 134) en de omgeving (n = 637) werden tot op species niveau geïdentificeerd door middel van de gevalideerde AFLP bibliotheek (hoofdstuk 3). *Staphylococcus chromogenes*, *S. haemolyticus*, *S. epidermidis* en *S. simulans* waren samen goed voor 81.3% van het totaal aantal CNS melkisolaten. Kwartieren werden beschouwd als CNS geïnfecteerd (positieve IMI status) wanneer uit twee van de drie opeenvolgende kwartiermelkstalen hetzelfde CNS AFLP type werd geïsoleerd. De species die IMI veroorzaakten waren *S. chromogenes* (n = 35 melkstalen met positieve IMI status), *S. haemolyticus* (n = 29), *S. simulans* (n = 14) en *S. epidermidis* (n = 6). De persisterende CNS IMI (n = 17) hadden een gemiddelde duur van 150 dagen. De meest voorkomende CNS species in de omgeving waren *S. equorum*, *S. sciuri*, *S. haemolyticus* en *S. fleurettii*. Zowel in de melk als in de omgeving werden in de CNS species distributie verschillen waargenomen tussen bedrijven, wat er sterk op wijst dat bedrijfsafhankelijke factoren invloed hebben op het handhaven van bepaalde CNS species op een bedrijf. De resultaten van deze veldstudie tonen aan dat de primaire reservoirs van CNS species die IMI veroorzaken kunnen variëren. Zo werden *S. chromogenes* en *S. epidermidis* bijvoorbeeld zelden gevonden in de omgeving, wat aangeeft dat andere reservoirs wellicht belangrijker zijn in de epidemiologie van deze species. Daarentegen werd voor *S. haemolyticus* en *S. simulans* een reservoir aangetoond in de stalomgeving, wat aantoont dat IMI met deze species mogelijk hun oorsprong hebben vanuit de omgeving.

Om te kunnen besluiten of CNS species zich gedragen als omgevings- of als uiergeadapteerde (besmettelijke) pathogenen is een beter inzicht nodig in de epidemiologie van individuele species en genotypes die voorkomen op melkveebedrijven. Momenteel is de kennis omtrent genetische diversiteit binnen CNS species die IMI veroorzaken beperkt. Bovendien zijn CNS die aanwezig zijn in de omgeving nog nooit genotypisch vergeleken met deze die IMI veroorzaken. In *Hoofdstuk 5* werd bij vier CNS species die werden geassocieerd met bovienne IMI, zijnde *S. haemolyticus*, *S. simulans*, *S. chromogenes*, en *S. epidermidis* (hoofdstuk 4) de genetische diversiteit bepaald. Voor epidemiologische doeleinden werden CNS genotypes afkomstig van melk vergeleken met die uit de omgeving en werd hun verspreiding binnen de bedrijven nagegaan. De genetische diversiteit werd bepaald door middel van twee moleculaire typeringmethoden, AFLP en random amplification of polymorphic DNA (RAPD) analyse. Zowel AFLP- als RAPD-typering toonden de grootste genetische diversiteit aan
onder de *S. haemolyticus* isolaten. Een grote variëteit aan genotypes werd gevonden onder de omgevingsisolaten, waarvan er verschillende konden gelinkt worden met IMI. Deze bevindingen bevestigen dat de omgeving kan functioneren als een mogelijke bron van *S. haemolyticus* IMI. Voor *S. simulans* werden eveneens in de omgeving verschillende genotypes gevonden, maar een link met IMI was minder duidelijk. Voor zowel *S. epidermidis* als *S. chromogenes* was de genotypische heterogeniteit beperkt en de sporadische genotypes uit de omgeving waren grotendeels dezelfde als die in de melk. De grotere clonaliteit binnen de *S. epidermidis* en *S. chromogenes* melkisolaten wijst erop dat specifieke genotypes zich waarschijnlijk verspreiden binnen bedrijven en geadapteerd zijn aan de uier. De resultaten van deze epidemiologische studie duiden erop dat zowel omgevingsbronnen als besmettelijke transmissie een rol kunnen spelen in de epidemiologie van CNS, maar dat hun relatief belang sterk kan verschillen al naargelang het species.

CNS zijn beduidend minder pathogeen dan het coagulase-positieve species *S. aureus*. Desalniettemin zijn CNS vaker resistent voor verschillende antimicrobiële middelen en biociden doordat ze mobiele resistentiegenen dragen. Daarnaast is ook aangetoond dat een aantal CNS species beschikken over virulentiefactoren zoals biofilmvorming, wat het koloniseren van en persisteren in de uier kan bevorderen. De opzet van de studie beschreven in **Hoofdstuk 6** was om de mogelijke rol van biofilmvorming, antibiotica- en biocideresistentie in het vermogen om IMI te veroorzaken te evalueren, door deze eigenschappen te vergelijken bij isolaten van verschillende oorsprong. Isolaten afkomstig van melk, speentoppen en de stalomgeving (n = 366), behorend tot 22 verschillende CNS species, werden geanalyseerd met PCR voor de detectie van de biofilmgeassocieerde genen *bap* en *icaA* en het methicilline-resistantiegen *mecA*. Daarnaast werd ook de fenotypische gevoeligheid voor vijf courant gebruikte antibiotica en twee speendipmiddelen bepaald van een selectie van 82 CNS isolaten behorend tot de species die algemeen IMI veroorzaakten (*S. chromogenes, S. epidermidis, S. haemolyticus, and S. simulans*) of die vaak vrijlevend voorkwamen in de omgeving (*S. equorum* en *S. sciuri*). De antimicrobiële minimale inhibitorische concentraties (MIC) voor enrofloxacin, erythromycin, gentamicin, cephalothin en oxacillin werden bepaald door middel van commerciële Etesten. Voor het bepalen van de minimale biocidale concentraties (MBC) van de twee speendipmiddelen (0,15% jood en 0,42% chlorhexidine) werd een nieuwe microdiluïtemethode geoptimaliseerd. De
bap, icaA en mecA genen werden significant vaker aangetroffen bij de CNS species afkomstig uit de omgeving dan in de IMI-veroorzakende species. Binnen de omgevingsgerelateerde species werd bap vaak gevonden bij S. equorum (97%) en S. xylosus (89%), terwijl icaA voornamelijk werd gevonden bij S. capitis (100%), S. xylosus (33%), en S. sciuri (29%). MecA werd gedetecteerd bij S. fleurettii (100%) en S. sciuri (94%). Binnen de IMI-gerelateerde species werd enkel in S. epidermidis icaA (4%) of mecA (33%) aangetroffen. Fenotypische resistentie werd voornamelijk gevonden voor oxacillin (16%) en erythromycin (23%), en resistentie werd ook meer gevonden in omgevings- dan in IMI-gerelateerde species. De isolaten die het minst gevoelig waren voor de jood- en chlorhexidinedip behoorden tot de IMI-gerelateerde species S. chromogenes en S. simulans. De resultaten van deze studie tonen aan dat biofilmgeassocieerde genen en antimicrobiële resistentie, inclusief het bezit van het mecA gen, meer voorkwamen bij omgevings- dan bij IMI-gerelateerde CNS species. Een verminderde gevoeligheid voor speenmiddelen zou mogelijk een rol kunnen spelen bij het vermogen van bepaalde CNS species om IMI te veroorzaken.

In Hoofdstuk 7 worden de algemene resultaten en enkele nieuwe inzichten in de epidemiologie van CNS besproken. Tenslotte worden ook de toekomstperspectieven van het onderzoek op CNS mastitis aan de orde gesteld.
CURRICULUM VITAE

Veerle Piessens was born in Kortrijk on May the 23rd in 1984. After obtaining a degree in Latin and Mathematics at Onze-Lieve-Vrouw van Vlaanderen Institute in 2002 in Kortrijk, she started her studies bachelor in Biology at the University of Ghent. She graduated with great honours as master in Biotechnology in 2006. Her master thesis was the result of her internship at the Center for Medical Genetics at the University Hospital of Ghent, where she worked on the analysis of genes involved in skeletal disorders.

Since April 2007 she was employed as a research associate at the Institute for Agricultural and Fisheries research (ILVO) at the Food Science and Technology Unit, working on a project on the control of the contamination level of broiler carcasses with Campylobacter sp. In November 2007, she started her PhD study on the epidemiology of CNS in milk and the farm environment, within the scope of the agricultural project “Improvement of the udder health and milk quality in Flanders: Research on relevant microbiota and associated factors”, funded by the agency for Innovation by Science and Technology (IWT-Vlaanderen, grant no. 60714).

During the four years of her PhD study, Veerle Piessens was author and co-author of several scientific publications in international journals, and attended national and international meetings, symposia, and congresses on udder health. She also guided a graduate and a university student in achieving their thesis.
BIBLIOGRAPHY

Publications in international journals


Abstracts, proceedings and oral presentations on international and national meetings

2008


2009


2010


Students


Katrien Verheyen. Studie van virulentie-eigenschappen van coagulase-negatieve staphylococcen (CNS) geassocieerd met bovine mastitis. 2009-2010. Faculty of Pharmaceutical Sciences, Ghent University, Belgium.
DANKWOORD

Na vier jaar een doctoraat met succes afronden, dat lukt geen mens zonder inspiratie of hulp van anderen. Ik wil dan ook iedereen die op enige wijze heeft bijgedragen tot het slagen ervan hartelijk bedanken en vereeuwigen met een vermelding in mijn boekje 😊.

Prof. De Vliegher, beste Sarne, uiteraard was jij de grote drijfveer achter het uitpluizen van het hele CNS verhaal. Al snapte ik aanvankelijk “the fuzz” rond CNS niet helemaal, gaandeweg ging ik wel inzien wat een fascinerende groep dit was. Sinds jouw eigen doctoraat zijn er heel wat puzzelstukjes bijgekomen, maar ook veel nieuwe interessante onderzoeksvragen gerezen: het was een plezier om hieraan bij te dragen! Je enthousiasme bij elke nieuwe vondst of publicatie was altijd een opsteker en werkte voor mij heel motiverend. Je kon perfect alles in perspectief plaatsen en me weer op weg zetten als ik vast zat, en dat is wat een goede promotor doet, waarvoor dank! Ook chapeau hoe je alles in goede banen leidt: het M-team lijkt wel elk jaar te verdubbelen en levert schitterend werk 😊.

Beste Els, als co-promotor op het ILVO heb je mij uiteraard het dichtst gevolgd. Ik vond het super hoe stipt je alles opvolgde, zodat ik nooit voor onaangename verrassingen kwam te staan en mijn deadlines haalde. Talrijke abstracts, publicaties, en verslagen heb je met veel geduld gelezen, en ik kon altijd bij je terecht met vragen en kleine problemen. Heel erg bedankt om zo betrokken te zijn!

Prof. Marc Heyndrickx en Prof. Luc De Vuyst, ook voor jullie een woordje van dank voor jullie bijdragen aan de publicaties. Jullie hebben het verloop van mijn onderzoek mee gestuurd en lieten vaak een ander licht schijnen op de resultaten. Daar heb ik zeker en vast veel van geleerd, bedankt!

Professor Zadoks, beste Ruth, uw talrijke publicaties rond de epidemiologie van mastitis pathogenen hebben me veel bijgeleerd en inspiratie bezorgd voor de interpretatie van mijn eigen resultaten. Ik was dan ook heel blij dat u de tijd heeft genomen om mijn werk zo grondig na te lezen. Ik weet zeker dat het eindresultaat er beter door geworden is.

Dear Suvi Taponen, as you were a “pioneer” in the epidemiological study of CNS, I learned a lot from your previous work. It was an interesting collaboration on the new
Dankwoord

species *Staphylococcus agnetis*, which - I think - is a really nice name and tribute. Thank you for revising my dissertation. Ook de overige leden van de examenjury, Prof. Patrick Butaye, Prof. Katleen Hermans en Prof. Ynte Hein Schukken, wil ik graag bedanken voor hun opmerkingen op het finale werk.

Mijn collega’s Bert en Gorik wil ik graag bedanken voor de vier jaar geslaagde samenwerking. Ik vond het altijt leuk om als groep ons werk te kunnen voorstellen, en zeker Atlanta was een fijne ervaring. Bert, je geduld en inzet tijdens het fameuze jaar vollenbak-stalen-nemen-en-ondertussen-ook-eerste-keer-vader-worden, respect! Gorik, onze vele gesprekken over “ons CNS-kes” waren altijd inspirerend en als lopende CNS-encyclopedie kwam je literatuurkennis vaak handig van pas 😊. Veel succes met het afronden van jullie doctoraten en veel geluk gewenst met al jullie toekomstige projecten!

Beste Karlien, bedankt om me zo goed te begeleiden bij de aanvang van het project. Fantastisch hoe je altijt nog tijd wist te maken naast je onderzoek en de buitenpraktijk om te helpen. Naar het einde toe heb ik ook erg veel aan je gehad, bedankt! Blij dat we het samen tot een goed einde hebben kunnen brengen! Ook de rest van het M-team: Sofie, Lars, Joren, Anneleen, en Kathelijne, bedankt om me altijt hartelijk te verwelkomen op den bureau, en voor jullie hulp op studiedagen en onze verdediging.

Nathan & Katrien, bedankt voor al jullie werk tijdens jullie stages. Het was een zeer leuke ervaring om jullie te mogen begeleiden in het labo en met het schrijven van jullie thesis.

Over naar mijn dierbare ILVO-collega’s uit de kelder waarmee ik dagelijks lief en leed (en vooral veel koekskes, taart en cake) mee heb gedeeld: Beste Ann en Jessy, duizend maal dank voor jullie vele goede raad en tips, EN het zo kundig runnen van de labo’s. Daardoor konden wij steeds werken in propere labo’s met aangevulde voorraden, toestellen die werken, plaats in de frigo’s en diepvriezen, enz. En al kunnen we zagen over de verplichte kuis, zo’n labo is gewoon pure luxe! Ook de rest van de kelder wil ik graag bedanken voor de leuke werksfeer, de behoorlijk hilarische middag- en koffiepauzes, de vele (al dan niet werk-gerelateerde 😊) tips en discussies: Valerie DJ, Els, Hadewig, Katrien, Joris, Pieter, Geertrui, Marijke, Isabelle, Céline, Séverine en Dorine. Saskia, Valerie en Karen, een speciale dankjewel voor de aangename tijd in ons “landschapsbureel” en het supporteren, ik supporter nu voor jullie! Ambroos, zonder jou was ik nooit bij het ILVO terecht gekomen. Van lab-partners aan de unief tot ILVO-collega’s, merci dat je er altijt voor me was. Veel geluk met jullie kersverse gezinnetje,
en hopelijk kunnen we nog vaak bijpraten over die goeien tijd 😊. Ook Leneke, merci voor je steun en je vriendschap, op het werk en daarnaast.

Hannetje, een welgemeende dankjewel voor je vriendschap al zo lang, je luisterend oor, de leuke reizen, het carpoolen, het gezellige naar-de-colruyt-gaan en koken als we samenwoonden in de Leeuwstraat, en al wat ik niet kan opsommen. Je bent een grote steun geweest als het moeilijk was, en een supervriendin op andere momenten.

Ook Jessika, bedankt voor de leuke uitstapjes om op tijd en stond eens de gedachten te verzetten, dat deed deugd. Ik wens je het allerbeste met jouw doctoraat en ik kom af he, als je daarna in Montpellier of op een anders Zuiders bestemming terecht komt 😊.

Lieven, je bent de voorbije drie jaar mijn beste vriend, mijn steun, mijn thuis, mijn plezier, mijn troost geweest, dankjewel om zo’n fantastische vriend te zijn! Je geduld en relativeringsvermogen kwamen goed van pas om mij op stress-momenten te kalmeren, en als dat niet volstond, dan zorgde je wel voor afleiding of comfort food op tafel 😊.

Mama en Frans, bedankt voor jullie aanmoedigingen. Papa, de wetenschappelijke nieuwsgierigheid heb ik van geen vreemde me dunkt 😊. Dankjewel om me te steunen tijdens mijn studies in Gent en erna! Marc en Anny, jullie ook bedankt voor de lieve gebaren en het aanleveren van vitamientjes uit jullie groententuin om goed te kunnen werken 😊.

Iedereen van harte bedankt om me te steunen en/of inspireren, ’t was een fijne leerrijke rit!

Veerle

Oktober 2011