ADVANCEMENTS IN THE UNDERSTANDING OF STAPHYLOCOCCAL
MASTITIS THROUGH THE USE OF MOLECULAR TOOLS

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MASTITIS THROUGH THE USE OF MOLECULAR TOOLS

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DEDICATION

I dedicate this to my husband, Eric Adkins, and my mother, Denice Condon. I am forever grateful for their eternal love and support.

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ABSTRACT

The objectives of this work were: 1) to complete a whole genome sequence of a *Staphylococcus chromogenes* isolate that is known to persist in the mammary gland of a cow, 2) to define a genetic target that could easily and reliably be used to differentiate *Staphylococcus agnetis* and *Staphylococcus hyicus* and to use this new assay along with pulsed-field gel electrophoresis (PFGE) strain typing to characterize a collection of non-*Staphylococcus aureus* coagulase positive staphylococci, 3) to determine if the recently described *S. aureus* Genotype B was present among previously characterized isolates from cases of bovine intramammary infection in the USA and to compare PFGE to the combination of ribosomal spacer PCR (RS-PCR) and virulence gene identification for strain typing of *S. aureus*, 4) to further the understanding of the association between body site colonization and intramammary infection before and after calving in heifers using molecular methods of speciation and strain typing, and 5) to describe the prevalence and distribution of *Staphylococcus* species on the teat and inguinal skin of dairy heifers throughout the heifer lifecycle using molecular speciation methods.

A draft genome sequence of *S. chromogenes* MU 970, a strain that was isolated from the right rear quarter of a Holstein cow for 16 consecutive months, was completed. Whole genome sequences for *S. chromogenes*, *S. hyicus*, and *S. agnetis* were then used to construct a multiplex PCR used to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*. The multiplex PCR was then used to characterize a collection of isolates and found that *S. agnetis* was much more prevalent than *S. hyicus*. Furthermore, *S. agnetis* can cause persistent infections and can potentially be contagious. Next, RS-PCR plus toxin gene

identification was explored as a possible strain typing method, however it was found to be less discriminatory than PFGE. Hence, PFGE was used as the strain typing method for the remainder of the dissertation research. The final two studies were conducted to characterize body site colonization with staphylococcal species and to explore the relationship of prepartum body site colonization to intramammary infections, both pre- and post-calving. MALDI-TOF was explored as a speciation method in these studies and was found to be accurate, rapid, and inexpensive. Overall, *S. chromogenes* was the most common species identified in prepartum mammary secretions and post-calving milk samples. Through PFGE strain typing, it was determined that many different strains of each species can be found on one heifer. Finally, it was determined that colonization of the inguinal region and teat skin was associated with age for *S. agnetis*, *S. chromogenes*, *Staphylococcus devriesei*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus lentus*, *Staphylococcus sciuri*, *Staphylococcus vitulinus*, and *Staphylococcus xylosus*. Overall, these studies demonstrate that the staphylococcal species and subspecies associated with IMI are extremely abundant and diverse on dairy farms.

CHAPTER ONE

INTRODUCTION

This dissertation is written in the alternative format. It consists of this introduction, a review of the literature, five manuscripts, and a conclusion. The five manuscripts are written in scientific publication format. Of the five manuscripts, two are published, one is under review, and two are formatted to be submitted to the Journal of Dairy Science. The statistical analysis conducted in Chapter 6 and Chapter 7 were done by a coinvestigator, Simon Dufour, at the University of Montreal.

Mastitis, an inflammation of the mammary gland typically caused by bacterial intramammary infection (IMI), is one of the most common and costly diseases of dairy cattle worldwide (Halasa et al., 2007). Many recent epidemiologic investigations have identified coagulase negative staphylococci (CNS) species as the predominant mastitis pathogens and the leading cause of subclinical mastitis on well-managed dairy farms (Pitkala et al., 2004, Tenhagen et al., 2006, Piepers et al., 2007, Schukken et al., 2009,). Furthermore, CNS are the most likely cause of IMI in heifers both pre and post-partum (Fox et al., 1995, Fox, 2009). Identification of CNS at the species-level is important due to differences in prevalence as well as epidemiologic and pathogenic differences between the various CNS species associated with mastitis (Vanderhaeghen et al., 2014a, b). Few studies have been conducted to determine the relationship between body site colonization

and IMI at the strain level (Thorberg et al., 2006, Taponen et al., 2008, Leroy et al., 2015). Furthermore, several knowledge gaps have been identified related to molecular speciation and subspecies of staphylococcal species. Therefore, the objectives of this work were: 1) to complete a whole genome sequence of a *Staphylococcus chromogenes* isolate that is known to persist in the mammary gland of a cow, 2) to define a genetic target that could easily and reliably be used to differentiate *Staphylococcus agnetis* and *Staphylococcus hyicus* and to use this new assay along with pulsed-field gel electrophoresis (PFGE) strain typing to characterize a collection of non-*Staphylococcus aureus* coagulase-positive staphylococci, 3) to determine if the recently described *S. aureus* Genotype B was present among previously characterized isolates from cases of bovine intramammary infection in the USA and to compare PFGE to the combination of ribosomal spacer PCR (RS-PCR) and virulence gene identification for strain typing of *S. aureus*, 4) to further the understanding of the association between body site colonization and intramammary infection before and after calving in heifers using molecular methods of speciation and strain typing, and 5) to describe the prevalence and distribution of *Staphylococcus* species on the teat and inguinal skin of dairy heifers throughout the heifer lifecycle using molecular speciation methods.

Chapter two is a review of the mastitis literature related to the identification and characterization of staphylococcal species, the epidemiology of CNS mastitis, and heifer mastitis. Several recent studies have demonstrated the importance of characterizing staphylococci at the species level using molecular tools. Also, some work has been done

to show subspecies variability. Very few studies have applied these tools to characterize non-*aureus* staphylococci related to heifer mastitis.

The first study (Chapter 3) established a draft genome sequence of *S. chromogenes* MU 970, a strain that was isolated from the right rear quarter of a Holstein cow for 16 consecutive months, between July 2005 and October 2006, with an associated geometric mean somatic cell count of 365,000 cells/ml. Overall, one plasmid was identified within the genome, which was found to contain an apparent transposable element similar to a *S. aureus* B-lactamase encoding transposon. A multidrug resistant efflux pump, an ORF with 41% identity to a predicted coagulase gene, and two biofilm associated protein genes were also identified. The genome information was deposited at GenBank, accession number JMJF01000001. Future research using this genome as a baseline may help further the understanding of within species variabilities.

The research in chapter four established a multiplex PCR that allows an inexpensive and efficient way of differentiating common species of CPS isolated from milk. The multiplex PCR was constructed through the use of whole genome sequences completed for *S. chromogenes*, *S. hyicus*, and *S. agnetis*, which were done by this MU research group. In this study, *S. agnetis* was found to be much more prevalent than *S. hyicus*, as only one *S. hyicus* isolate was identified. This study demonstrates that some strains of *S. agnetis* can cause persistent infections and can potentially have contagious behavior. The

described multiplex PCR can be used in the future to identify *S. agnetis* and *S. hyicus* on dairy farms.

The study covered in chapter five was conducted to determine if an inexpensive and fast stain typing method could potentially be applied to staphylococcal species associated with bovine intramammary infections. Recent studies from Switzerland used ribosomal spacer (RS)-PCR to characterize *Staphylococcus aureus* isolated from bovine milk samples and found various strain types, classified as genotypes, of *S. aureus* possessing different virulence factors. Several genotypes were identified, with Genotype B (GTB) and Genotype C (GTC) being identified commonly. Genotype B isolates were found to be highly contagious and were also considered to have increased pathogenicity. In order to determine if this method was appropriate for the characterization of other staphylococcal species, this study was conducted to first determine if *S. aureus* GTB was present among previously characterized isolates from cases of bovine IMI in the USA and to compare PFGE to this described method. Overall, based on RS-PCR banding patterns, isolates were characterized into five different genotypes, however none of the isolates were characterized as GTB. All identified genotypes were found to have consistent toxin profiles. The discriminatory power of PFGE was 1.0 and for RS-PCR it was 0.46. These data suggest that PFGE is more discriminatory than RS-PCR for strain-typing *S. aureus* isolates of bovine origin. Although PFGE is more time consuming and expensive than PCR-based techniques, a higher discriminatory power is important to help determine the contagiousness of a strain and to define the epidemiology of *S. aureus*, and other staphylococcal species, within a given herd.

The research in chapter six furthered the understanding of non-aureus *Staphylococcus* (NAS) intramammary infections in heifers in the periparturient period and the relationship of prepartum NAS intramammary infection, post-calving intramammary infections, and body site colonization through molecular speciation and PFGE strain typing. This study demonstrated the complexity of understanding the molecular epidemiology of the relevant NAS species, as many different strains of each species can be found on one heifer. One important finding in this study was the impact a prepartum IMI can have on post-calving IMI status, and in the case of *S. chromogenes*, on somatic cell score (SCS) status. Therefore, if interventions or prevention strategies are pursued in the future, targeting the prepartum period is likely required.

The research in chapter seven was conducted to further the understanding of body site colonization in heifers throughout their lifecycle. The research in chapter six determined that teat and inguinal colonization were the most important with regards to relationship to IMI. Therefore, the focus of this study was to describe the prevalence and distribution of *Staphylococcus* species on the teat and inguinal skin of dairy heifers. Colonization of the inguinal region and teat skin was associated with age for *S. agnetis*, *S. chromogenes*, *Staphylococcus devriesei*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus lentus*, *Staphylococcus sciuri*, *Staphylococcus vitulinus*, and *Staphylococcus xylosus*. The probability of *S. chromogenes* and *S. agnetis* colonization on the teat and inguinal region increased with age while the probability of *S. devriesei*

and *S. haemolyticus* decreased with age. This study provides further insight into the ecology of staphylococcal species on the dairy farm.

The appendix contains detailed materials and methods for sample collection and staphylococci isolation. It also contains details pertaining to the molecular methods used throughout these studies.

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CHAPTER TWO

LITERATURE REVIEW

1.1 Introduction

Mastitis, an inflammation of the mammary gland typically caused by bacterial intramammary infection (IMI), is one of the most common and costly diseases of dairy cattle worldwide (Halasa et al., 2007). The economic impact of mastitis is due to losses related to treatment, impaired production, premature culling, and changes in milk quality that affect finished product yield. Mastitis therapy accounts for the largest proportion of antimicrobial drugs used in dairy production, with 68-80% of use being related to udder health (Pol and Ruegg, 2007, Kuipers et al., 2016).

In general, the disease appears in two forms, either clinical or subclinical mastitis.

Clinical mastitis is characterized by visible changes in the milk, mammary gland, and/or the cow due to generalized illness, and can have a long lasting negative effect on milk production. Subclinical mastitis is characterized by a lack of visible changes to the milk, but an increase in somatic cell count (SCC) and suboptimal milk production. Production losses due to subclinical mastitis have a log-linear relationship between somatic cell count and test day milk yield (Bartlett et al., 1990). However, more recent work has shown that milk production does not improve completely after recovering from subclinical mastitis, making the previously reported log-linear relationship a potential underestimate (St Rose et al., 2003).

Mastitis results in a significant change in the level of most of the major milk components, including protein, lactose, and fat (Kitchen, 1981). These changes are thought to be primarily due to impaired synthetic and secretory activity of the udder epithelial cells resulting in a decrease in casein and lactose levels. During mastitic events, blood capillary permeability changes and tight junctions between epithelial cells open. This results in a change in ion levels, including an increase in sodium and chloride and a decrease in potassium (Kitchen, 1981), as well an increase in proteins from the blood in the milk (Peaker, 1975). These changes effect finished dairy product quality and yield, as mastitis results in alterations in the flavor of pasteurized milk and increases rennet clotting time, decreases curd firmness, and reduces starter activity in cheese manufacture, all of which lower cheese yields (Kitchen, 1981).

The main focus of mastitis control in the 1970s-1990s was on the contagious major pathogens, primarily focusing on *Streptococcus agalactiae* and *Staphylococcus aureus* (Myllys et al., 1998). The five point plan for mastitis control was first introduced in the 1960s, primarily focusing on control of contagious pathogens through 1) maintaining a properly functioning milking machine, 2) dipping teats in an effective post-milking germicide, 3) appropriate therapy of clinical mastitis, 4) treating every quarter of every cow at dry off with intramammary antibiotics, and 5) culling chronically infected cows (Neave et al., 1969). The plan was later expanded to a 10 point plan, adding points to encourage regular monitoring as well as control for environmental mastitis including: maintaining a clean, dry comfortable environment, good record keeping, maintaining biosecurity, monitoring udder health status, and periodic review of the herd's mastitis

control program (National Mastitis Council, 2009, Middleton et al., 2014). The mastitis prevention program put forth by the National Mastitis Council has resulted in improved control of contagious mastitis pathogens (National Mastitis Council, 2009). Since that time, the proportion of IMIs associated with coagulase-negative *Staphylococcus* (CNS) have increased (Myllys et al., 1998).

Many recent epidemiologic investigations have identified CNS species as the predominant mastitis pathogens and the leading cause of subclinical mastitis on well-managed dairy farms (Pitkala et al., 2004, Tenhagen et al., 2006, Piepers et al., 2007, Schukken et al., 2009). Therefore, the focus of this literature review is on CNS species, including a broad overview of *Staphylococcus* species associated with intramammary infection, diagnostic methods used to speciate and strain type staphylococcal species, the epidemiology of CNS mastitis, and the role of CNS in heifer mastitis.

1.2 *Staphylococcus* species associated with bovine intramammary infections

Staphylococcal species are Gram-positive, non-motile, facultative anaerobes that are spherical and range from 0.5-1.5 μm in diameter. These organisms are usually catalase positive and most will grow in the presence of 10% NaCl between 18 and 40°C (Kloos, 1986). There are approximately 47 species and 23 subspecies of staphylococci that have been characterized to date (Becker et al., 2014). The genus can be divided into two broad groups, coagulase-positive staphylococci (CPS) and CNS, based on their ability to convert fibrinogen to fibrin (Chapman et al., 1934).

Staphylococcus aureus is considered to be a major mastitis pathogen, while all other *Staphylococcus* spp. are considered minor mastitis pathogens. *Staphylococcus aureus* can be distinguished from other *Staphylococcus* spp. of bovine origin based on phenotypic traits, including being coagulase positive in combination with the presence of hemolysis on blood agar plates. A range of colony pigments have been observed from grey to yellow-orange (Kloos, 1986). Many different strains of *S. aureus* exist with differing abilities to spread within herds (Smith et al., 1998), elevate SCC, cause clinical mastitis, cause persistent infections (Zadoks et al., 2000, Haveri et al., 2005), and result in milk production losses (Middleton and Fox, 2002).

Three non-*aureus* CPS species have been isolated from bovine milk, including *Staphylococcus intermedius*, *Staphylococcus hyicus*, and *Staphylococcus agnetis*. *Staphylococcus hyicus* and *S. agnetis* are coagulase variable species, with some isolates being found to be CPS and others CNS (Kloos, 1986, Taponen et al., 2012). Most strains of *S. intermedius* and *S. aureus* have indistinguishable hemolytic patterns on blood agar. Lack of pigment can be used to help differentiate non-hemolytic *S. hyicus* and hemolytic *S. intermedius* from *S. aureus*, however some strains of *S. aureus* are also non-pigmented (Roberson et al., 1996). Very limited data are available on the prevalence of non-*aureus* CPS species on dairy farms. In one study, which included 21 herds, the prevalence of coagulase-positive *S. hyicus* was low, 0.6% of all cows and 2% of primiparous cows at parturition (Roberson et al., 1996). However, the percentage of CPS IMI due to *S. hyicus* varied considerably between low prevalence *S. aureus* herds and high prevalence *S. aureus* herds. *Staphylococcus hyicus* represented 30% of CPS isolates from herds with a

low prevalence of *S. aureus* and only 1.8% of the CPS isolates from herds with a high prevalence of *S. aureus*. One issue with the Roberson et al. (1996) study is that it was done prior to the discovery of *S. agnetis*, a coagulase variable species that is phenotypically identical to *S. hyicus* (Taponen et al., 2012). Hence, it is unknown whether these isolates would now be classified as *S. agnetis*. Since the identification of *S. agnetis*, little has been done to characterize the prevalence and epidemiology of *S. agnetis* and *S. hyicus* (Taponen et al., 2012). *Staphylococcus intermedius* appears to be an extremely rare cause of bovine mastitis with the Roberson et al. (1996) study finding only one isolate among 487 CPS isolates from 22 dairy herds. That isolate was found in a herd with a low prevalence of *S. aureus*. With the discovery of *Staphylococcus pseudintermedius* in 2005 (Devriese et al., 2005), it is also unclear whether the isolate reported by Roberson et al. (1996) would now be classified differently.

Within the *Staphylococcus* genus there are many different coagulase-negative species with a diversity of phenotypic characteristics. Species identification using phenotypic approaches can be very laborious, as it requires a large number of biochemical tests. Approximately 10 species are frequently found in bovine milk samples with the most frequently found species being *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, and *Staphylococcus xylosus* (Piessens et al., 2011, Vanderhaeghen et al., 2015). Overall, as mastitis pathogens, CNS is the most common cause of subclinical IMI (Piepers et al., 2007). Coagulase-negative staphylococcal species that are found in milk have been classified as

skin-associated opportunists, although at the time of this classification, sound data on their habitats was lacking (Pyorala and Taponen, 2009).

Although CNS has been well characterized at the group level, accurate species identification and subspecies characterization of staphylococcal species is paramount to understanding the ecology, epidemiology, and significance of each of these organisms.

1.3 Speciation and subtyping of staphylococcal species

1.3.A Traditional methods of diagnosis and characterization of staphylococcal species associated with bovine intramammary infection

The identification of CNS to the species level using traditional biochemical testing methods can be costly and time consuming. Overall, CNS species can be characterized by separating them into two groups, based on susceptibility to novobiocin (Devriese et al., 1985). In cattle, novobiocin-sensitive species tend to colonize the teat canal and are associated with clinical and subclinical mastitis. The novobiocin-resistant species are found on the teat skin and in the environment, less often being associated with IMI (White et al., 1989, Devriese et al., 1994). Further tests used to differentiate the prevalent species that cause bovine mastitis include the DNase test, protease test, inhibition of delta hemolysin, sensitivity to deferroxamine, and sensitivity to fosfomycin (Devriese et al., 1994).

Phenotypic speciation of staphylococcal isolates can also be done more rapidly using commercial identification kits, such as API Staph ID (BioMerieux) and Staph-Zym (Rosco). These tests were developed primarily for human clinical use (Thorberg and Brandstrom, 2000). API Staph ID was previously recommended by the National Mastitis Council for differentiation of CNS species (Hogan, 1999); however the typeability and accuracy of these systems for identification of CNS isolates from cows is not optimal (Thorberg and Brandstrom, 2000, Taponen et al., 2006, Park et al., 2011). Using these methods, 11-59% of isolates from bovine milk samples are classified as unidentified (Thorberg and Brandstrom, 2000, Sampimon et al., 2009c). The accuracy of API Staph ID was 77% when compared to conventional biochemical methods (Thorberg and Brandstrom, 2000), 76% when compared to 16S rRNA gene sequencing (Park et al., 2011), and 41% when compared to *rpoB* gene sequencing (Sampimon et al., 2009c). Specifically, problems have been encountered with the commercial kits in the identification of *S. chromogenes*, *S. haemolyticus*, and *S. simulans*, all of which are common species causing bovine IMI (Sampimon et al., 2009c, Park et al., 2011). In one study, 29-45% of these key species were identified incorrectly or could not be identified when using API Staph ID (BioMerieux, Paris, France; Thorberg and Brandstrom, 2000).

Phenotypic speciation methods are based on metabolic activity and morphological features, which might show variability due to environmental changes such as culture conditions, sub-culturing, and storage (Sauer and Kliem, 2010). Furthermore, variability in preparation, incubation procedures, and reading of the test can lead to inconsistencies, especially when conducted by inexperienced personnel (Thorberg and Brandstrom,

2000). With these problems in mind, it has been recommended that phenotypic systems should be abandoned for scientific and routine use in bovine CNS species identification (Vanderhaeghen et al., 2015).

1.3.B The use of molecular techniques to speciate staphylococcal species of bovine origin

Sequencing of housekeeping genes is considered the most reliable method for speciation of bacteria (CLSI, 2007), with the 16S rRNA gene being the most universal target (Patel, 2001). The usefulness of 16S rRNA gene sequencing is limited when applied to certain staphylococcal species because of the high degree of similarity between closely related species, with a reported mean similarity of 97.4% (Gribaldo et al., 1997, Becker et al., 2004, Shah et al., 2007). Therefore, several different genotypic speciation methods have been described for speciation of staphylococci including amplified fragment length polymorphism (AFLP; Taponen et al., 2006), ribotyping (Carretto et al., 2005), transfer RNA-intergenic spacer PCR (Supre et al., 2009), *gap* PCR-RFLP (Park et al., 2011) and DNA sequencing of housekeeping genes (Heikens et al., 2005). The latter technique has targeted several different genes, including *rpoB* (β subunit of RNA polymerase; Drancourt and Raoult, 2002), *tuf* (elongation factor Tu; Hwang et al., 2011), *sodA* (superoxide dismutase A; Poyart et al., 2001), *gap* (glceraldehyde-3-phosphate dehydrogenase; Ghebremedhin et al., 2008), *dnaJ* (chaperone DnaJ; Shah et al., 2007) and *cpn60/hsp60/groEL* (heat shock protein 60; Kwok et al., 1999). Although accurate, gene sequencing can be time consuming and expensive, especially at times when multiple genes have to be sequenced to conclusively identify a species. Another limitation to

sequence based methods is the dependence on publicly accessible databases without controlled input of gene sequence data, which can result in faulty or redundant sequence entries, outdated nomenclature, and potential lack of quality assurance (Becker et al., 2004).

Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry is a technology now being used for routine identification of bacteria in many fields, including the food industry, public health, environmental analysis, and clinical microbiology laboratories (Teramoto et al., 2007). Recently, several groups have used MALDI-TOF to speciate CNS isolates of bovine origin (Barreiro et al., 2010, Tomazi et al., 2014, Elbehiry et al., 2016). The method is considered to be a simple and low cost technique with high-throughput and rapid species level identification. MALDI-TOF is based on spectral analysis of primarily ribosomal proteins (Ryzhov and Fenselau, 2001), which are the most abundant cellular proteins and present in all organisms. Many reports indicate that the technique is more accurate in species identification than those based on traditional phenotypic or biochemical tests. In one study, all isolates yielded reliable identification by MALDI-TOF and species assignments by MALDI-TOF and *tuf* gene sequencing were congruent in all cases, indicating 100% accuracy (Carpaij et al., 2011). Using PCR-RFLP as a comparator, an agreement of 98% was found (Tomazi et al., 2014). Although fast and inexpensive, MALDI-TOF does have a few limitations. The method has been shown to have a lower diagnostic sensitivity, with one study finding a 95.4% sensitivity when compared to PCR-RFLP methods (Tomazi et al., 2014). Furthermore, because of the library based approach, if an organism is not within the

library, it will be classified as unidentifiable. One study looking at mastitis pathogens found 18% (6/33) of isolates to be unidentifiable (Banach et al., 2016).

Through the increased use of molecular methods to speciate CNS isolates, several new *Staphylococcus* spp. have been identified in recent years, including *S. devriesei* (Supre et al., 2010) and *S. agnetis* (Taponen et al., 2012). These new species have been found to be phenotypically similar to others, and even using some molecular speciation methods, specifically 16S rRNA gene sequencing, thus challenging to definitively identify (Supre et al., 2010, Taponen et al., 2012). To date, no standard molecular methodology has been widely accepted for the identification of bacterial agents that cause bovine mastitis. With the lower cost of whole genome sequencing, new targets may be identified that help differentiate these difficult to identify species.

1.3.C Strain typing of staphylococcal species of bovine origin

Strain typing of bacteria is used to characterize organisms at the subspecies level to better understand sources, transmission routes, biological relationships, and virulence characteristics (Zadoks et al., 2011). Molecular fingerprinting can also be used to determine persistence of infection. For example, with regards to CNS species, several studies have shown that some species of CNS tend to cause persistent infections; however these studies have not uniformly used strain-typing techniques to confirm that infections with the same species over time within a cow or mammary quarter actually have the same fingerprint (strain-type) (Aarestrup and Jensen, 1997, Thorberg et al., 2009, Supre et al., 2011). Because of the very high incidence of CNS IMI, finding the

same CNS species on two different occasions is not sufficient to define a chronic IMI (Dufour et al., 2012).

Several DNA-based approaches have been used to study staphylococcal species, with the most work being done on the characterization of *S. aureus*. These methods include random amplified polymorphic DNA (RAPD) analysis (Myllys et al., 1997), ribotyping (Richardson et al., 1994), pulsed-field gel electrophoresis (PFGE; Zadoks et al., 2000), ribosomal spacer PCR (RS-PCR; Jensen et al., 1993), and binary typing (van Leeuwen et al., 1999). PCR-based DNA fingerprinting methods have been previously shown to cluster large groups of unrelated isolates together. For example, in one study, PCR-based DNA fingerprinting was used and found isolates from several herds in several distinct regions could be categorized as the same type with approximately half of the isolates in the study characterized as one of two types (Matthews et al., 1994). Conversely, PFGE tends to find strains that are unique to a herd (Joo et al., 2001, Middleton et al., 2002a, b). Gene sequence-based techniques include multilocus sequence typing (MLST; Enright and Spratt, 1999), multiple locus variable number tandem repeat analysis (MLVA; Sabat et al., 2003), *spa*-typing (Shopsin et al., 1999), and whole genome sequencing (Salipante et al., 2015). The discriminatory power of PFGE for strain-typing *S. aureus* has been previously described, and ranges from 0.83 to 0.8887 (Ikawaty et al., 2009, Du et al., 2014, Silveira-Filho et al., 2014). When comparing several strain-typing methods to characterize *S. aureus* isolates of bovine origin, PFGE had the highest discriminatory power, followed by MLST, MLVA, and then *spa*-typing (Ikawaty et al., 2009). Of these methods, PFGE is generally considered to be the ‘gold standard’ for the epidemiological

typing of microorganisms with whole genome sequencing the most likely to replace it in the future (Spinali et al., 2015). To date, a comprehensive comparison of DNA-based strain-typing methods has not been done for CNS species. Several studies have used amplified fragment length polymorphism (AFLP) or AFLP with RAPD for strain-typing of CNS species, however the discriminatory power of these methods has not been determined (Taponen et al., 2007, Piessens et al., 2012).

Recently, MALDI-TOF has been explored as a strain-typing method (MALDI typing); however the utility of this approach has not been completely elucidated. Some issues with this method include the lack of guidelines for data interpretation (Spinali et al., 2015). Furthermore, the same bacteria can give different mass spectra under different culture conditions or different chemical extraction methods. Therefore, it is important to have well controlled growth media and standardized sample preparation procedures in order to obtain reproducible mass spectra (Fenselau and Demirev, 2001, Chen et al., 2008).

Through the use of the described phenotypic and genotypic speciation techniques, the epidemiology of CNS mastitis has been evaluated. Furthermore, subtyping has been used to help characterize persistence of intramammary infections and in a few cases, determining the relationship between extramammary colonization and intramammary infection. Below is a review of the current knowledge on the epidemiology of CNS species.

1.4 Coagulase-negative staphylococcal mastitis

As a group, CNS are the most prevalent bacteria found in milk samples collected from dairy cows and heifers in many areas of the world (Tenhagen et al., 2006, Piepers et al., 2007, Schukken et al., 2009). In general, this group of organisms induces mild subclinical mastitis as measured by a modest increase in milk SCC between that observed for non-infected quarters and *S. aureus* infected quarters (Schukken et al., 2009). In general, SCC associated with CNS species usually remains below 500,000 cells/ml (Djabri et al., 2002, Fry et al., 2014). However, when assessing persistent CNS infections specifically, the geometric mean SCC was higher, at 657,600 cells/ml (Taponen et al., 2007).

Reports on CNS being associated with clinical mastitis can be found; however descriptions of prevalence are highly variable. One reported that about 52% of clinical mastitis cases were caused by CNS (Jarp, 1991), but a more recent study showed a much lower occurrence, at 5.1% of samples submitted from 106 dairy farms in 10 Canadian provinces (Olde Riekerink et al., 2008). In a Finnish study, half of the IMIs caused by CNS were clinical; however the majority of these involved very mild signs (Taponen et al., 2006). In other studies, no CNS species were found to be associated with clinical mastitis cases (Supre et al., 2011).

Identification of CNS at the species-level is important due to differences in prevalence as well as epidemiologic and pathogenic differences between the various CNS species associated with mastitis (Vanderhaeghen et al., 2014a, b). With regards to prevalence, most studies find *S. chromogenes* to be the predominant species, causing between 31-48% of all CNS IMI (Taponen et al., 2008, Supre et al., 2011, Fry et al., 2014, De

Visser et al., 2016b). One study, in which 11 herds in Sweden were sampled, found *S. epidermidis* to be the most prevalent species (Thorberg et al., 2009). Other common species identified include *S. simulans*, *S. xylosus*, and *S. haemolyticus* (Pyorala and Taponen, 2009, Sampimon et al., 2009a, Fry et al., 2014). Distribution of CNS species causing IMI are herd specific (Supre et al., 2011), with one study showing *S. chromogenes* to be the only species found in all 13 commercial herds assessed (De Visser et al., 2016a), whereas another study found no species to be identified in all of the 11 herds assessed (Thorberg et al., 2009). In herds with IMIs caused by more than one CNS species, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. xylosus* tend to be among the dominant species in a given herd (Thorberg et al., 2009). Distribution of species also varies with age group, with *S. chromogenes* being more often diagnosed in first-parity cows than in older cows and *S. epidermidis* being found mainly in older cows (Thorberg et al., 2009).

Staphylococcus chromogenes, *S. epidermidis*, *S. simulans*, and *S. xylosus* have been associated with persistent infections (Piessens et al., 2011, Supre et al., 2011, Fry et al., 2014). In one study, *S. chromogenes* caused more persistent IMIs than transient IMIs, and *S. xylosus* was slightly more common in persistent IMIs than transient IMIs (Supre et al., 2011). A North American study found the median number of days between first and last positive sample to be between 15-28 days depending on species; however, some CNS IMIs were found to persist as long as 340 days (Fry et al., 2014). In a European study, the mean duration was much longer, with *S. chromogenes* IMI lasting ~148 d (range 89-303 d) and *S. simulans* IMI lasting 176 d (range 140-212 d) (Supre et al., 2011).

Staphylococcus chromogenes has also been found to persist over the dry period in some infected mammary quarters (Supre et al., 2011). Unfortunately, most studies have not used strain-typing to define persistence and therefore may be overestimating the true prevalence of persistent CNS IMIs.

Mean milk SCC has been shown to be significantly higher than the mean SCC of uninfected control quarters for 8/20 species, including *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, *S. epidermidis*, *Staphylococcus warneri*, *Staphylococcus capitis*, and *S. hyicus* (Fry et al., 2014). It has also been shown that *S. chromogenes*, *S. simulans*, and *S. xylosus* can have a substantial effect on milk SCC, with effects comparable to *S. aureus* (Supre et al., 2011). In a North American study, *S. chromogenes* and *S. simulans* were found to have a significantly higher mean SCC compared to *S. xylosus* and *S. cohnii* (Fry et al., 2014). Several studies have found that *S. simulans* causes a more severe mastitis and is often more prevalent among the CNS species that cause clinical mastitis. (Myllys, 1995, Waage et al., 1999). Conversely, when using the California Mastitis Test (CMT) score as a measure of inflammation, 95% of IMI associated with *S. cohnii*, *Staphylococcus kloosii*, *Staphylococcus saprophyticus*, *S. sciuri*, and *S. xylosus* were found to be associated with a low CMT score suggesting that they might be less pathogenic than some of the aforementioned staphylococcal species (Thorberg et al., 2009). Overall, a high degree of variability has been noted within a given species for milk SCC suggesting that either there are subspecies differences in pathogenicity or differences in host response to IMI (Fry et al., 2014).

The aforementioned differences in persistence of IMI and also variation in the resultant inflammatory responses to IMI, suggests that some species and strains of CNS may be better host-adapted while others are environmental opportunists (Piessens et al, 2011). Different environments in which CNS have been found in abundance include floor samples, air samples, used bedding samples, and bovine body sites (Taponen et al., 2008, Piessens et al., 2011, De Visscher et al., 2014). It has been demonstrated that *S. chromogenes* and *S. epidermidis* seem to be more adapted to the mammary gland, predominately being isolated from milk, and rarely being isolated from the bovine environment (Piessens et al., 2011, Piessens et al., 2012). Unlike *S. chromogenes*, *S. epidermidis* is rarely isolated from bovine skin but is one of the most prevalent species on human skin and may be transmitted from milkers to cows (Thorberg et al., 2006). *Staphylococcus haemolyticus* and *S. simulans* can be isolated from both milk and the environment (Piessens et al., 2011). For *S. haemolyticus*, similar AFLP types were observed among isolates originating from the environment and from IMI, potentially indicating the environment is the source for IMI with this species (Piessens et al., 2011). This hypothesis is corroborated by the fact that the overall genetic similarity of *S. haemolyticus* isolates using AFLP was low suggesting multiple sources rather than a point-source as may be expected with a contagious pathogen that is host-adapted and spreads cow-to-cow (Piessens et al., 2012). Similarly, *Staphylococcus equorum*, *S. sciuri*, *S. saprophyticus*, and *S. cohnii* are more commonly present in environmental habitats and seem to behave more like environmental opportunists (Piessens et al., 2011, De Visscher et al., 2014). Another niche that has been further assessed is the skin of the milker's hands and gloves worn during milking, which were found to frequently harbor

Staphylococcus auricularis, *S. cohnii*, *S. equorum*, *S. haemolyticus*, and *Staphylococcus hominis* and less frequently harbor *S. capitis*, *S. epidermidis*, *Staphylococcus fleurettii*, *Staphylococcus nepalensis*, *S. sciuri*, and *S. warneri* (De Visscher et al., 2014).

The true impact of CNS IMI on milk yield and quality is still under debate. Several studies have documented that cows and heifers infected with CNS had a slightly higher milk production at test day than did non-infected cows and heifers, despite an increase in test day SCC (Schukken et al., 2009, Piepers et al., 2010). When comparing cows with transient and persistent subclinical CNS mastitis, higher milk production has been found among persistently infected cows (Thorberg et al., 2009). Furthermore, it has been found that cows affected with clinical mastitis caused by CNS produced 2.3-2.7 kg/d more milk in the month before clinical symptoms appeared than did the healthy control group (Grohn et al., 2004). After diagnosis, there was no statistically significant difference in milk yield between the clinical mastitis group and healthy control; however, because the cows with CNS mastitis were higher producers before the onset of mastitis, they suffered a large production drop in comparison to cows not infected (Grohn et al., 2004). It has been hypothesized that high producing animals are potentially more sensitive to CNS than low producing animals and therefore true milk production losses caused by CNS may have been underestimated (Grohn et al., 2004, Compton et al., 2007a). Further assessment has found that the positive association between CNS IMI and milk yield was only marginally confounded by the higher genetically determined milk production levels and could only be partially explained by the lower incidence of clinical mastitis in CNS infected heifers compared with non-infected heifers (Piepers et al., 2013). To date, the

published studies on milk yield have all been done at the cow-level and not the quarter-level, and therefore the changes in milk yield in the infected quarter have not specifically been assessed (Piepers et al., 2010)

Additionally, a negative association between CNS IMI and the number of clinical mastitis cases encountered during the first lactation has been documented (Matthews et al., 1990, Matthews et al., 1991, Lam et al., 1997, Piepers et al., 2010). It has also been shown that heifers infected with CNS in early lactation showed a lower risk of being culled in the first lactation than non-infected control heifers (Piepers et al., 2010). A potential mechanism for the protective effect of some CNS species on preventing IMI with other pathogens is the production of bacteriocins by these protective species. Bacteriocins produced by Gram-positive bacteria make up a heterogeneous group of ribosomally synthesized antimicrobial peptides and proteins (De Vuyst and Leroy, 2007). In vitro, some *S. chromogenes* isolates (2/10) that originated from teat apices of dairy heifers have been shown to inhibit the growth of some Gram-positive major mastitis pathogens, including *S. aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*; however, these same isolates were not able to inhibit growth of *Escherichia coli* (De Vliegher et al., 2004c). Another study found antibacterial activity in 13% of CNS strains originating from the teat apex skin of dairy cows (Braem et al., 2014) based on the *in vitro* deferred antagonism test (Tagg and Bannister, 1979). Further work exploring the prevalence and potential use of bacteriocins in mastitis prevention is needed before complete conclusions can be drawn about their role in prevention of IMI.

In summary, although the true impact of CNS species is under review, it is likely highly variable depending on the specific species involved. Based on this review, *S. chromogenes*, *S. epidermidis*, *S. simulans*, and *S. xylosus* are the species most associated with increased SCC and persistent infections. In heifers, CNS are the major cause of IMI both pre- and post-calving. Again, although the true impact of CNS in heifers is debatable, the impact of mastitis and elevated SCC in heifers is well studied and reviewed below.

1.5 Heifer mastitis

It might be assumed that heifer mammary glands should be free of IMI and mastitis prior to calving. After all, primiparous heifers have not experienced daily milking and therefore have not been exposed to contagious pathogens transmitted during milking time. Likewise, they have not experienced the effects of milking vacuum and its potential deleterious effects to the teat end. For most of their lives, the mammary gland has been immature and therefore had less physical contact with the environment (Fox, 2009). Nevertheless, mastitis in heifers is a common finding with potential negative impacts on future productivity.

Several studies have shown that the incidence of subclinical mastitis in heifers is high, with a prevalence around 18-27% (De Vlieghe et al., 2012, Archer et al., 2013).

Intramammary infections in heifers can be detected either before or after calving (Trinidad et al., 1990a). In a study conducted in New Zealand, 18.5% of pre-calving and 21.5% of post-calving heifer mammary quarters were found to have a positive bacterial

culture result (Compton et al., 2007b). In Belgium, 35% of early lactation heifers were found to have subclinical mastitis based on a composite milk SCC > 150,000 cells/ml, and approximately 25% of all quarters of heifers around the time of calving were culture positive for an IMI (Piepers et al., 2010).

The incidence of clinical mastitis in heifers in the first two weeks after calving is higher than in cows (Barkema et al., 1998, Valde et al., 2004, Compton et al., 2007a). During the first month of lactation, heifers have been found to have a higher incidence of clinical mastitis than second-parity cows, and nearly as high as third-parity cows (Valde et al., 2004). Overall, the cumulative incidence of clinical mastitis is lower in 3 and 4 year old cows than in 2 year old heifers (McDougall et al., 2007). The distribution of bacterial isolates from cases of clinical mastitis can vary depending on age, for example, heifers have been found to have a lower incidence of *S. aureus* compared to older animals, with one study showing that the risk of *S. aureus* being isolated from the mammary gland increased from 11.8% for 2 year olds up to 48% for 8 year olds (McDougall et al., 2007). In general, primiparous heifers are more likely to be infected with CNS at the beginning of lactation, which is different from multiparous cows, which in general get CNS IMI later in lactation (Taponen et al., 2007).

Coagulase-negative staphylococci are the most likely cause of IMI in heifers both pre and postpartum (Fox et al., 1995, Fox, 2009). Prevalence of CNS IMI prepartum has ranged from 15.7% (Oliver and Mitchell, 1983) up to 45% (Oliver et al., 2003). Another report indicated that approximately 72% of heifers and 35% of mammary quarters had a CNS

IMI between days 1 and 4 post calving. However, many of these IMIs were found to be of short duration, as only 27% of quarters were CNS culture positive at days 5-8 post-partum (Piepers et al., 2010). The reported rapid decline in prevalence of infected quarters likely reflects the transient nature of CNS in early lactation heifers, which has been demonstrated by others (Myllys, 1995). Overall, periparturient IMI that are cleared shortly after calving have a less harmful effect on future productivity compared with IMI that persist into lactation (De Vliegher et al., 2004a, 2005b). The elevation in SCC in mammary secretions collected before calving and shortly after from CNS-infected quarters compared to non-infected quarters implies that the infection does provoke an inflammatory response in the udder of heifers (Trinidad et al., 1990a, Barkema et al., 1999). However, with regard to those found in early lactation, the geometric mean SCC of quarters infected with CNS decreases in the first 3 days after calving, starting with a geometric mean SCC of $1,000 \times 10^3$ cells/ml at calving which declines to 170×10^3 cells/ml at the sixth milking after calving, (Barkema et al., 1999). This decline in SCC is similar to what is noted in culture negative heifers, however culture negative SCC is lower at both time points, with a geometric mean SCC decrease from 306×10^3 cells/ml at calving to 42×10^3 cells/ml at the sixth milking (Barkema et al., 1999).

Heifers with prepartum CPS IMI range from 0.4-14.9% as compared to IMI at parturition, which ranges from 0.6% to 8% (Oliver et al., 1992, Roberson et al., 1994a, Myllys, 1995, Oliver et al., 2003). A decrease in post-partum prevalence was evaluated in one study in which heifers were sampled multiple times in the immediate post-partum period. In that study, the overall prevalence of heifers with quarters that were culture

positive for *S. aureus* decreased between samplings at 1-4 days in milk (DIM) and 5-8 DIM, suggesting some spontaneous elimination, similar to what has been documented for CNS IMI (Piepers et al., 2010). This does not fit with the more persistent character typically found with *S. aureus* (Roberson et al., 1994a).

Risk factors for *S. aureus* IMIs in heifers have been evaluated. One risk factor for *S. aureus* IMI in heifers was the housing system, with a higher prevalence in heifers housed in tie-stall barns (Bludau et al., 2016). Heifers with teat orifices colonized by *S. aureus* were 3.3 times more likely to have an IMI with *S. aureus* at parturition than heifers that were not colonized at the teat orifices (Roberson et al., 1994b). However, when using PFGE, some research has shown that *S. aureus* strains from bovine teat skin are different from milk strains (Zadoks et al., 2002). Conversely, others have shown that 47% of the strains found on the teat and udder skin were the same strains isolated from heifer mammary secretions (Middleton et al., 2002b). Horn flies have also been identified as a risk factor for the transmission of *S. aureus* in heifers (Gillespie et al., 1999, Piepers et al., 2011, Anderson et al., 2012). Identical DNA fingerprints have been found among *S. aureus* isolated from horn flies and those from heifer mammary secretions (Gillespie et al., 1999, Anderson et al., 2012). Application of fly control prior to calving has been associated with a reduced risk for IMI with contagious mastitis pathogens (Nickerson et al., 1995, Piepers et al., 2011). Moderate to excessive udder edema has also been identified as a risk factor for IMI with contagious major pathogens (Piepers et al., 2011).

In general, well managed dairy herds have been found to have better overall udder health in heifers in early lactation (De Vliegher et al., 2004b). For example, the presence of CNS IMI in heifers has been associated with poor heifer hygiene (Piepers et al., 2011). However, heifers differ from multiparous cows with respect to non-lactating period, production level, and nutrition before calving and therefore have different risk factors for development of IMI. Several studies have identified potential risk factors for heifer mastitis, including environmental and management factors as well as body site colonization (Roberson et al., 1994b, De Vliegher et al., 2004b, Svensson et al., 2006, Piepers et al., 2011, De Visscher et al., 2016b). Overall, it is reported to be a multifactorial disease that is influenced by climate, season, geographic location, and genetic background (Fox et al., 1995). Management factors, such as social stress, poor environmental hygiene, and inadequate nutrition have been associated with IMI in first lactation heifers (Piepers et al., 2011).

Specific management factors which have been correlated to a reduction in heifer subclinical mastitis include removal of supernumerary teats, housing late gestation heifers with lactating cows, and day and night grazing of lactating cattle (Santman-Berends et al., 2012). Floor type in the calving area was correlated with heifer SCC in one study, with heifers calving on slatted floors having a reduced odds of a high SCC over heifers calving on non-slatted floors (De Vliegher et al., 2004b). Slatted floors had drier bedding, which potentially corroborates with another study showing that poor hygiene of the calving area was associated with an increased prevalence of elevated SCC in heifers (Peeler et al., 2000). Bedding type has also been found to be a risk factor, with

mammary quarters of primiparous cows bedded on new sand having greater time to a clinical mastitis episode than quarters of those heifers bedded on recycled sand or deep bedded manure solids (Rowbotham and Ruegg, 2016). In another study, heifers reared at an off-site specialized rearing facility had a lower risk of environmental major pathogen mastitis (Bludau et al., 2016). Reasons for this may be because these specialized farms pay more attention to young stock (Bludau et al., 2016). With respect to season, in Belgium, heifers calving in April to June had a higher risk of having an elevated SCC, as opposed to heifers calving in the other seasons (De Vliegher et al., 2004b). In the USA, IMI at parturition in heifers were found to be more prevalent in the summer, primarily because of a relative increase in IMI caused by both *S. aureus* and CNS (Fox et al., 1995).

Several studies have evaluated nutritional risk factors for heifer mastitis. One study found that 11 to 16 month old heifers fed increasing amounts of concentrate feed (grains & plant seeds) had an increased risk of a $\text{SCC} \geq 200,000$ cells/ml at first test milking (Svensson et al., 2006). Feeding more roughage to calves at weaning was found to be associated with a reduced risk of veterinary treated clinical mastitis between -7 and 30 d relative to calving, as was feeding higher proportions of grain in the concentrate portion of the ration to heifers from 11 to 23 months of age or at 4 months of pregnancy (Svensson et al., 2006). The use of silage as the only roughage for young calves and the introduction of the lactation ration one week or less before calving were both associated with an increased risk of $\text{SCC} \geq 200,000$ cells/ml at first herd monthly test day milking. Also, an increased growth rate in calves from birth to weaning was associated with

decreased risk of veterinary treated clinical mastitis at -7 to 30 days relative to calving (Svensson et al., 2006).

An additional risk factor for CNS mastitis in heifers and cows is colonization of the skin (De Visscher et al., 2016b, Taponen et al., 2008). Coagulase-negative staphylococci are part of the normal skin flora of animals (Devriese and De Keyser, 1980, Devriese et al., 1985) and have been isolated from different body sites of cows and heifers (White et al., 1989, Taponen et al., 2008). However, teat skin colonization has been the primary focus of most studies (Braem et al., 2013, De Visscher et al., 2016b), with the thought that bacteria that have accumulated on the teat apex are more likely to enter the mammary gland.

Most of the literature on body site colonization to date has been done on lactating cows, which needs to be reviewed to further understand the relationship between body site colonization and IMI. In lactating cows, the total bacterial species diversity of the teat apex has been analyzed using culture-dependent and culture-independent analysis (Braem et al., 2012, Braem et al., 2013). Overall, CNS species were the most commonly identified and frequently isolated bacteria from the teat apices, with 59% of all isolates being CNS and 97.2% of all sampled teat apices found to be culture positive for CNS (Braem et al., 2013). Interestingly, higher bacterial species diversity was detected for CNS using culture-dependent analysis compared with culture-independent analysis in which denaturing gradient gel electrophoresis of PCR amplicons targeting the V3 region of the 16 rRNA gene was used. The CNS microbiota lining the teat apex was

found to be composed of a wide variety of species, with 15 different species identified (Braem et al., 2013).

Beyond the lack of data on CNS body site colonization, even fewer studies have been conducted to determine the relationship between body site colonization and IMI at the strain level (Thorberg et al., 2006, Taponen et al., 2008, Leroy et al., 2015). Again, the majority of data available has been collected on lactating cows. One study investigated the role of extramammary body sites in CNS IMI in lactating cows, without defining lactation number of sampled cattle, on a single Finnish dairy farm (Taponen et al., 2008). Swabs were collected from the perineum, udder skin, teat apices, and streak canal and identified to the species-level using the API Staph ID 32 test and strain-level by PFGE. The results of the study indicated that some CNS species were common on extramammary sites, but rarely caused mastitis, including *S. equorum*, *S. sciuri*, *S. saprophyticus*, and *S. xylosus*. On the other hand, *S. chromogenes* was the most common species causing mastitis and was also frequently isolated from extramammary sites. Five out of ten *S. chromogenes* PFGE strain-types were identified in milk and body sites. These findings help confirm the supposition that *S. chromogenes* is part of the skin flora and serves as an opportunistic mastitis pathogen. Overall, it was concluded that the origin of CNS mastitis may vary, depending on the infecting CNS species (Taponen et al., 2008).

With regards to heifers specifically, the prevalence of teat canal and teat skin colonization in unbred and primigravid heifers with CNS has also been reported to be high. Overall,

teat apices of pregnant heifers have been found to mainly be colonized by *S. chromogenes*, followed by *S. haemolyticus*, *S. devriesei*, and *S. equorum* (De Visscher et al., 2016b). Generally, 20-62% of sampled heifers have been found to harbor *S. chromogenes* on the teat skin (White et al., 1989, De Visscher et al., 2016b) with 43% of all teat canal samples (Trinidad et al., 1990a) and 10% of teat apices colonized by *S. chromogenes* (De Visscher et al., 2016b). With respect to age of the heifers, more than one-third of heifers between 20.1 and 28 months of age and half of heifers older than 28 months of age have been found to have at least one teat apex colonized by *S. chromogenes*. Overall, the chance of heifers having at least one teat apex colonized increased significantly with age (De Vlieghe et al., 2003). Further work has found that prepartum teat apex colonization with *S. chromogenes* increases the likelihood of *S. chromogenes* IMI in the corresponding quarter at parturition (De Visscher et al., 2016a), which is similar to *S. aureus* (Roberson et al., 1994b), another host-adapted species. Interestingly, unlike *S. aureus*, teat apex colonization with some strains of *S. chromogenes* may actually be protective as colonization was associated with a decreased incidence of mastitis, defined as an SCC $\geq 200,000$ cells/ml, in early lactation (De Vlieghe et al., 2003). When compared to cultures of the teat apices of lactating cows, more diversity in species distribution has been found in prepartum samples of teat apices. Also, one of the predominant species found in prepartum samples, *S. devriesei*, was rarely identified or absent in lactating cow samples (Braem et al., 2013, De Visscher et al., 2014).

Coagulase-negative staphylococcal species isolated from bovine body sites and streak canals of nulliparous heifers, defined as cattle that have never given birth, have also been rarely investigated (White et al., 1989). In one study, *S. xylosus* was the predominant species found on many body sites, including the nares, hair coat, vagina, and teat skin. However, the predominant species in the streak canal was *S. chromogenes*. Interestingly, as heifers aged, the recovery of *S. xylosus* and *S. sciuri* decreased in most groups, whereas *S. chromogenes* and *S. warneri* populations increased. Results of the White (1989) study suggested that changes in microflora are due to both age and housing factors. One downfall to the White (1989) study was that *Staphylococcus* species were identified based on the results of a modified biochemical identification scheme rather than by molecular methods.

Risk factors for the colonization of teat apices with CNS have been evaluated by De Visscher (2016). Dirty teat apices were significantly more likely to be colonized with CNS in general and *S. equorum*, in particular. Slightly dirty teat apices had higher odds of being colonized with *S. chromogenes* compared to very clean teat apices. Higher ambient temperature increased the odds of being colonized with *S. devriesei*, whereas the opposite was true for *S. equorum* (De Visscher et al., 2016b).

First lactation mastitis can have a lasting impact on heifer productivity with heifers having an increased SCC early in the first lactation having an elevated SCC (De Vlieghe et al., 2004a) and decreased milk production throughout the entire first lactation (Coffey et al., 1986, De Vlieghe et al., 2005b). One study found that a one unit increase in the

LnSCC in early lactation was on average associated with a decrease in milk yield of 0.13 kg/d later in lactation (De Vliegher et al., 2005b). Furthermore, a more recent study showed that increased SCC between 5 and 30 DIM of the first lactation negatively impacted lifetime milk yield (Archer et al., 2013). Mastitis in heifers is also associated with increased culling rates, with each unit increase in the LnSCC in early lactation being associated with an 11% increase in culling hazard (De Vliegher et al., 2005a). Increased heifer culling resulted in increased rearing costs (De Vliegher et al., 2012).

Treating pregnant heifers with intramammary dry or lactating cow antibiotics reduces the prevalence of IMI at calving (Trinidad et al., 1990b, Middleton et al., 2005, Borm et al., 2006) and increases milk production in the first lactation (Oliver et al., 2003, Sampimon et al., 2009b). Early work examined the use of dry cow antibiotics and found a reduction in the prevalence of IMI in the treated group compared to the control group, however some concerns with antimicrobial residues were noted with 2.9% of quarters that were treated 3 months before calving testing positive for residues at calving (Trinidad et al., 1990b). A more recent study investigated the use of prepartum dry cow therapy found a decrease in the prevalence of major mastitis pathogens, as positive samples between day 0 and day 10-14 decreased more in the treatment group (5.5%) than in the control group (4.8%) (Sampimon et al., 2009b). However, one major downfall in that study is that residue testing was not conducted. Others have examined the use of lactating cow antibiotics given 7 days prior to expected parturition (Oliver et al., 1992). When compared to the use of dry cow antibiotics, lactating cow antibiotics had a higher overall efficacy in the reduction of IMIs (Trinidad et al., 1990b, Oliver et al., 1992). This

difference may be due to timing of antimicrobial treatment, difference in drugs being used, and time when IMIs occur in heifers. With an increase in milk production and lower SCC, a more recent study evaluating lactating cow antibiotics found an economically beneficial associated with antibiotic treatment of pregnant heifers (Oliver et al., 2003). Systemic prepartum treatment with penethamate hydriodide of end-term heifers has been assessed, which resulted in fewer IMI at the quarter level in early lactation. However, long-term effects on milk production, udder health, and culling hazard during later lactation were not detected (Passchyn et al., 2013). Prepartum systemic antimicrobial therapy is not warranted in herds with low prevalence of heifer mastitis, but may help herds classified as having low-yielding heifers (Passchyn et al., 2013).

Although many benefits have been noted with prepartum antibiotic treatment of heifers, some major concerns exist, including safety and residues (Trinidad et al., 1990b, Sampimon et al., 2009b). Intramammary treatment of nonlactating heifers can be hazardous for both farm personnel and heifers (Sampimon et al., 2009b). If a Gram-positive spectrum antimicrobial is used, the number of cases of clinical mastitis caused by Gram-negative bacteria has been shown to be significantly higher in the treated quarters (Sampimon et al., 2009b) or treated heifer, in the case of systemic treatment (Passchyn et al., 2013). The use of antibiotics in heifers before calving should be carefully considered due to concerns surrounding appropriate extra label drug use, milk residues, and antimicrobial resistance.

1.6 Conclusions

The data presented above demonstrate that staphylococcal mastitis is a prevalent disease. Historical data on the ecology and epidemiology of staphylococcal mastitis, particularly mastitis caused by different species of CNS is clouded by the fact that, prior to the use of molecular speciation methods, some of the identified organisms were likely misclassified. It is also clear that staphylococci are an important cause of heifer mastitis. This dissertation sought to answer some of the gaps in our understanding of staphylococcal mastitis by addressing the following:

- 1) *Staphylococcus chromogenes* is clearly the most prevalent cause of CNS mastitis.

In addition, it can cause elevations in milk SCC, persist in the mammary gland, and in some instances perhaps have a protective effect against major pathogen IMI. Hence, the first objective of this thesis was to use next generation DNA sequencing to construct a whole genome sequence of a *S. chromogenes* mastitis isolate known to persist in the mammary gland of the cow from which it was isolated.

- 2) Given the difficulties of differentiating *S. agnetis* from *S. hyicus* and also their close relationship with *S. chromogenes*, the second objective was to define a genetic target that could be easily and reliably used to differentiate *S. agnetis* and *S. hyicus* and to use the new assay along with pulsed-field gel electrophoresis strain-typing to characterize a collection of non-*S. aureus* coagulase-positive staphylococci isolated from bovine milk samples from 15 dairy farms in Washington and Idaho. A further objective was to compare the total number of *S.*

aureus, *S. agnetis*, and *S. hyicus* isolates identified from each farm. This work was aided by the publication of whole genome sequences for a bovine strain of *S. agnetis* and *S. hyicus*, respectively, by my mentors and collaborators.

- 3) It is clear from the literature review above, that our complete understanding of the ecology and epidemiology of staphylococcal IMI in cows and heifers is hampered by the lack of a rapid, repeatable, and highly discriminatory method for subspecies classification of staphylococci. Hence, the third objective was to examine alternative methods to staphylococcal strain-typing (RS-PCR and virulence gene identification) using *S. aureus* isolates as the initial benchmark because these techniques are described elsewhere for this genus and species. The overall goal was to determine if the recently described *Staphylococcus aureus* Genotype B was present among previously characterized isolates from cases of bovine intramammary infection in the USA and to compare pulsed-field gel electrophoresis (PFGE) to the combination of ribosomal spacer PCR (RS-PCR) and virulence gene identification for strain-typing of *S. aureus*. If the RS-PCR typing method was found to be equally as discriminatory as PFGE, this method could then be applied to other staphylococcal species.
- 4) Overall, based on the literature review, CNS species are an important and highly prevalent cause of heifer mastitis. Unfortunately, there is a paucity of data regarding the ecology and epidemiology of CNS mastitis in heifers in North America as defined using molecular speciation and subspecies classification

methods. Therefore, the fourth objective was to clearly understand the association between body site colonization and IMI before and after calving in heifers using molecular methods of CNS speciation and subtyping.

- 5) Finally, as established in literature review, there is a clear lack of knowledge on when heifers become colonized with CNS species of importance in heifer mastitis. Therefore, the fifth objective was to describe the prevalence and distribution of *Staphylococcus* species on the teat and inguinal skin of dairy heifers throughout the heifer lifecycle on one farm using molecular speciation methods.

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CHAPTER THREE

DRAFT GENOME SEQUENCE OF *STAPHYLOCOCCUS CHROMOGENES* MU 970, ISOLATED FROM A CASE OF CHRONIC BOVINE MASTITIS

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Running title: *Staphylococcus chromogenes* genome

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Coagulase-negative staphylococcal species are a common cause of subclinical bovine mastitis with *Staphylococcus chromogenes* being one of the most frequently identified species in these cases. The draft genome sequence of a *S. chromogenes* isolate (MU 970) recovered from milk of a cow with a chronic intramammary infection is reported herein.

Staphylococcus chromogenes is a Gram-positive, nonhemolytic, yellow/orange coccus. This organism was originally considered a subspecies of *Staphylococcus hyicus*; however it is now classified as a separate species (Hajek V, 1986). *S. chromogenes* has been found to be the most prevalent coagulase-negative staphylococcal (CNS) species associated with subclinical intramammary infection (IMI) in dairy cattle in several studies (Sampimon et al., 2009, Piessens et al., 2011, Fry et al., 2014). *S. chromogenes* has also been associated with persistent infections and elevated milk somatic cell count (Supre et al., 2011). However, little is known about the genetic basis for pathogenesis of this organism. Hence, a draft genome sequence of *S. chromogenes* MU 970 was determined. This strain was isolated from the right rear mammary quarter of a Holstein cow at the University of Missouri Dairy (with ACUC approval) for 16 consecutive months, between July 2005 and October 2006. The first and last isolate recovered from this IMI had identical pulsed-field gel electrophoresis banding patterns. The geometric mean somatic cell count associated with this IMI was 365,000 cells/ml.

Next generation sequencing (454 Titanium) was performed at The Genome Institute, Washington University, St. Louis, MO. Newbler assembly (Roche) of 178,110 reads resulted in 38 contigs (largest 558,330 base pairs; N50 258 kb). The genome of 2,344,537 bp was covered at a sequencing depth of 24.7 fold and comprised 36.54% G+C. Auto-annotation of the contigs was performed at the National Center for Biotechnology Information using the PGAP pipeline. A total of 2311 ORFs and 60 tRNAs were annotated using GeneMarkS+. Five pseudogenes were verified though Sanger sequencing. Analysis of contig ends predicted that 6 RNA operons are present.

One plasmid sequence (19.3 kb) was identified in the genome of isolate MU 970. This plasmid contains an apparent transposable element with >99% identity to *Staphylococcus aureus* Tn552, a well characterized 6.5-kb β -lactamase encoding transposon (Rowland and Dyke, 1990), as well as a novel glycosyltransferase gene.

A gene predicted to encode a multidrug resistance efflux pump was identified. A phytoene dehydrogenase encoding gene, with 67% identity to the *S. aureus* dehydrosqualene desaturase gene, was also found and is expected to contribute to the observed pigmentation.

Putative virulence factor genes recognized included *sasH*, which has previously been identified in *S. aureus* and determined to code for a cell wall associated adenosine synthase that converts adenosine nucleotides into adenosine. Adenosine is a strong immunomodulator, helping staphylococci to escape phagocyte induced killing

(Thammavongsa et al., 2009). Also, two biofilm associated protein genes and two ORFs with 34% and 37% identity to the *S. aureus* fibronectin binding protein A were identified. This genome contains a gene encoding a putative superantigen-like protein, one gene encoding an IgG binding protein, and an ORF with 41% identity to a predicted coagulase of *Staphylococcus pseudintermedius*.

The data reported here represent the first *S. chromogenes* genome sequence available. These data should provide useful information for future studies on the pathogenesis of *S. chromogenes* IMI in the bovine as well as studies to identify possible targets for therapeutic or vaccine interventions. This draft genome has been deposited at DDBJ/EMBL/GenBank under the accession JMJF01000001.

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CHAPTER FOUR

SPECIATION AND STRAIN-TYPING OF *STAPHYLOCOCCUS AGNETIS* AND *STAPHYLOCOCCUS HYICUS* ISOLATED FROM BOVINE MILK USING A NOVEL MULTIPLEX PCR AND PULSED-FIELD GEL ELECTROPHORESIS

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Running Head: Characterization of *S. agnetis* and *S. hyicus*

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ABSTRACT

Staphylococcus hyicus and *Staphylococcus agnetis* are two coagulase variable staphylococcal species that can be isolated from bovine milk and are difficult to differentiate. The objectives of this study were to characterize isolates of bovine milk origin from a collection that had previously been characterized as coagulase positive *S. hyicus* based on phenotypic speciation methods and to develop a PCR-based method for differentiating *S. hyicus*, *S. agnetis*, and *S. aureus*. Isolates (n=62) were selected from a previous study in which milk samples were collected from cows on 15 dairy farms. For the present study, isolates were coagulase tested and speciated using housekeeping gene sequencing. A multiplex PCR to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus* was developed. Pulsed-field gel electrophoresis was conducted to strain type isolates. Based on gene sequencing, 44/62 of the isolates were determined to be either *S. agnetis* (n=43) or *S. hyicus* (n=1). Overall, 86% (37/43) of *S. agnetis* isolates were found to be coagulase positive at 4 hours. On eight farms (53%), over 20% of the coagulase positive staphylococcal isolates were found to be *S. agnetis*. Strain-typing identified 24 different strains. Six strains were identified more than once and from multiple cows within the herd. Three strains were isolated from cows at more than one time point, with 45-264 days between samplings. These data suggest that *S. agnetis* is likely more prevalent on dairy farms than *S. hyicus*. Also, some *S. agnetis* isolates in this study appeared to be contagious and associated with persistent infections.

INTRODUCTION

Staphylococci are the most common bacteria isolated from the cow's mammary gland and are frequently associated with subclinical or mild clinical mastitis (Pitkala et al., 2004, Tenhagen et al., 2006, Sampimon et al., 2009a). Staphylococci can be differentiated into groups using the coagulase test. Hemolytic, coagulase positive isolates are often presumptively identified as *Staphylococcus aureus*, a so-called "major" contagious mastitis pathogen, and the coagulase negative staphylococci are frequently grouped together as "minor" pathogens (Hogan, 1999).

Currently, there are two coagulase variable staphylococcal species that have been associated with bovine mastitis, *Staphylococcus hyicus* and *Staphylococcus agnetis*. *Staphylococcus hyicus* was the first staphylococcal species to be described as coagulase variable, with coagulase production being found in approximately 24-56% of strains (Kloos, 1986). The coagulation reaction of *S. hyicus* was described as often having weak activity, requiring 18-24 hours for detection (Kloos, 1986). *Staphylococcus hyicus* has been associated with chronic intramammary infections (IMI) that cause subclinical or mild clinical mastitis in dairy cattle (Roberson et al., 1996, Gillespie et al., 2009). Historically, *S. hyicus* was found to be one of the most common organisms isolated from mammary gland secretions and teat canal keratin samples from primigravid heifers (Trinidad et al., 1990). When identified as a coagulase negative staphylococcal (CNS) species, the reported prevalence of *S. hyicus* on dairy farms has been highly variable, with some researchers finding it to be one of the most prevalent species identified (Gillespie et al., 2009), while being rarely identified by others (Sampimon et al., 2009a,

Fry et al., 2014b). Coagulase positive *S. hyicus* has been described as a low prevalence mastitis pathogen and has been found to be a greater problem in herds with a low prevalence of *S. aureus* IMI (Roberson et al., 1996). *Staphylococcus agnetis* is a newly described species that is genetically very similar to *S. hyicus* making it difficult to differentiate from *S. hyicus* with both routine phenotypic and genotypic tests. It was first reported in 2012 associated with subclinical and mild clinical bovine mastitis (Taponen et al., 2012).

The phenotypic tests used to differentiate the staphylococcal species have likely lead to classification errors. Prior to 1986, *Staphylococcus chromogenes* and *S. hyicus* were not recognized as different staphylococcal species (Hajek V, 1986), and phenotypic classification methods such as API 20 (API Test, bioMérieux, Hazelwood, MO, USA) have been shown to have low specificity in the detection of *S. hyicus* (Zadoks and Watts, 2009). Only since 2012 has *S. agnetis* been recognized as a species and mastitis agent (Taponen et al., 2012), and thus prior to this date most non-*aureus* coagulase positive *Staphylococcus* (CPS) were likely classified as *S. hyicus*. Further, from a practical standpoint, when a staphylococcal species is identified as coagulase positive, it is often presumptively identified as *S. aureus*, and thus *S. hyicus* and *S. agnetis* may be misclassified by practitioners or dairy farmers performing their own milk microbiology (Roberson et al., 1992, Roberson et al., 1996), particularly since approximately one fifth to one fourth of *S. aureus* isolates from bovine mastitis do not present any detectable beta-hemolytic activity in primary culture (Boerlin et al., 2003).

Recently, genotypic methods to identify staphylococcal species have been described as being superior to phenotypic methods (Zadoks and Watts, 2009). Phenotypic speciation methods have been found to be unreliable and irreproducible for some species of staphylococci, possibly due to variable expression of the phenotypic traits and potentially related to subjective assessment of test results if an automated system is not used (Stager and Davis, 1992, Heikens et al., 2005). Analysis of 16S rRNA gene sequence is the most commonly used method for identification and classification of bacteria (Becker et al., 2004). However, the usefulness of 16S rRNA gene sequencing is limited when applied to certain staphylococcal species because of the high degree of similarity between closely related species (Gribaldo et al., 1997, Becker et al., 2004). Therefore, several different genotypic speciation methods have been described for staphylococcal speciation including amplified fragment length polymorphism (AFLP) (Taponen et al., 2006), ribotyping (Carretto et al., 2005), transfer RNA-intergenic spacer PCR (Supre et al., 2009), *gap* PCR-RFLP (Park et al., 2011) and DNA sequencing of housekeeping genes (Heikens et al., 2005). The latter technique has targeted several different genes, including *rpoB* (Drancourt and Raoult, 2002), *tuf* (Hwang et al., 2011), and *cpn60* (Kwok et al., 1999). Although accurate, gene sequencing can be time consuming and expensive, especially at times when multiple genes have to be sequenced to conclusively identify a species.

When using 16S rRNA gene sequencing, *S. agnetis* isolates were found to exhibit 99.7% identity to *S. hyicus* ATCC 11249^T and 99.1% identity to *S. chromogenes* ATCC 43764 (Taponen et al., 2012). Using the recommended cut-off value of 98.7% to differentiate a

species (Jousson et al., 2007), these results would be inconclusive. When using *rpoB* gene sequencing with a cut-off of $\geq 97\%$ for identification of these species, *S. agnetis* can be differentiated from the swine origin type strain of *S. hyicus* (ATCC 11249^T) as these isolates only exhibit 93.5% identity. However, the partial sequence of the *S. agnetis rpoB* genes are 99% similar to some bovine-origin *S. hyicus* strains, e.g. strain KSOS1-02, a *S. hyicus* isolated from bovine milk, thus leading to further confusion in making a definitive diagnosis. It is unknown if the *rpoB* gene of *S. hyicus* isolated from the bovine is different from that of swine origin or if there is an error in the database, which has been previously reported (Woo et al., 2009). The *tuf* gene can be used to differentiate *S. agnetis* and *S. hyicus* when following the recommended cut-off values of greater $\geq 98.0\%$ identity with $> 0.8\%$ separation between species (Hwang et al., 2011). However, if this gene is not the primary gene used to speciate staphylococcal isolates, several gene sequencing reactions may need to be performed before arriving at a definitive identification. Hence, a rapid, accurate, and inexpensive method to differentiate coagulase positive staphylococci isolated from bovine milk would improve diagnostic accuracy and allow a true understanding of the epidemiology of *S. hyicus*, *S. agnetis*, and *S. aureus* on dairy farms.

The authors recently published whole genome sequences for *S. hyicus* (Calcutt et al., 2015), *S. agnetis* (Calcutt et al., 2014), and *S. chromogenes* (Fry et al., 2014a).

Examination of these whole genome sequences identified unique gene sequences that were postulated to be good diagnostic targets for differentiating *S. hyicus* from *S. agnetis* isolated from bovine milk using a multiplex PCR platform. Hence, the objectives of this study were to: 1) develop a simple multiplex PCR-based assay to differentiate *S. hyicus*,

S. agnetis and *S. aureus*, and 2) use this assay along with pulsed-field gel electrophoresis strain-typing to characterize a collection of non-*S. aureus* coagulase positive staphylococci isolated from various bovine origin samples from 15 dairy farms in Washington and Idaho. A further objective was to compare the total number of *S. aureus*, *S. agnetis*, and *S. hyicus* isolates identified from each farm.

MATERIALS AND METHODS

Isolates and phenotypic characterization

Banked coagulase positive, non-*S. aureus* staphylococcal isolates (n = 62) collected from whole herd milk samplings and milk samples of cows and heifers at calving on 15 dairy farms in Washington and Idaho as part of a previous study (Middleton et al., 2002b) to evaluate different cattle importation practices on the prevalence of *S. aureus* IMI were utilized for the present study. In the original study, all lactating cattle had composite mammary foremilk samples (aliquots of milk from all four mammary quarters comingled in a single sample vial) aseptically collected at approximately 4-monthly intervals over a one year period. Additional composite foremilk samples were taken when cattle were purchased and from cows and heifers at the time of calving. During sample processing to identify cows with *S. aureus* IMI, all coagulase positive staphylococci were stored in phosphate buffered glycerol at -20°C. All coagulase positive isolates were then identified to the species-level using a commercial biochemical test strip (API STAPH, bioMerieux, Inc., Hazelwood, MO, USA). Isolates were either identified as *S. aureus* or *S. hyicus* by

this method. The focus of the present study was the isolates originally identified as coagulase positive *S. hyicus*.

Isolates were sub-cultured from the phosphate buffered glycerol storage media onto brain heart infusion agar slants (Remel, Lenexa, KS, USA) at Washington State University and shipped chilled to the University of Missouri for further characterization. At the University of Missouri, isolates were grown on Columbia Blood Agar (CBA) (Remel, Lenexa, KS, USA) for approximately 24 h at 37°C and representative colonies of each isolate were coagulase tested using rabbit plasma (Thermo Scientific, Lenexa, KS, USA). Coagulase results were recorded after 4 hours and 24 hours of incubation at 37°C.

Confirmation of species identification by housekeeping gene sequence analysis

A single colony was picked from CBA and inoculated into 100 µl of 1X Tris-EDTA (Promega, Madison, WI, USA) for lysate preparation. Standard PCR was performed to amplify *rpoB* (Drancourt and Raoult, 2002) and *tuf* (Hwang et al., 2011) genes. The PCR for *rpoB* gene identification and subsequent sequencing for species identification was done on all isolates and the PCR for *tuf* gene identification and subsequent sequencing was only conducted on isolates determined to be either *S. agnetis* or *S. hyicus*. The resultant PCR products were purified using a commercial kit (Invitrogen, Carlsbad, CA, USA) and submitted to the University of Missouri DNA Core Facility for Sanger sequencing. Sequences were compared to the GenBank database using the publicly available nucleotide-BLAST algorithm (www.ncbi.nlm.nih.gov). For *rpoB* analysis,

species identity was confirmed if the sequence had $\geq 97\%$ identity with a single species of *Staphylococcus* in the GenBank database (Mellmann et al., 2006). For *tuf* gene analysis, species were confirmed if sequence identity was $\geq 98.0\%$ with $> 0.8\%$ separation between species (Hwang et al., 2011). Once species were identified, the total numbers of each CPS species identified from herd cultures and cattle at the time of calving or purchase on each farm were determined.

Multiplex PCR development

A novel multiplex PCR was developed to differentiate *S. hyicus* from *S. agnetis* by comparing the whole genome sequences for *S. agnetis* CBMRN 20813338 (Calcutt et al., 2014) and *S. hyicus* ATCC 11249^T (Calcutt et al., 2015). The Artemis Comparison Tool (Wellcome Trust Sanger Institute, Hinxton, UK) was used to identify regions of difference between the two genomes. Based on these genome-wide comparisons, primer sets were designed to amplify a partial segment of the *aroD* gene for *S. hyicus* and *S. agnetis* (Table 1). Standard multiplex PCR, including sets of *aroD* gene primers to identify *S. hyicus* and *S. agnetis*, respectively, and a *nuc* gene primer set to identify *S. aureus* (Graber et al., 2007), was performed using the following thermocycler conditions; 94°C for 15 minutes followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 minute. The run was completed at 72°C for 5 minutes and then held at 4°C. After amplification, electrophoresis was completed using a 1% agarose gel (Gold Biotechnology, Olivette, MO, USA) and visualized by UV trans-illumination. A 100 bp ladder was used to determine PCR product size.

Testing the specificity of the novel multiplex PCR assay to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*

The isolates obtained from Washington State University (test isolates) and identified as *S. agnetis* or *S. hyicus* by housekeeping gene sequence were analyzed using the novel multiplex PCR described above. Positive controls included *S. agnetis* CBMRN 20813338, *S. hyicus* ATCC 11249^T, and *S. aureus* ATCC 29740. Additional *S. agnetis* strains were also included as controls including the type strain DSM 23656^T (6 VT), the reference strain DSM 23658 (55 OE), and two strains (43 OE and 100 VT) reported in the original description of *S. agnetis* (Taponen et al., 2012). Given that *S. chromogenes* is genotypically similar to both *S. hyicus* and *S. agnetis*, two strains of *S. chromogenes* (strain ATCC 43764^T and MU 970) were tested to ensure that the multiplex PCR did not misclassify *S. chromogenes* as *S. agnetis*, *S. hyicus*, or *S. aureus*. All PCR products that amplified with the *aroD* gene primer sets were purified and sequenced to verify amplification of the targeted gene and to compare sequences.

Accession numbers

The *aroD* gene fragment sequences from the isolates included in the study were submitted to NCBI GenBank, Accession numbers KT750097-KT750140.

Pulsed-field gel electrophoresis

To understand species variation within the tested isolates, pulsed-field gel electrophoresis was performed as previously described (Middleton et al., 2002a). Briefly, *Sma*I (Invitrogen, Carlsbad, CA, USA) digests of staphylococcal DNA were separated by PFGE in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, Hercules, CA) immersed in 0.5% TBE (TRIS, Boric Acid, EDTA) buffer at 15°C for 20 hours at 6V/cm with a 5-50 s pulse time on a CHEF DRIII PFGE machine (BioRad, Hercules, CA, USA). A lambda ladder molecular weight size standard (BioRad, Hercules, CA, USA) was run in the first and last lane of each gel. Completed gels were stained with ethidium bromide and photographed using UV trans-illumination. For interpretation of PFGE results, isolates that had indistinguishable banding patterns based on having the same number of bands and corresponding bands of the same apparent molecular weight were considered to be the same strain (Tenover et al., 1995). A unique strain name was assigned to each PFGE profile that was identified.

Data analysis

All data were descriptive with isolate species and strain type information expressed as absolute numbers and percentages. *S. aureus* prevalence data from the previous study were used to compare the relative importance of the coagulase positive, non-*S. aureus* staphylococci among the CPS IMIs in each herd. Sequenced products were aligned and compared through the construction of a neighbor joining dendrogram based on the multiple alignment similarity matrix using computer software, (Bionumerics, version 7.5, Applied Maths, Kortrijk, Belgium).

RESULTS

A total of 62 isolates that had been previously identified as coagulase positive *S. hyicus* based on coagulase testing and API STAPH (Middleton et al., 2002b) were studied.

Based on *rpoB* gene sequencing, 18 of the isolates were found to be *Staphylococcus* spp. other than *S. hyicus* or *S. agnetis*, including *S. chromogenes* (n = 8), *S. aureus* (n = 5), *Staphylococcus hominis* (n = 2), *Staphylococcus epidermidis* (n = 1), *Staphylococcus simulans* (n = 1), and *Staphylococcus xylosus* (n = 1) leaving 44 isolates for further study.

A total of 43 of the 44 isolates were found to have *rpoB* gene sequences that were 99% homologous with both *S. agnetis* and *S. hyicus*, and one isolate was found to have 99.8% sequence homology to *S. hyicus* and 93.5% sequence homology to *S. agnetis*. Based on the aforementioned definitions for species identification, 43 of the isolates could not be definitively speciated using *rpoB* gene sequence analysis and one isolate was determined to be *S. hyicus*. Using *tuf* gene sequencing, all 44 isolates were identified to the species-level with 43 of the isolates being identified as *S. agnetis* and 1 isolate, the same isolate identified by *rpoB* gene sequence, being identified as *S. hyicus*. Coagulase tests on the 44 test isolates revealed that 42 of the 44 isolates were coagulase positive. Two isolates were found to be coagulase negative, including one *S. agnetis* isolate and the single isolate identified as *S. hyicus*. A total of 86% (37/43) of the *S. agnetis* isolates were found to be coagulase positive at 4 h. The remaining *S. agnetis* isolates (12%; 5/43) exhibited a delayed coagulation and were found to be coagulase negative at 4 h and positive at 24 h.

The *tuf* gene sequencing results were used as the definitive identification method against which the newly designed multiplex PCR was tested. All 44 *S. agnetis* test isolates and the four *S. agnetis* control isolates each produced a single ~293 bp band on gel electrophoresis following multiplex PCR (Figure 1), whereas the single isolate that was identified as *S. hyicus*, based on *rpoB* and *tuf* gene sequencing, and the *S. hyicus* type strain (ATCC 11249^T) each produced a single ~ 425 bp band. Sanger sequencing and DNA alignment of the *aroD* gene fragments found 99-100% homology of all isolates identified as *S. agnetis* (Figure 2). Sanger sequencing of the single *S. hyicus* test isolate *aroD* gene showed 99% homology with the *S. hyicus* type strain (ATCC 11249^T) *aroD* gene sequence. Alignment of the *S. agnetis* and *S. hyicus* gene fragments that were produced using the multiplex PCR showed that the fragments were only about 58% similar. No electrophoretic band was detectable when the two *S. chromogenes* control isolates were tested using the multiplex PCR.

Overall, all 15 farms had at least one cow with a CPS IMI (Table 2). Of these farms, 9 had at least some cows that were infected with *S. aureus* and some cows infected with coagulase positive *S. agnetis*. On 5 farms, no *S. agnetis* was isolated and on 1 farm (Farm #4) the only recorded CPS infection was with *S. agnetis*. On all farms (n = 9) where *S. aureus* and *S. agnetis* IMI were recorded, *S. aureus* IMI was more prevalent among the CPS IMI than *S. agnetis*, comprising 57-98% of the CPS IMI. The prevalence of *S. agnetis* among the CPS IMI on each farm that recorded both *S. aureus* and *S. agnetis* IMI ranged from 2-43% of the cases. Over half of the farms (53%; 8/15)

had > 20% of the CPS IMI being *S. agnetis*. The only *S. hyicus* isolate was from Farm #2.

Pulsed-field gel electrophoresis was completed on all 44 non-*S. aureus* staphylococcal isolates, which included the single coagulase negative *S. agnetis* isolate and the *S. hyicus* isolate. Two of the coagulase positive *S. agnetis* isolates were not typable and produced consistent smearing of the electrophoretic bands. Among the 42 typable isolates, 24 different PFGE banding patterns were identified. Overall, 18 strains were only identified once and six were identified more than one time (Table 3). One of the strains found only once was the *S. hyicus* isolate, which had a different banding pattern from all of the *S. agnetis* isolates. Additionally, one of the strains only identified once was the coagulase negative *S. agnetis*. Of the 6 strain types that were identified more than once, all 6 were identified from more than one cow within the herd. Also, 3 strains were associated with persistent infections, with each of the 3 strains being isolated from at least one cow at more than one time point. The time between samplings and isolation of the same strain ranged from 45- 264 d.

DISCUSSION

To the authors' knowledge, this is the first study to characterize a collection of coagulase positive *S. agnetis* isolates from dairies in the U.S., and to develop a simple method to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*. In the original description of *S. agnetis*, 13 isolates originating from 13 cows on 8 dairies in southern Finland were characterized,

including 12 isolates from bovine subclinical or mild clinical mastitis and one isolate from a teat apex (Taponen et al., 2012). Of those original 13 *S. agnetis* isolates, 3 were found to have a delayed coagulase positive reaction and the remaining 10 were coagulase negative. The delayed coagulase reaction of the previously reported *S. agnetis* isolates (Taponen et al., 2012) matched the coagulase description originally given to *S. hyicus* (Kloos, 1986). In contrast, in the present study most (86%) of the isolates were tube coagulase positive at 4 hours. The isolates used in the present study originated from a study in which the primary goal was to identify *S. aureus* (Middleton et al., 2002b), but the study protocol included isolation and storage of all coagulase positive staphylococci during the initial screening of all milk samples. Coagulase negative staphylococci were not saved for further characterization, and thus could not be evaluated in the present study. In the original study, the banked CPS isolates were speciated using biochemical methods and only those isolates identified as *S. aureus* were utilized for further study at the time.

The data presented here demonstrates that coagulase positive *S. agnetis* can be prevalent among CPS IMI in some herds and could be confused with *S. aureus* especially since the majority of isolates clotted rabbit plasma after 4 h of incubation similar to *S. aureus* and different from the delayed coagulation typically described for *S. hyicus* (Kloos, 1986, Taponen et al., 2012). Misclassification could impact management decisions such as unnecessary culling based on the belief that the cow has *S. aureus* and is a contagious reservoir for infection of other cows.

Based on the original phenotypic speciation of the isolates used in this study, 18 isolates were originally misclassified as *S. hyicus*. When genotypic speciation was performed, the 18 isolates were found to be staphylococcal species other than *S. hyicus* or *S. agnetis*. Some of this error could be due to storage error or loss of the initial organism during the recovery and sub-culturing of the isolates. Some of the error is also likely due to misclassification reported to occur with phenotypic speciation, as such methods can lead to a higher number of unidentified or misidentified staphylococcal isolates (Sampimon et al., 2009b). When using phenotypic methods, *S. chromogenes* can be misidentified as *S. hyicus* (Bes et al., 2000), as was seen with 8/18 (44%) of the misclassified isolates in this study. Although phenotypic methods have been shown to be inaccurate for non-*S. aureus* staphylococcal species (Park et al., 2011), previous reports have shown the API system to be satisfactory in the identification of *S. aureus* (Jasper et al., 1985). Therefore, in this study, the *S. aureus* isolate identities were not further confirmed using genotypic methods.

The *aroD* gene encodes for 3-dehydroquinate dehydratase, an enzyme involved in the shikimate pathway used to synthesize chorismic acid, a central precursor for aromatic compounds (Servos et al., 1991). This is a housekeeping function for the cell and therefore the gene should be highly conserved. Selection of a highly conserved gene as a target for speciation is important to allow for broad applicability and consistency in results. Based on the gene sequence analyses reported herein, the *aroD* gene fragment used for the multiplex PCR appears to be highly conserved with over 99% homology found within all *S. agnetis* isolates studied, including isolates from 10 different U.S.

farms that were comprised of 23 different PFGE strains types as well as four isolates from Finland.

Because *S. hyicus* was rarely found in this study, we are unable to determine how conserved the *aroD* gene is in this species. However, when the single study isolate was compared to a type strain isolate of swine origin, it was found to have 99% sequence homology, suggesting that a conserved region was selected. It should be further noted that the only *S. hyicus* isolate harvested from cow's milk was not coagulase positive, but it was included in the analyses to demonstrate that the multiplex PCR could differentiate a field *S. hyicus* strain from *S. agnetis* and *S. aureus*. Further, it was included in the PFGE analyses to demonstrate that the electrophoretic banding pattern was different from all of the *S. agnetis* isolates. Similarly, the coagulase negative *S. agnetis* was included in the PFGE analysis to demonstrate that it was genotypically different from the coagulase positive variants. Note that the data in Table 2 does not include the coagulase negative *S. agnetis* isolate or the *S. hyicus* isolate.

Overall, assessment of CPS organisms identified on the studied farms found that *S. agnetis* can make up a sizable percentage of all CPS species found in some herds, with over 20% of the CPS species being identified as *S. agnetis* on over half the farms. Furthermore, based on PFGE, some strains of *S. agnetis* appear to act as contagious strains based on the fact that the same PFGE strain type was isolated from the milk of multiple animals on a single farm. Also, some strains appear to cause a persistent

infection, lasting over 150 days in 3 cows. Cow-level somatic cell count and milk yield data were not collected in the original study and thus it cannot be determined whether the reported strains have an important impact on udder health. The potential for contagious spread does raise concern that some strains of coagulase positive *S. agnetis* may be important udder pathogens in some herds, but further studies would be needed to confirm this possibility.

The multiplex PCR described herein, provides an inexpensive, accurate method to differentiate three species of staphylococci that can be difficult to differentiate based on phenotype alone. Further, preliminary evidence is provided that suggests the multiplex PCR has promise for differentiating *S. chromogenes* from coagulase negative variants of *S. hyicus* and *S. agnetis*.

One limitation of this study was the lack of a comparable number of *S. hyicus* field isolates, and the lack of a coagulase positive *S. hyicus* as a comparator for coagulase positive *S. agnetis* in the PCR test. This may be due to *S. hyicus*, and in particular coagulase positive *S. hyicus*, truly being a rare species on dairy farms or just rare on the farms within this study. Several other recent studies, in which speciation was done using genotypic methods, have found the prevalence of coagulase negative *S. hyicus* to be very low, ranging from 0.5% to 1.3% (De Visscher et al., 2016, Taponen et al., 2016). The data could also be biased because the original protocol for isolate storage only included coagulase positive isolates. Thus, the prevalence and importance of coagulase negative

S. hyicus and *S. agnetis* among staphylococcal IMI on these farms cannot be evaluated.

The original misclassification of the two coagulase negative isolates as coagulase positive (n = 1 *S. hyicus* and n = 1 *S. agnetis*) may be due to the banked isolate not being the originally recorded CPS isolate or differences in interpretation of a weak or delayed coagulase test result.

In conclusion, within these herds, *S. agnetis* was much more prevalent than *S. hyicus*.

Based on PFGE, some strains of *S. agnetis* were shown to potentially have contagious behavior and cause persistent infections. The use of the described multiplex PCR allows inexpensive and efficient differentiation of the common species of CPS isolated from milk, and preliminary evidence suggests it may also potentially aid in differentiating CNS variants of *S. agnetis* and *S. hyicus* from each other and from *S. chromogenes*.

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Table 1. Primers used for the multiplex PCR to speciate and differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*

<i>Gene</i> ¹	<i>Primer</i>	<i>Sequence 5'-3'</i>	<i>Amplicon size (bp)</i>	<i>Positive species</i>
<i>aroD</i>	aroD-HyF	TAT GGT GTC GAC	425	<i>S. hyicus</i>
		CAA TCG AAG GCT		
<i>aroD</i>	aroD-HyR	ACC CTA TAG CCC	293	<i>S. agnetis</i>
		GCT TAC TT		
<i>aroD</i>	aroD-AgF	CGC ATG AGA GAC	664	<i>S. aureus</i>
		CAA TAC GCT		
<i>aroD</i>	aroD-AgR	TAG GAC GTA TAG		
		AGG TGG		
<i>nuc</i> ²	nuc-S	CTG GCA TAT GTA		
		TGG CAA TTG TT		
<i>nuc</i> ²	nuc-AS	TAT TGA CCT GAA		
		TCA GCG TTG TCT		

¹ *aroD* = 3-dehydroquinase dehydratase gene; *nuc* = thermonuclease gene

²Graber et al. (2007)

Table 2. Total number and percentage of coagulase positive *Staphylococcus* (CPS) isolates identified within each herd from whole herd milk samplings and milk samples collected at calving or purchase. The CPS isolates were characterized as *S. aureus*, *S. agnetis*, or *S. hyicus* based on *rpoB* and *tuf* sequencing and multiplex PCR. No CPS *S. hyicus* were identified in this study.

Herd	Number of CPS isolates	<i>S. aureus</i> ^a	<i>S. agnetis</i>
1	25	18 (72%)	7 (28%)
2	23	16 (70%)	7 (30%)
4	1	0	1 (100%)
5	7	4 (57%)	3 (43%)
6	1	1 (100%)	0
7	14	14 (100%)	0
8	17	13 (76%)	4 (24%)
9 ^b	10	9 (90%)	0
10	115	113 (98%)	2 (2%)
11	10	8 (80%)	2 (20%)
12 ^b	20	13 (65%)	5 (25%)
13	24	24 (100%)	0
14	40	40 (100%)	0
15	32	22 (69%)	10 (31%)
16	51	50 (98%)	1 (2%)
Total	351	309 (88%)	42 (12%)

^a Total number and percentage of *S. aureus* isolates per farm are slightly different from the original data (31) because 5 isolates from farms 1 (n=1), 14 (n=3), and 15 (n=1) that were originally identified as *S. hyicus* using the biochemical test strips (API Staph) were identified as *S. aureus* based on housekeeping gene sequence. These data were thus added to the original *S. aureus* totals for the farms of origin.

^b Three non-*S. aureus* staphylococcal isolates were not recovered from storage media and were therefore missing from the dataset. One isolate from Herd 9 and 2 isolates from Herd 12 were not recovered.

Table 3. Summary of pulsed-field gel electrophoresis results for all strains identified more than once on a farm including number of cows infected with each strain and number of cows identified with the strains at multiple sampling points.

Herd number ^a	Total # of <i>S.</i> <i>agnetis</i> isolates within herd	Strain ID	# of isolates within strain (% of total <i>S. agnetis</i> isolates within herd)	# of cows with strain	# of cows with the strain at multiple time points	Days between sample collections
1	8	A	2 (25%)	2	-	-
5	3	B	2 (67%)	2	-	-
8	4	C	3 (75%)	2	1	177
11	2	D	2 (100%)	2	-	-
12	5	E	5 (100%)	4	1	264
15	10	F	10 (100%)	6	3	45, 47, and 153, respectively

^a Herds were only included in this table if a strain was identified more than one time. If a herd is not listed, all strains within that herd were unique based on a PFGE electrophoretic pattern with at least one band difference.

Figure 1. Multiplex PCR amplification profiles. The M lanes contain a 100 bp molecular size ladder marker. Lane 1 contains *S. chromogenes* ATCC 43764^T; Lane 2 contains *S. chromogenes* MU 970; Lane 3 contains coagulase negative *S. agnetis* DSM 23656^T; Lane 4 contains coagulase positive *S. agnetis* 43OE; Lane 5 contains *S. hyicus* ATCC 11249^T; Lane 6. *S. aureus* ATCC 29740. The N lane contains a negative control (no DNA included in reaction mix).

Figure 2. Comparison and alignment of *aroD* gene sequences from all *S. agnetis* isolates. The dendrogram shows that all isolates were at least 99.25% homologous. The dendrogram includes the *S. agnetis* type strain (DSM 23656^T (6VT)), the reference strain DSM 23658 (55 OE), and two strains (100 VT and 43 OE) originally isolated from cows in Finland (Taponen et al, 2012). A consensus sequence is at the top of the figure, with all other isolates below. All nucleotide bases in agreement with the consensus sequence are noted by a vertical bar (|) and any single nucleotide changes are noted by the presence of the variant nucleotide base present within that column.

CHAPTER FIVE

COMPARISON OF VIRULENCE GENE IDENTIFICATION, RIBOSOMAL SPACER PCR, AND PULSED FIELD GEL ELECTROPHORESIS FOR STRAIN-TYPING *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CASES OF SUBCLINICAL BOVINE MASTITIS IN THE USA

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Running Head: Strain-Typing *S. aureus* of Bovine Origin

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ABSTRACT

Staphylococcus aureus is one of the most important pathogens causing contagious mastitis in dairy cattle worldwide. The objectives of this study were to determine if the recently described *Staphylococcus aureus* Genotype B was present among previously characterized isolates from cases of bovine intramammary infection in the USA and to compare pulsed-field gel electrophoresis (PFGE) to the combination of ribosomal spacer PCR (RS-PCR) and virulence gene identification for strain-typing of *S. aureus*. The hypothesis was that isolates that were previously characterized as contagious would be identified as Genotype B and that the two strain-typing methods would be comparable. Isolates were selected from a collection of *S. aureus* isolates from eight dairy farms. Mammary quarter milk somatic cell count (SCC) and *N*-acetyl- β -D-glucosaminidase (NAGase) activity were known and used to evaluate strain pathogenicity. RS-PCR with conventional gel electrophoresis was performed and PCR was used for toxin gene identification. RS-PCR patterns were associated with a specific virulence gene pattern, as previously reported. Five RS-PCR banding patterns were identified. None of the isolates were characterized as Genotype B. No association between RS-PCR types and milk SCC was found, however milk NAGase activity was significantly higher in milk from mammary glands infected with RSP-type-1 than those infected with RSP-type-2. The discriminatory powers were 1.0 and 0.46 for PFGE and RS-PCR, respectively. These data suggest that Genotype B may have a limited geographic distribution and PFGE is more discriminatory than RS-PCR performed with conventional gel electrophoresis for strain-typing *S. aureus* isolates of bovine origin.

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens causing contagious mastitis in dairy cattle worldwide (Piepers et al., 2007, USDA-APHIS, 2008, Abera et al., 2012). Although *S. aureus* is considered a contagious intramammary pathogen and spread from cow-to-cow during milking, strains of this agent are most often farm specific and there is generally a predominant strain in a herd (Joo et al., 2001). Several methods have been used to characterize and determine genetic subtypes or strain-types of this pathogen, allowing identification of the predominant contagious strains. Methods commonly used to strain-type *S. aureus* include pulsed-field gel electrophoresis (PFGE) (Middleton et al., 2002a), *spa*-typing (Shopsin et al., 1999), and multilocus sequence typing (MLST) (Smith et al., 2005). Although labor intensive, PFGE is considered the gold standard strain-typing method for this genus and species (Bannerman et al., 1995). Currently, an accurate, fast, and inexpensive tool enabling one to determine which strains are important and likely to be a significant problem for a farm is not readily available.

Recent studies from Switzerland used ribosomal spacer (RS)-PCR to characterize *S. aureus* isolated from bovine milk samples and found various strain types, classified as genotypes, of *S. aureus* possessing different virulence and pathogenicity factors (Fournier et al., 2008, Boss et al., 2011, Syring et al., 2012). Several genotypes were identified, with Genotype B (GTB) and Genotype C (GTC) being identified commonly, while other genotypes (GTOG) were found rarely (Fournier et al., 2008). Overall, Genotype B isolates were found to be highly contagious, as GTB was the sole strain-type observed in herds having significant *S. aureus* problems where prevalence of *S. aureus*

intramammary infection (IMI) ranged from 65% -87% of the herd (Fournier et al., 2008, Graber et al., 2009). Genotype B isolates were also considered to have increased pathogenicity, as SCC for this type was significantly higher than for GTC or GTOG. Further, GTC and GTOG only caused infections in one to three cows per farm and were therefore not considered very contagious (Fournier et al., 2008, Graber et al., 2009). It was also shown that there was an association between RS-PCR genotype and virulence gene patterns. Specifically, GTB was characterized by the presence of *S. aureus* enterotoxin A (*sea*) and D (*sed*), and by a polymorphism within the leukotoxin E gene (*lukE*) caused by a point mutation, classified as *lukEB* (Fournier et al., 2008).

Ribosomal spacer PCR has been described as a technique used to rapidly identify bacteria and, in the case of *Staphylococcus* species, has been found to show significant intraspecies variation (Jensen et al., 1993). The method is completed by using a set of primers to amplify the spacer regions between the 16S and 23S genes of the rRNA genetic loci. The spacer region exhibits a large degree of sequence and length variations at the level of genus and species. Also, within a single genome, there frequently are multiple rRNA genetic loci, and regions found within these loci also show variations in length and sequence. This diversity is due, in part, to variations in the number and type of tRNA sequences found within the spacers (Brosius et al., 1981, Loughney et al., 1982). Ribosomal spacer PCR has previously been compared to PFGE (Kumari et al., 1997), and, when applied to the investigation of a methicillin resistant *S. aureus* outbreak in humans, RS-PCR was found to be as discriminatory as PFGE. However, it was noted that RS-PCR failed to distinguish isolates with minor genetic differences. Research

comparing RS-PCR and PFGE to characterize *S. aureus* isolated from bovine intramammary infections (IMI) is limited, including one study done on a group of *S. aureus* isolates collected from Holstein-Friesian cows from Northern Italy that found PFGE to have a slightly higher discriminatory value than RS-PCR, 0.8943 and 0.8236, respectively (Cremonesi et al., 2015). However, in that study, a miniature electrophoresis system was used, rather than traditional gel electrophoresis. Therefore, the objectives of this study were to determine if *S. aureus* GTB was present among previously characterized isolates from cases of bovine IMI and to compare pulsed-field gel electrophoresis (PFGE) to the combination of ribosomal spacer PCR (RS-PCR) using conventional gel electrophoresis and virulence gene identification for strain-typing *S. aureus*. RS-PCR with conventional gel electrophoresis was chosen over the method described by Fournier et al. (Fournier et al., 2008) because the laboratory equipment needed for this technique is available in many diagnostic laboratories around the world, making the method less expensive and more universally available to dairy farmers and researchers. The hypothesis was that isolates that were previously characterized as contagious would be identified as Genotype B and that the two strain-typing methods would be comparable.

MATERIALS AND METHODS

Isolates were selected from a cryopreserved collection of *S. aureus* strains isolated from the milk of cows on eight dairy farms in the USA (Middleton et al., 2002a). These isolates were used because of the large amount of information that had previously been collected pertaining to these specific isolates, including strain-type based on PFGE,

prevalence of each strain within herd, and mean milk SCC and *N*-acetyl- β -D-glucosaminidase (NAGase) activity in mammary quarters infected with each strain. The isolate collection included 35 PFGE strain-types of *S. aureus*, some of which were isolated from multiple cows within a herd while others were isolated from only one cow. At least one isolate within each PFGE strain-type was selected for characterization. If more than one cow was affected with a single PFGE strain, up to three representative isolates were randomly selected from each farm within a given strain-type. Selected isolates were sub-cultured onto brain heart infusion agar slants (Remel, Lenexa, KS, USA) at Washington State University and shipped chilled to the University of Missouri for further characterization.

At the University of Missouri, isolates were grown on Columbia Blood Agar (CBA) (Remel, Lenexa, KS, USA) and confirmed to be coagulase positive staphylococci based on colony morphology, a positive catalase test, and a positive coagulase test (Remel, Lenexa, KS, USA). Genomic DNA was extracted using DNAeasy Tissue Kit (Qiagen, Valencia, CA, USA). Standard PCR was performed to identify the presence or absence of the *nuc* gene in order to confirm the isolates as *S. aureus* (Brakstad et al., 1992). Standard PCR was also used to confirm the presence or absence of the leukotoxin E (*lukE*) gene and enterotoxin genes, including *sea*, *sed*, *seg*, *sei*, and *sej*. All primers and PCR running conditions were used as previously described (Fournier et al., 2008, Boss et al., 2011). For strain-typing by RS-PCR, previously described methods were used (Jensen et al., 1993). DNA from known GTB isolates were used as positive controls for both standard PCR and RS-PCR, including *S. aureus* 54365/2, 133783/58, 187173/86, 175994/259, and

170649/92 (Cremonesi et al., 2015). All RS-PCR DNA products were separated using 1% agarose gel electrophoresis and bands were visualized and photographed with UV transillumination.

For interpretation of the RS-PCR results, two patterns were considered different when one or more band difference was noted. A unique name (RSP-type) was given to each RS-PCR banding pattern that was identified. For interpretation of PFGE results, isolates that had indistinguishable banding patterns, based on having the same number of bands and corresponding bands of the same apparent molecular weight, were considered to be the same strain (Tenover et al., 1995). A unique strain name was given to each PFGE strain that was identified.

In order to compare the discriminatory power of these two methods, an index of discrimination based on Simpson's index of diversity was used according to the formula,

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s x_j(x_j - 1), \text{ where } N \text{ is the total number of strains in the sample}$$

population, s is the total number of types described, and x_j is the number of strains belonging to the j th type (Hunter and Gaston, 1988). To make this calculation, one isolate from each farm was used ($n = 8$), as the calculation is based on the ability to discriminate between epidemiologically unrelated isolates. Isolates were selected from each farm for the calculation using Microsoft Excel (2013) random number generator.

Toxin gene prevalence, genotype, and PFGE strain-type data were expressed as absolute numbers and percentages. For all statistical analyses, a commercially available software package was used (Sigmaplot 13.0, SyStat Software, Inc., San Jose, CA, USA).

Prevalence data were compared using the Chi-square or Fisher's Exact test as appropriate. For the purposes of statistical analysis of SCC, the linear score somatic cell count (LSCC) was used to achieve normality. A one-way analysis of variance (ANOVA) for normally distributed data (LSCC) and a Kruskal-Wallis ANOVA on ranks (NAGase), respectively, were used to compare differences in SCC and NAGase between RS-PCR genotypes. Significance was declared at $P < 0.05$.

RESULTS

Overall, staphylococcal enterotoxin (SET) genes were uncommonly identified among these isolates, with 82% (53/65) of the isolates testing negative for all five of the tested SET genes (Table 1). The most common toxin genes identified were *lukE* (100%) and *sei* (10.7%; 7/65). Enterotoxin genes *sed* and *sej* were not identified in any of the isolates. The *seg* gene was only identified in isolates from Farm 2, while the *sea* gene was only detected in isolates from Farms 5, 6, and 7. Only 12/65 (18.5%) isolates were found to have more than one toxin gene, with five isolates being positive for *lukE* and *sea*, four isolates being positive for *lukE* and *sei*, and three isolates being positive for *lukE*, *sei*, and *seg*. None of the isolates had the enterotoxin gene profile (*sed*, *sej*, and *sea*) commonly found among GTB isolates (Fournier et al., 2008).

Based on RS-PCR banding patterns, isolates were characterized into five different RSP-types. (Figure 1). All identified RSP-types were found to have consistent toxin profiles. RSP-type-1 was identified on the majority of farms (6/8) and all isolates were only positive for *lukE*. RSP-type-2 was found on 3 different farms and had the toxin profile of *lukE* and *sea*. RSP-type-3 was found on three different farms and had the toxin profile of *lukE* and *sei*. RSP-type-4 was identified three times and all three isolates were from the same farm and had identical toxin profiles, including *lukE*, *sei*, and *seg*. RSP-type-5 was also only found on one farm, farm 3, and all isolates were only positive for *lukE*, which is the same toxin profile as RSP-type-1. The majority of isolates were characterized as RSP-type-1 (50/65; 76.9%). The prevalence of RSP-types 2, 3, 4, and 5 did not differ from each other ($P = 0.68$). The prevalence of RSP-type-1 was significantly higher than all other RSP-types ($P < 0.01$). Based on RS-PCR banding patterns and toxin gene profiles, none of the isolates were characterized as GTB (Fournier et al., 2008). Milk somatic cell count was not statistically significantly different between the different RSP-types ($P \geq 0.08$; Table 1). Milk NAGase activity was significantly higher for RSP-type-1 when compared to RSP-type-2 ($P = 0.03$).

The discriminatory power of PFGE was 1.0 and for RS-PCR it was 0.46. A total of 35 different strain-types were identified based on PFGE, as compared to five with RS-PCR (Table 2). With PFGE, 4/35 (11.4%) strains were identified on more than one farm. With RS-PCR, 3/5 (60%) RSP-types were found on more than one farm and this included the predominant type, RSP-type-1.

DISCUSSION

In this study, no isolates were characterized as the previously described GTB. Overall, very few RS-PCR patterns were identified with the majority of isolates being categorized as RSP-type-1, and RSP-type-1 was found in 75% of the herds evaluated. The finding of dominant RSP-types is similar to previous reports that used RS-PCR and toxin profiles to strain-type isolates (Fournier et al., 2008). This finding is in contrast to other studies that used PFGE strain-typing to differentiate isolates and demonstrated that the majority of strain-types are unique to one herd (Joo et al., 2001), which is similar to the PFGE data in this study (Middleton et al., 2002a). Two RSP-types, RSP-type-4 and RSP-type-2, could be classified as a contagious strain based on the findings of this study. All three RSP-type-4 isolates were from the same farm, had the same toxin profile and same PFGE pattern, while all five RSP-type-2 isolates had the same toxin profile and same PFGE pattern, but were found on three different farms. Based on RS-PCR alone, RSP-type-1 would be considered highly contagious, being found very commonly on 6 of the 8 farms included in the study. However, when including PFGE data, some of the RSP-type-1 isolates would be considered contagious, such as the 63 isolates that were found to be PFGE strain-type 32 from farm 7. However, there are other PFGE strain-types that were characterized as RSP-type-1 that were only found once on a farm, such as strain types 34, 35, 37, and 38 that were also found on farm 7. Using RS-PCR alone would have grouped all of these isolates as one type, which could lead to the presumption of the majority of cows being infected with a highly contagious strain potentially leading to excessive culling of cattle. Pulsed-field gel electrophoresis, while more time-consuming to perform, provided greater discrimination among strains and thus the potential for culling

cattle with sporadic (non-host-adapted or less contagious) strain-types could be prevented.

Unlike previous reports, this study was unable to find an association between RSP-types and strain pathogenicity as measured by milk SCC. However, NAGase activity was significantly higher in mammary glands infected with RSP-type-1 than those infected with RSP-type-2. Milk NAGase activity is an indicator of tissue damage, as it is released into the milk from mammary epithelial cells and, to a lesser extent, from milk somatic cells (Fox et al., 1988, Kitchen et al., 1978), and SCC is a measure of mammary gland inflammation. Previous reports have indicated that GTB is not only highly contagious but also associated with increased pathogenicity based on SCC being significantly higher than for GTC or GTOG (Fournier et al., 2008). Milk from glands infected with GTB isolates have been found to have a geometric mean somatic cell count of 7.85×10^5 cells/ml (Fournier et al., 2008), corresponding to a LSCC of 6.1, which is lower than the median LSCC for all RSP-types found in this study except RSP-type-2 and RSP-type-3. Milk NAGase activity was not assessed in previous studies using RS-PCR, but did provide an indication of differences in pathogenicity between strain-types in the present study.

Very few enterotoxin genes were identified in this study, which is similar to two previous studies conducted in the USA (Oliveira et al., 2011, Haran et al., 2012) and contrary to a third study (Srinivasan and Oliver, 2012). Previous work has shown that *sea* negative

results were observed in 100% of non-GTB strains and *sea* positive results were observed in 77% of GTB isolates (Fournier et al., 2008). Given that the detection of the gene encoding for SEA is an important determinant for identifying GTB and this gene was not detected in three prior studies from different geographical locations in the USA and at a low prevalence in the present study, suggests that GTB is not prevalent on U.S. farms. Other studies have found *sec* to be the most commonly identified enterotoxin gene in the northern USA (Oliveira et al., 2011, Haran et al., 2012). In contrast, a study conducted in southern USA found *sem* and *sen* to be the most common enterotoxin genes identified (Srinivasan and Oliver, 2012). These specific enterotoxin genes were not tested for in this study due to a lack of association with the previously described GTB (Fournier et al., 2008). Studies from other parts of the world have identified different prevalences of enterotoxin genes, suggesting possible geographic differences (Zschock et al., 2005, Silveira-Filho et al., 2014). Because of these differences, typing isolates by virulence gene identification in combination with RS-PCR likely does not represent a universally applicable rapid method for identifying contagious and pathogenic *S. aureus*.

There was not a sufficient number of enterotoxin producing strains in this study to test the association between the presence of toxin genes and RS-PCR types or PFGE strain-type. However, previous research done in the USA found no correlation between PFGE strain-types and enterotoxin profiles (Srinivasan and Oliver, 2012). Overall, the lack of detection of enterotoxin genes meant there was insufficient data to determine whether there was a statistically significant association between toxin gene presence and SCC or NAGase activity. Based on the previously published data not demonstrating a

statistically significant relationship between PFGE strain-type and SCC or NAGase activity (Middleton et al., 2002a), and placing that information in the context of these strains infrequently having toxin genes, suggests that the toxin genes screened for are likely not important determinants of udder inflammation for these strain-types. Other reports have found the presence of virulence genes, specifically *spa* and *sej*, to be risk factors for subclinical mastitis and suggest that strain characteristics and the expression of specific combinations of virulence factors could result in the development of subclinical mastitis (Zecconi et al., 2006). However, in that study all isolates harbored at least one enterotoxin gene and most had two or more, which is in contrast to the findings of this study.

The discriminatory power of PFGE for strain-typing *S. aureus* has been previously described, and ranges from 0.83 to 0.8887 (Ikawaty et al., 2009, Du et al., 2014, Silveira-Filho et al., 2014). When comparing several strain-typing methods to characterize *S. aureus* isolates of bovine origin, PFGE had the highest discriminatory power, followed by multilocus sequence typing (MLST), multiple locus variable number tandem repeat analysis (MLVA), and then *spa*-typing (Ikawaty et al., 2009). Furthermore, PCR-based DNA fingerprinting methods have been previously shown to cluster large groups of unrelated isolates together. For example, in one study, PCR-based DNA fingerprinting was used and found isolates from several herds in several distinct regions could be categorized as the same type with approximately half of the isolates in the study characterized as one of two types (Matthews et al., 1994). Conversely, PFGE tends to find strains to be unique to a herd (Joo et al., 2001, Middleton et al., 2002a, b). In the

present study, the discriminatory power of PFGE was higher than previously described. This was likely due to the small number of herds in the comparison, because a very stringent cut off value (100% similarity) was used to define a strain-type, and because PFGE strain-type was part of the selection criteria for this study. Other studies have used a 70-84% similarity as strain cut-off values (Boerema et al., 2006, Du et al., 2014), which would decrease the discriminatory power while allowing grouping of isolates that may not be epidemiologically related. Isolates with genetically indistinguishable restriction patterns can be considered the same strain-type, while isolates that are closely related may have a single genetic alteration (point mutation, insertion, or deletion) which can result in two to three band differences (Tenover et al., 1995). One recent study found RS-PCR to have a similar discriminatory power to PFGE, with values of 0.8236 and 0.8943, respectively (Cremonesi et al., 2015). A higher discriminatory power for RS-PCR may have been identified in that study because RS-PCR results were used as part of the selection criteria for isolates that were compared using different strain typing methods. Furthermore, in that study, a chip electrophoresis system was used, which can determine smaller differences in banding patterns than conventional gel electrophoresis. Conventional gel electrophoresis was used herein to determine the utility of the technique as applied in a reasonably well-equipped veterinary diagnostic laboratory thus making the results relevant to every day usage in aiding farmers to make management decisions.

A few limitations should be noted in this study. One is related to the equipment used in this study. It has been recommended to use a miniaturized electrophoresis system (Agilent Technologies) for best resolution, data evaluation, and data management of RS-

PCR results (Boss et al., 2016). The suggested system can be expensive and not necessarily available in all labs conducting PCR, which may be considered a limitation of this technique. Because this system was not used, the isolates in this study could not be classified using the previously described genotyping system (Fournier et al., 2008). However, to allow proper comparison of the genotype of interest, a GTB control was used. Another limitation in this study is sample size. Increasing the number of farms and number of isolates would allow for further assessment of prevalent genotypes found within the USA and determine if GTB is truly absent from USA dairy farms. This limitation is also likely a factor in some of the calculations, such as discriminatory power, and these values would likely change with further isolate characterization from a larger number of farms.

In conclusion, GTB was not identified in this group of isolates. It is possible that GTB may be localized to some European countries, as previous studies have identified this genotype in Austria, Belgium, France, Germany, Italy, and Switzerland but did not find it in Ireland, Slovenia, Macedonia, Norway, the Netherlands, or Sweden (Cosandey et al., 2016). Further work on the geographical distribution of this virulent strain of *S. aureus* is needed. In this study, RS-PCR patterns were associated with a specific virulence gene pattern, as previously reported (Fournier et al., 2008). Also, these data suggest that PFGE is more discriminatory than traditional methods of RS-PCR for strain-typing *S. aureus* isolates of bovine origin. Although PFGE is more time consuming and expensive than PCR-based techniques, a higher discriminatory power is important to help determine the contagiousness of a strain and to define the epidemiology of *S. aureus* within a given

herd. Few enterotoxin genes were identified in the isolates studied herein, which corroborates the findings from other studies conducted in the USA. Further studies are needed to identify a simple method that could be applied universally to allow identification of virulent or contagious *S. aureus* strains.

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Table 1. Distribution of *S. aureus* enterotoxin genes, median (range) for NAGase activity, median (range) linear score SCC (LSCC) identified within each RSP-type, and number (percent) of isolates possessing the different enterotoxin genes within each RSP-type.

RSP-TYPE (N=65)	MEDIAN NAGase (RANGE)	MEDIAN LSCC (RANGE)	<i>sea</i>	<i>sed</i>	<i>sei</i>	<i>sej</i>	<i>seg</i>	<i>lukE</i>
1 (N = 50)^a	17.8 (2.3-363.6) ^a	8.1 (2.5-12.5)	0	0	0	0	0	50 (100%)
2 (N = 5)^b	3.04 (2.6-10.4) ^b	4.8 (2.5-7.8)	5 (100%)*	0	0	0	0	5 (100%)
3 (N = 4)^b	8.3 (4.3-18.3)	5.4 (4.8-5.8)	0	0	4 (100%)	0	0	4 (100%)
4 (N = 3)^b	14.2 (8.0-46.5)	7.8 (5.0-11.9)	0	0	3 (100%)	0	3 (100%)	3 (100%)
5 (N = 3)^b	23.3 (5.6-23.7)	6.8 (4.8-8.1)	0	0	0	0	0	3 (100%)
TOTAL (N = 65)			5 (7.7%)	0	7 (10.8%)	0	3 (4.6%)	65 (100%)

^{a-b} Values with different superscripts within column differ from each other ($P < 0.05$).

*Percentages of isolates possessing the different SET genes are the number of isolates with a given SET gene detected divided by the row total, e.g., 5 of 5 isolates identified by RSP-type-2 as possessed the *sea* gene (100%).

Table 2. Distribution of *S. aureus* PFGE strain-types and RS-PCR types identified within each farm.

Farm	Number of isolates	¹ PFGE stain ID	² Number of isolates identified on farm	³ N=	⁴ RSP-type
1	8	1	15	3	1
		2	4	2	1
		3	5	3	1
2	3	4	14	3	4
3	4	5	1	1	5
		6	5	2	5
		7	1	1	3
4	14	7	1	1	3
		8	19	3	1
		9	4	2	1
		10	17	3	1
		11	1	1	1
		12	1	1	3
		16	1	1	1
		17	1	1	1
		19	1	1	1
5	10	21	4	3	1
		22	4	3	1
		23	1	1	2
		24	1	2	1
		26	1	1	1
6	9	23	4	3	2
		27	10	2	1
		28	1	1	1
		29	2	1	1
		30	1	1	1
		31	1	1	1
7	13	8	9	3	1
		23	2	1	2
		31	1	1	1
		32	63	3	1
		34	1	1	1
		35	1	1	1
		37	1	1	1
		38	1	1	1
		39	1	1	1

8	4	40	6	1	1
		41	1	1	1
		42	1	1	1
		43	1	1	3
		35 PFGE	210 isolates	65	5 RSP-
TOTAL	65 isolates	strains		isolates	types

¹PFGE strain-types displayed in bold font were found on more than one farm.

²Reference: Middleton JR, Fox LK, Gay JM, Tyler JW, Besser TE. 2002. Influence of *Staphylococcus aureus* strain-type on mammary quarter milk somatic cell count and *N*-acetyl- β -D-glucosaminidase activity in cattle from eight dairies. J Dairy Sci 85:1133-40.

³ Number of isolates characterized within PFGE strain-type; note that only one RSP-type was found within each PFGE strain-type.

⁴RSP-type-1 was found on six farms, RSP-type-2 and RSP-type-3 were found on three farms, RSP-type-4 and RSP-type-5 were each found on one farm.

Figure 1. Gel electrophoresis of all RSP-types. Lanes labelled O are 100 base pair (bp) ladders with the 500 bp bands labelled. Lane 1 is RSP-type-4. Lane 2 is RSP-type-3. Lane 3 is RSP-type-1. Lane 4 is RSP-type-5. Lane 5 is RSP-type-2. Lane 6 is the GTB positive control. Lane 7 is the negative control.

CHAPTER SIX

RUNNING TITLE: STAPHYLOCOCCAL CHARACTERIZATION IN HEIFERS

MOLECULAR CHARACTERIZATION OF NON-AUREUS *STAPHYLOCOCCUS*
SPP. FROM HEIFER INTRAMAMMARY INFECTIONS AND BODY SITES

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ABSTRACT

The purpose of this study was to investigate non-*aureus Staphylococcus* (NAS) intramammary infections (IMI) in periparturiant heifers and determine the relationship of prepartum body site colonization with prepartum IMI and post-calving IMI using molecular speciation and strain-typing methods. Primiparous heifers were enrolled at approximately 14 days prior to expected calving date. Prepartum mammary quarter secretions and body site swabbing samples (teat skin, inguinal skin, muzzle, and perineum) were collected. Post-calving, mammary quarter milk samples were collected for culture and SCC enumeration. For prepartum body site samples, up to 10 staphylococcal colonies were saved for further characterization. Staphylococcal isolates were speciated using matrix-assisted laser desorption ionisation-time of flight mass spectrometry or PCR amplification and sequencing of the *rpoB* or *tuf* gene. Pulsed-field gel electrophoresis was used to strain-type isolates. Overall, *Staphylococcus chromogenes*, *Staphylococcus agnetis*, and *Staphylococcus simulans* were the most common species identified in prepartum mammary secretions while *S. chromogenes*, *Staphylococcus xylosus*, and *S. agnetis* were the most common species found in post-calving milk samples. The most common species identified from body site samples were *S. chromogenes*, *S. xylosus*, and *Staphylococcus haemolyticus*. Prepartum mammary secretions that were culture positive for *S. agnetis*, *S. chromogenes*, and *Staphylococcus devriesei* had significantly increased odds of having an IMI with the same species post-calving, and a *S. chromogenes* IMI either prepartum or post-calving was associated with higher SCS after calving when compared to culture negative quarters. Among heifers identified with a NAS IMI either prepartum or post-calving, heifers that had teats

colonized with *S. agnetis* or *S. chromogenes* had significantly increased odds of having the same species found in the prepartum mammary secretions. Heifers with the inguinal region colonized with *S. chromogenes* were at increased odds of having *S. chromogenes* isolated from a prepartum mammary secretion sample. Heifers with prepartum teat skin colonized with *S. chromogenes*, *S. simulans*, and *S. xylosus* were at increased odds of having an IMI with the same species post-calving. Overall, 44% of all heifers with a *S. chromogenes* IMI around the time of parturition were found to have the same strain on a body site. Based on PFGE, a high level of strain diversity was found.

Key words: heifer, non-*aureus Staphylococcus* spp., MALDI-TOF, Pulsed-field gel electrophoresis

INTRODUCTION

Staphylococci are the most common bacteria isolated from cow's milk (Piepers et al, 2007, Sampimon et al, 2009, Thorberg et al, 2009). For decades, staphylococci that infect the mammary gland have been divided into *Staphylococcus aureus* and coagulase negative staphylococci (CNS) based on the coagulase test. While this division works reasonably well in practice for differentiating IMI with the potentially more pathogenic and contagious *S. aureus* from the less pathogenic CNS, there are some species of staphylococci that are coagulase variable, such as *Staphylococcus hyicus* and *Staphylococcus agnetis* (Taponen et al, 2012). When these coagulase variable staphylococci are included in studies, non-*aureus* *Staphylococcus* spp. (NAS) is likely a better term than CNS (Condas et al., *submitted*). However, the majority of previous research has focused specifically on CNS.

Coagulase negative *Staphylococcus* spp. are the most common cause of intramammary infections (IMI) in heifers both pre and postpartum (Fox et al., 1995, Fox, 2009, De Vlieghe et al., 2012). The percentage of CNS- infected quarters in heifers post-calving has been found to be approximately 35-37% (Rajala-Schultz et al., 2009, Piepers et al., 2010, De Visscher et al., 2016a). When using molecular techniques to speciate CNS, the most common species identified in heifers include *Staphylococcus chromogenes*, *Staphylococcus simulans*, and *Staphylococcus xylosus* (De Visscher et al., 2016a), which are considered among the most important CNS species as they have all been shown to have a more substantial effect on udder health and milk quality than other CNS species (Supre et al., 2011, Fry et al., 2014). Mammary quarters infected at parturition with the

most important CNS species can have a geometric mean SCC above 400×10^3 cells/ml, while quarters infected with less-relevant CNS species have been found to have an SCC that does not differ from uninfected quarters (De Visscher et al., 2016a). Increased SCC early in the first lactation has been shown to have lasting negative effects on milk quality and production, including being associated with an elevated SCC during the first lactation (De Vlieghe et al., 2004a) and decreased milk production throughout the entire first lactation (Coffey et al., 1986, De Vlieghe et al., 2005b). Furthermore, an increased SCC between 5 and 30 DIM of the first lactation can negatively impact lifetime milk yield (Archer et al., 2013). Mastitis, in general, in heifers has also been associated with increased culling with each unit increase in the LnSCC in early lactation being associated with an 11% increase in culling hazard (De Vlieghe et al., 2005a).

Coagulase negative staphylococci are part of the normal skin flora of animals (Devriese and De Keyser, 1980, Devriese et al., 1985) and have been isolated from different body sites of cows and heifers, from udder secretions and milk, and from the dairy environment (White et al., 1989, Taponen et al., 2008, Piessens et al., 2011). With regard to body site colonization, teat skin colonization has been the primary focus of most studies (De Vlieghe et al., 2003, Braem et al., 2013, De Visscher et al., 2016b). Overall, teat apices of pregnant heifers have been found to mainly be colonized by *S. chromogenes*, followed by *Staphylococcus haemolyticus*, *Staphylococcus devriesei*, and *Staphylococcus equorum* (De Visscher et al., 2016b). Generally, 20-62% of sampled heifers have been found to harbor *S. chromogenes* on the teat skin (White et al., 1989, De Visscher et al., 2016b). Furthermore, teat apex colonization with *S. chromogenes*

increases the likelihood of *S. chromogenes* IMI in the corresponding quarter at parturition (De Visscher et al., 2016a), which is similar to *Staphylococcus aureus* (Roberson et al., 1994), another host-adapted species (species in which the bovine is a major reservoir). Although some research points to the negative effects of teat apex colonization with *S. chromogenes* and its relationship to IMI, other studies have found a potential protective effect of teat apex colonization with *S. chromogenes* as some strains of *S. chromogenes* display antibacterial activities *in vitro* (Braem et al., 2014, De Vlieghe et al., 2004b) and heifer quarters with prepartum colonization of the teat apex with *S. chromogenes* have been found to have an SCC < 200,000 cells/ml in early lactation (De Vlieghe et al., 2003).

Although there is ample evidence that some of the most important NAS species can be found on the teat apex and in the mammary gland of early lactation heifers, little work has been done to strain-type NAS species identified on body sites of prepartum heifers to determine if the same strain is found in the mammary gland either before or after calving. Furthermore, little work has been done to characterize colonization of body sites other than the teat end and the relationship of such colonization to IMI with the exception of a study by White et al. (1989) for CNS and previous studies on *S. aureus* (Matos et al., 1991, Roberson et al., 1994, Middleton et al., 2002b), another host-adapted species. Of note, the White study only described body site colonization and not the relationship to IMI and used phenotypic methods for speciation which may have led to misclassification of some CNS species based on recent evidence on the poor typeability of phenotypic methods (Zadoks and Watts, 2009). Therefore, the purpose of this study was to

investigate NAS IMIs in periparturient heifers and determine the relationship of body site colonization, prepartum NAS IMI, and post-calving NAS IMI using molecular speciation and pulsed-field gel electrophoresis (PFGE) strain-typing. As discussed above, most previous studies have focused on CNS; however, all NAS species were included in this study regardless of coagulase reaction.

MATERIALS AND METHODS

Herd and heifer selection

Late gestation primiparous heifers (n = 100), including both Guernsey (n = 9) and Holstein (n = 91) breeds, at the University of Missouri research dairy farm were enrolled between April 2014 and April 2015. Heifers were enrolled in the study at approximately 14 d prior to expected calving date. During the study, the farm milked a median number of 178.5 (range: 171-193) Holsteins and 26 (range: 25-28) Guernsey cows, and had a geometric mean bulk tank SCC of 206,000 cell/ml with an average 305-day milk production of 10,844 kg. All heifers included in the study were raised on the farm. Cows and heifers on the farm calved year-round with the exception of a planned reduction in calving from July to September. The study was approved by the University of Missouri Animal Care and Use Committee.

Sampling collection

Pre-calving mammary quarter secretions and post-calving mammary quarter milk samples were aseptically collected from the cohort heifers. Prior to sample collection, teats were treated with a germicidal teat dip, stripped, dried with an individual towel, and

then scrubbed with a cotton pad soaked in 70% isopropyl alcohol. Pre-calving secretion samples were collected approximately 14 d prior to expected calving date. All pre-calving mammary quarter secretion samples were taken after body site swabbing samples had been collected. Post-calving mammary quarter foremilk samples were collected twice within the first 10 days of lactation for bacterial culture and somatic cell counting. Samples were chilled on ice for transport to the laboratory. Samples for bacterial culture were stored at -20°C until bacteriological examination was performed. Milk samples for SCC enumeration were placed in vials containing a 2-bromo-2-nitropropane-1,3-diol preservative tablet (Broad Spectrum Microtabs II, D&F Control Systems Inc., Dublin, CA), stored at 4°C, and shipped once weekly to a commercial laboratory (Mid-South Dairy Records, Springfield, MO).

Body site swabbing samples were collected once, at the prepartum sampling time point. All heifers were restrained in a chute for sample collection. Gloves were worn at all times and were changed between each sample collected. Prior to sample collection, visible debris was removed. Body site swabbing samples were collected by gently rubbing a dry sterilized electrostatic dusters (Swiffers®) over the entire surface of each site. A total of four body site samples were collected and included one composite sample of all four teats, one composite sample of both inguinal regions, one muzzle sample, and one sample from the perineum. Body sites selected for sampling was based on sites previously examined for *S. aureus* (Roberson et al, 1994, Middleton et al, 2002). All body site swabbing samples were chilled on ice for transport to the laboratory and processed the same day.

Laboratory procedures

Prepartum mammary secretions and post-calving milk samples were thawed at room temperature (22°C) and 10 µl of the respective samples were evenly spread onto half a Columbia blood agar (CBA) plate and incubated at 37°C for 24 h, followed by 24 h at room temperature (22°C). Plates were read at 24 and 48 h. Primary identification was determined according to the National Mastitis Council guidelines (Hogan et al, 1999). Briefly, potassium hydroxide test was used to differentiate Gram-negative from Gram-positive bacteria. Staphylococcal species were presumptively identified based on colony morphology, a positive catalase reaction, and results of the coagulase test. A mammary quarter was defined as having a prepartum IMI with a given NAS species when that species was recovered from the single prepartum mammary quarter secretion sample at a concentration ≥ 100 cfu/ml. A mammary quarter was defined as having a post-partum IMI with a given NAS species if that species was recovered from one or both (i.e. parallel interpretation) post-calving quarter-milk samples at concentration ≥ 100 cfu/ml (Dohoo, et al, 2011,b). An IMI was defined as a mixed infection when 2 different bacterial colony types were present, and contaminated when 3 or more different bacterial colony types were present. All isolates presumed to be staphylococcal species were stored in phosphate buffered glycerol at -80°C until further processing.

Milk SCC were determined at a commercial laboratory (Mid-South Dairy Records, Springfield, MO, USA) using an automated counter (Somacount 300, Bentley Instruments, Chaska MN, USA).

Body site swabbing samples were mixed with 10 ml of sterile saline, agitated, and 50 μ l of the resultant solution was plated on Mannitol Salt Agar (MSA). Plates were incubated at 37°C for 24 h. At 24 h, MSA plates were read and up to 10 presumptively identified staphylococcal colonies, including at least one of each morphologically distinct colony type, were sub-cultured onto CBA. All isolates were stored in phosphate buffered glycerol at -80°C until further processing. All staphylococcal isolates from pre- or post-calving IMI were speciated as below; however, because the overall objective was to determine the relationship between body site colonization and pre- or post-calving IMI, only body site isolates that originated from heifers with a pre- or post-calving IMI were subjected to further speciation.

All staphylococcal isolates that were subjected to speciation were initially speciated using matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (Tomazi et al., 2014). Samples were processed on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with flex control software (Bruker Daltonics). The MALDI-TOF target plate was prepared using the plate extraction method (Bruker Daltonics). All isolates were run in duplicate. The plate extraction method was performed by spotting one colony of bacteria on the MALDI-TOF target plate and overlaying it with 1 μ l of 70% formic acid. Once dried, the spot was overlaid

with 1 µl of matrix solution, consisting of α -cyano-4-hydroxy-cinnamic acid diluted in a solution of 50% acetonitrile and 2.5% trifluoroacetic acid. The bacterial test standard (Bruker Daltonics) was used to calibrate the instrument prior to running test samples. Peaks produced by each sample were compared with entries in the Biotyper database and the University of Missouri (MU) laboratory custom database (see below) using the standard parameters of the pattern-matching algorithm. The MALDI-TOF manufacturer's identification cut-off value for species-level identification was a score ≥ 2 on at least one of the duplicate samples and was used to make a diagnosis. Any score < 2 was classified as inconclusive for species identification.

An in-house validation of the MALDI-TOF was performed by speciating the first 770 isolates from the study by housekeeping gene sequence (see below) and MALDI-TOF. The 770 isolates represented 17 species (Table 1). When a score of ≥ 2 was obtained, MALDI-TOF correctly identified 72.9% of the isolates overall and a range of 0-100% of isolates within each species. Because three of the commonly isolated NAS species (*S. agnetis*, *S. devriesei*, and *S. haemolyticus*) had a low identification accuracy ($< 74\%$) either because they were not included in the manufacturer's database or the included database isolate yielded a poor identification accuracy for the tested bovine isolates, a single representative isolate of each of these three species was added to the MU laboratory custom database from the authors' isolate collection. All three isolates were added to the custom database according to the manufacturer's instructions in which 100 known isolates of a given species that had previously been identified using *rpoB* (Drancourt and Raoult, 2002) or *tuf* (Hwang et al., 2011) gene sequencing were run

against the reference isolates added to the custom database to verify accurate identification. After the creation of the custom database, MALDI-TOF accurately identified 96.1% of the 770 isolates overall with a range of 0-100% of isolates within each species (Table 1).

For MALDI-TOF validation, and for all isolates where MALDI-TOF yielded inconclusive results, isolates were speciated using PCR amplification and subsequent Sanger sequencing of a portion of either the *rpoB* or *tuf* gene. Briefly, isolates were cultured on CBA at 37°C for 24 h and a single representative colony was inoculated into 100 µl of 1X Tris-EDTA (Promega, Madison, WI, USA) for lysate preparation. Standard PCR was performed to amplify a portion of the *rpoB* (Drancourt and Raoult, 2002) or *tuf* (Hwang et al., 2011) gene. The resultant PCR products were purified using a commercial kit (Invitrogen, Carlsbad, CA, USA) and submitted to the University of Missouri DNA Core Facility for Sanger sequencing. Sequences were compared to the GenBank database using the publicly available nucleotide-BLAST algorithm (www.ncbi.nlm.nih.gov). For *rpoB* analysis, species identity was confirmed if the sequence had $\geq 97\%$ identity with a single species of staphylococcus in the GenBank database (Mellmann et al., 2006). For *tuf* gene analysis, species were confirmed if sequence identity was $\geq 98.0\%$ with $> 0.8\%$ separation between species (Hwang et al., 2011). Any isolate identified as *S. agnetis* or *S. hyicus* by MALDI-TOF was speciated using the *aroD* gene multiplex PCR (Adkins et al, *submitted*). Isolates that could not be speciated by MALDI-TOF or housekeeping gene sequencing were identified as unidentified NAS.

Heifers which had a prepartum mammary quarter secretion or post-calving milk sample and a body site sample with isolates of the same species identified within heifer were selected for strain-typing by PFGE. All body site, mammary secretion, and milk sample isolates within heifer were strain-typed. For heifers colonized or infected with *S. agnetis*, *S. devriesei*, *S. haemolyticus*, *S. sciuri*, *S. simulans*, and *S. xylosus* all colonized/infected heifers were studied. Due to the high prevalence of *S. chromogenes* colonization/infection and the cost of PFGE, only a random sample of heifers (60%) with a *S. chromogenes* IMI were studied with strain-typing. A total of 60% of heifers with a *S. chromogenes* IMI were selected because *S. chromogenes* IMIs represented 60% of all quarters with a NAS IMI that had a corresponding body site sample of the same species identified. Heifers with *S. chromogenes* colonization/infection were randomly selected using Microsoft Excel (2013) random number generator. All strain-typing was performed using PFGE as previously described (Middleton et al., 2002a). Briefly, *Sma*I (Invitrogen, Carlsbad, CA, USA) digests of staphylococcal DNA were separated by PFGE in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, Hercules, CA) immersed in 0.5% TBE (TRIS, Boric Acid, EDTA) buffer at 15°C for 20 h at 6 V/cm with a 5-50 s pulse time on a CHEF DRIII PFGE machine (BioRad, Hercules, CA, USA). *Salmonella* BAA-664 was used as a molecular weight size standard and was digested using *Xba*I (Invitrogen, Carlsbad, CA, USA). Standards were included in the first, middle, and last lane of each gel. Completed gels were stained with ethidium bromide and photographed using UV trans-illumination. Images were analyzed using Bionumerics 7.5 (Applied Math, Belgium) using Dice similarity coefficient and an UPGMA

dendrogram type. Tolerance settings included optimization at 0.5% and position tolerance at 1.0%. Strain types were determined by using a threshold cut point of 100% similarity.

Statistical analyses

First, the association between prepartum colonization of a given body site with a given NAS species and prepartum quarter NAS IMI status was investigated. To achieve this, generalized mixed models using a logit link function and with cow treated as random intercept were used. The outcomes in these models were always whether or not a quarter was infected by a given NAS species at the prepartum sampling. Different models were, therefore, used for each of the NAS IMI species recovered in the prepartum mammary quarter secretion sample. Fixed predictors investigated were inguinal, teat, muzzle, and perineum region colonized with NAS. These predictors were included one at a time in the model (i.e. unconditional associations). Models were run using Proc GLIMMIX and using a Gauss-Hermite quadrature approximation method (i.e. method=quad) in SAS 9.4 (Cary, NC, USA). Then, the association between prepartum colonization of a given body site with a given NAS species and post-calving quarter NAS IMI status was investigated. To achieve this, the same methodology previously described was used, but with outcomes being post-calving quarter NAS IMI status. The association between prepartum quarter NAS IMI status and post-calving NAS IMI status was investigated using a similar methodology, but with outcomes being post-calving quarter NAS IMI status and the only predictor being the prepartum quarter NAS IMI status.

Finally, the impact of prepartum and post-calving quarter NAS IMI statuses on post-calving somatic cell score (SCS) was investigated. To achieve this, two linear mixed models (i.e. one for prepartum and one for post-calving quarter NAS IMI status) were used. In one model each, the prepartum NAS IMI status or the post-calving quarter NAS IMI status (e.g. no NAS IMI, *S. agnetis* IMI, *Staphylococcus auricularis*, etc.) was used as sole predictor of post-calving SCS. In these models the natural logarithm of the SCS observed on the second post-calving sampling was used as the outcome, as the maximum sensitivity and specificity for detecting IMI with SCC is reached at a threshold of 100,000 cells/ml for samples evaluated 5 d post calving (Sargeant et al, 2001). The natural logarithm of this second SCS measurement rather than the raw SCS was used to meet the assumptions of normality and homoscedasticity of the residuals. These assumptions were verified using quantile plot of the residuals and plots of predicted values against residuals. Statistically significant results for all analyses were defined as a $P \leq 0.05$.

RESULTS

Prepartum IMI and its relationship to post-calving IMI and milk SCS

All 100 heifers completed the study. Overall, 83% (n = 83) of heifers and 30% (122/400) mammary quarters had an NAS species identified in a prepartum secretion and/or milk sample from at least one mammary quarter at ≥ 1 of the sample time points (prepartum or

post-calving [Sample 1 or Sample 2]), while 17% (n =17) of heifers were NAS negative at all time points. Among the 100 heifers, 23% (92/400) of prepartum mammary quarter secretions from 47 heifers, 16% (62/400) of post-calving quarter milk samples from 41 heifers at Sample 1, and 13% (52/400) of post-calving quarter milk samples from 38 heifers at Sample 2 had an NAS species isolated. At the species level, 17 different NAS species were identified among all the quarter mammary secretions and milk samples. The most common NAS species identified in prepartum mammary samples in order of prevalence were *S. chromogenes*, *S. agnetis*, *S. simulans*, and *S. devriesei* (Table 2). In post-calving milk samples, the most common NAS species identified was *S. chromogenes*, followed by *S. xylosus*, *S. agnetis*, and *S. simulans* (Table 2). Among the prepartum secretions and post-calving milk samples, *Staphylococcus arlettae*, *S. auricularis*, *S. equorum*, *Staphylococcus lentus*, *S. sciuri*, and *Staphylococcus vitulinus* were only identified in post-calving milk samples.

There was a significantly increased odds of having a post-calving IMI with the same species of NAS that a heifer was infected with in a prepartum secretion when the mammary quarter was infected with *S. agnetis* (OR = 193.5; 95% CI: 19.2, >999), *S. chromogenes* (OR = 20.6; 95% CI: 7.4, 57.5), or *S. devriesei* (OR =78.6; 95% CI: 4.2, >999). With regard to the association of IMI either pre- or post-calving with early lactation SCS, having a *S. chromogenes* IMI either prepartum ($P = 0.05$) or post-calving ($P < 0.01$) was associated with higher SCS in the second milk sample post-partum (median 8 d; range 6-10 d post-partum) when compared to mammary quarters that were culture negative for an IMI at all time points (Table 3). Overall, a high degree of

variability was found in SCS, either when comparing the effect of prepartum IMI (Figure 1) or post-calving IMI (Figure 2) on quarter milk SCS at the second post-calving milk sampling.

Body site colonization and its relationship to pre- or post-calving IMI

A total of 2,304 staphylococcal isolates were recovered from 332 body site samples that originated from the 83 heifers in which a NAS IMI was identified in at least one quarter either pre- or post-calving. Due to the replication of a single species on the same body site, 1,532 replicates among the 2,304 isolations of NAS were removed from the data set for the purposes of analysis, leaving 772 occurrences of body site colonization with a NAS. All *S. aureus* isolations (n = 14) were also removed from the analyses, leaving 758 total occurrences of body site colonization with a NAS (Table 1).

At least one NAS species was identified from all 83 teat skin samples. No NAS were isolated from 7.2% (24/332) of the other body site samples, including 4.8% (4/83) of inguinal skin samples, 4.8% (4/83) of muzzle samples, and 19.3% (16/83) of perineum samples. The median number of species identified from the inguinal skin, teat skin, muzzle, and perineum was 2 (range: 0-5), 2 (range: 1-7), 2 (range: 0-7), and 1 (range: 0-5), respectively. Because multiple species could colonize a given body site, of the 758 occurrences of body site colonization with a given NAS, 214 (28%), 213 (28%), 197 (26%), and 134 (18%) were on the teat skin, muzzle, inguinal skin, and perineum, respectively.

Overall, 18 different NAS species were identified from the body site samples (Table 2). The most common species identified from all the body sites was *S. chromogenes* followed by *S. xylosus*, *S. haemolyticus*, *S. sciuri*, and *S. devriesei*. The most common species identified from inguinal samples was *S. chromogenes* followed by *S. haemolyticus*, *S. xylosus* and *S. devriesei*. The most common species identified on the teat skin was *S. chromogenes*, followed by *S. haemolyticus*, *S. devriesei*, and *S. xylosus*. The most common species identified from the muzzle samples was *S. xylosus*, followed by *S. equorum*, *S. sciuri*, and *S. chromogenes*. The most common species identified from the perineum was *S. chromogenes*, followed by *S. haemolyticus*, *S. xylosus*, and *S. equorum*. *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus gallinarum*, and *Staphylococcus pasteurii* were all associated with either a pre- or post-calving IMI, but were not isolated from any of the studied body sites, while *S. saprophyticus*, *Staphylococcus cohnii*, *Staphylococcus nepalensis*, *Staphylococcus succinus*, and *Staphylococcus kloosii* were all identified on body sites, but were not isolated from a pre- or post-calving IMI.

Among heifers identified with a NAS IMI in either the pre- or post-calving periods (n=83), heifers that had teat skin colonized with *S. agnetis* or *S. chromogenes* had a significantly increased odds of having the same NAS species isolated from a prepartum IMI (Table 4). Heifers colonized with *S. chromogenes* on the inguinal skin had an increased odds of having a prepartum *S. chromogenes* IMI. Additionally, heifers with teat skin colonized with *S. chromogenes*, *S. simulans*, and *S. xylosus* had increased odds of

having a post-calving IMI with the same NAS species (Table 5). Colonization of the muzzle or perineum with any species was not found to increase odds of a pre- or post-calving IMI.

Relationship of pre- and post-calving IMI and body site colonization confirmed by PFGE

For PFGE strain-typing, 44 heifers that had an IMI diagnosed on a prepartum secretion sample or post-calving milk sample were studied. This included all heifers with an IMI caused by *S. xylosus*, *S. haemolyticus*, *S. agnetis*, *S. simulans*, *S. devriesei*, *S. sciuri*, and 60% of heifers with a *S. chromogenes* IMI (Table 5). Heifers with an IMI caused by *S. auricularis*, *S. gallinarum*, *S. pasteurii*, *S. lentus*, *S. vitulinus*, *S. arlettae*, and *S. equorum* were not included because there were no body site isolates of the same species that caused an IMI. Because the objective was to identify all strains within a given species, all isolates of the same species from a given body site or IMI were strain-typed. Hence, all replicates within heifer were studied. From the 44 heifers, a total of 551 NAS isolates from body sites and pre- or post-calving IMI were available for PFGE (Table 6). Among these isolates, 40 (7.2%) were classified as untypeable by PFGE based on unreadable banding pattern on repeated analysis, leaving 511 isolates from 42 different heifers with 69 mammary quarter IMI (Table 6)

Overall, within heifer strain diversity was highest for *S. chromogenes*, with a median number of strains identified per heifer of 6 (range: 2-14). An example of this diversity is

illustrated in Figure 3. The median number of strains was 5 (range: 2-10) for *S. xylosus*, 4 (range: 1-13) for *S. haemolyticus*, 2 (range: 1-3) for *S. simulans*, and 2 (range: 1-2) for *S. agnetis*. All heifers found to be colonized with *S. devriesei* (n = 2) or *S. sciuri* (n = 1) each had two strains/heifer. Overall, 44% of all IMI caused by *S. chromogenes* were found to be the same strain as a body site isolate (Table 7). Body sites that were colonized with the same strain of *S. chromogenes* that caused an IMI included the teat skin, the inguinal skin, and the perineum. On some heifers, the same strain that was associated with an IMI was found in multiple locations, either on both the teat and inguinal or on both the inguinal and perineum. A total of 67% of *S. agnetis* strains associated with an IMI were found to be the same strain as an isolate found on the teat skin. Of the quarters that had a *S. simulans* IMI, 40% were found to be the same strain as those found on the teat skin. A total of 20% (n = 1) of *S. haemolyticus* associated with an IMI were found to be the same strain as that found on inguinal skin. One *S. devriesei* isolate that was associated with an IMI was found to be the same strain as that which was found on the teat skin and perineum. For quarters with a *S. xylosus* (n = 5) or *S. sciuri* (n = 1) IMI, no body site isolates were determined to be the same strain as those found in the mammary gland.

DISCUSSION

To the best of the authors' knowledge, this is the first report to use both molecular speciation and strain typing of NAS species from IMIs and body sites of heifers in the peripartum period. Previous reports have identified that prepartum teat apex colonization with *S. chromogenes* increases the likelihood of *S. chromogenes* IMI in the corresponding

quarter at parturition (De Visscher et al., 2016b), however no subspecies strain-typing was conducted in that study. At the species-level for *S. chromogenes* specifically, similar results were identified in this study, however colonization of the inguinal skin with *S. chromogenes* also increased the odds of finding *S. chromogenes* IMI at the prepartum mammary sampling. Furthermore, through PFGE strain typing, it was identified that 44% of *S. chromogenes* isolated from the teat and/or inguinal skin were found to be 100% similar to those associated with a periparturient IMI, which is similar to what has previously been found with *S. aureus* (Middleton et al., 2002b). Based on these findings, teat and inguinal skin colonization should be included when investigating the role of body site colonization on the epidemiology of *S. chromogenes* IMI. Additionally, colonization of the teat skin with *S. simulans* and *S. xylosus* prepartum increased the odds of finding that same species associated with a NAS IMI post-calving. Strain-typing found that when a body site was colonized with the same strain of *S. simulans* as that associated with an IMI, it was always found on the teat skin. Conversely, no body site isolates were found to be similar to the isolates associated with an IMI in the case of *S. xylosus*.

Prepartum IMI with *S. agnetis*, *S. chromogenes*, or *S. devriesei* significantly increased the odds of having an IMI post-calving with the same NAS species. Previous reports have identified *S. chromogenes* and *S. hyicus* to be the more prevalent bacteria isolated from non-pregnant and primigravid heifer's mammary gland secretions (Trinidad et al., 1990). At the time of that study, *S. agnetis* (Taponen et al., 2012) and *S. devriesei* (Supre et al., 2010) had not been classified and therefore were not identified. Unlike the previous

report, *S. hyicus* was not identified in the present study, however since *S. agnetis* and *S. hyicus* are phenotypically indistinguishable (Taponen et al., 2012), it is possible that isolates previously reported as *S. hyicus* were truly *S. agnetis*. Prepartum IMI with *S. chromogenes* was found to have an effect on post-calving SCS. Because of the impact a prepartum IMI can have on post-calving IMI status, and in the case of *S. chromogenes* on SCS status, if interventions or prevention strategies are pursued in the future, targeting interventions in the prepartum period will likely be needed.

Although CNS are considered to be teat skin opportunists, the results of this study showed that not all species commonly found on the skin of the heifer are commonly associated with IMI. For example, *S. saprophyticus*, *S. cohnii*, *S. sciuri*, and *S. nepalensis* were commonly found on body sites, but were rarely, if ever, found to be associated with IMI. Furthermore, a few species that were rarely associated with IMI were never identified on a body site, including *S. epidermidis*, *S. hominis*, *S. warneri*, *S. gallinarum*, and *S. pasteurii*. Although *S. epidermidis* is often identified as one of the more prevalent CNS species associated with IMI (Thorberg et al., 2009, Piessens et al., 2011, Fry et al., 2014) and found in bulk tank milk samples (De Visscher et al., 2017), in general, this species is more common in older cows than heifers (Thorberg et al., 2009). Furthermore, others reported that *S. epidermidis* was rarely found on the teat apices of prepartum heifers (De Visscher et al., 2016b). In contrast, *S. epidermidis* has been found to be prevalent on human skin and it has been postulated that, when it is found in cases of IMI, it may have been transmitted from milkers' hands to cows (Thorberg et al., 2006).

In agreement with what has been previously documented in lactating cows (Taponen et al., 2008), *S. chromogenes* was found very commonly both on body sites and as a cause of IMI in the present study. Furthermore, the PFGE strain-typing in the present study confirmed that in 44% of cases the same strain that was found on body sites was causing an IMI in the same heifer, further confirming previous reports suggesting that *S. chromogenes* is a host-adapted NAS species (Piessens et al., 2011, De Visscher et al., 2016a).

Overall, a diverse population of staphylococcal species was identified to be associated with IMIs and body sites. For body sites specifically, 18 different NAS species were identified. Evaluation of the teat end microbiome has found that NAS species are the most frequently isolated and most commonly present species with only minor occurrences of other genera (Braem et al, 2013). The microbiome of the other body sites, including the inguinal region, muzzle, and perineum, have not been evaluated. In this study several samples were identified as NAS negative, with the majority of negatives being samples of the perineum. It is possible that these samples were not truly NAS negative, as overgrowth of other bacterial species may have prevented the identification of colonies of interest. Using culture independent methods may allow full assessment of the NAS populations in these samples and also help determine the interactions between different bacterial populations. Future studies evaluating the microbiome of these body sites may help further characterize the interactions of body site colonization and IMIs.

For many of the dominant species identified, a high level of strain diversity was found among isolates collected from body site samples and IMIs from within one heifer based on the results of PFGE. On one heifer, up to 14 different strains of *S. chromogenes* were identified and on a different heifer, up to 13 different strains of *S. haemolyticus* were found. This high level of strain diversity may make it very challenging to truly determine the origin of an IMI if only a few isolates of these species are strain-typed and may also be the reason why only 44% of all *S. chromogenes* IMIs were found to be the same as an isolate that originated from a body site, as some strains may have been missed. However, it is also possible that the majority of *S. chromogenes* IMIs are not related to a body site isolate. The high level of strain diversity may be related to the stringent cut-off of 100% similarity as the definition of a strain. Other studies have used a 70-84% similarity value as a cut point to define *S. aureus* strain-types (Boerema et al., 2006, Du et al., 2014) and, although this will allow more grouping of isolates, it will also potentially group isolates that may not be epidemiologically related. A 100% similarity cut point was used so that isolates with genetically indistinguishable restriction patterns should be considered the same strain-type and isolates that are closely related, which may have a single genetic alteration resulting in two or three band differences when examined by PFGE, would be considered a different strain (Tenover et al., 1995). Although PFGE is considered the gold standard for strain-typing of staphylococcal species (Bannerman et al., 1995), whole genome sequencing has demonstrated that PFGE is prone to some false-positive and false-negative results, and therefore suggests that a new gold standard is needed (Salipante et al., 2015). Furthermore, PFGE is costly and time consuming and therefore a

cheaper and faster strain typing alternative would be beneficial to the efficient study of NAS subspecies diversity on dairy farms.

Overall, the MALDI-TOF validation confirmed that this method is a quick, easy, and accurate way to speciate many of the staphylococcal species isolated from bovine samples (Tomazi et al, 2014). After adding three isolates to the MU laboratory custom database, MALDI-TOF correctly identified 96.1% of the validation isolates. Also, MALDI-TOF was not found to misclassify isolates. However, for some NAS species, such as *S. auricularis*, a large percentage of the isolates were categorized as unidentified (Table 1). One downfall of this method is that it is unable to differentiate *S. hyicus* and *S. agnetis*. Even after adding a *S. agnetis* isolate to the custom database, MALDI-TOF could not differentiate the two species. Therefore, this method should not be used to speciate *S. hyicus* and *S. agnetis* isolates. These species can easily be differentiated using *tuf* gene sequencing or *aroD* gene multiplex PCR (Adkins et al, *submitted*).

The IMI definition of ≥ 100 cfu/ml was selected for this study to increase the sensitivity of detecting an IMI, which according to Dohoo et al. (2011), maximizes sensitivity of detecting an IMI at 80.9%. A pitfall of the increased sensitivity is a lower specificity, which at the selected threshold of ≥ 100 cfu/ml was reported to be 86.7% (Dohoo et al., 2011). Therefore, both false positives and false negatives are expected with the definitions of IMI used in the present study. Although *S. chromogenes* IMI was found to be associated with an increase in SCS in early lactation compared to culture negative

quarters, false positives may explain why some of the SCS for prevalent species, specifically *S. simulans* and *S. xylosus*, were not found to be different from culture negative quarters as previously reported by others (De Visscher et al., 2016a). Furthermore, some culture negative control quarters had a relatively high SCS, which could be due to the low sensitivity of bacterial culture. The overall high degree of variability in milk SCS within a given species has been previously reported (Fry et al., 2014), which may be related to a relatively low number of isolates within some of the NAS species categories in the present study, differences in pathogenicity between bacterial strains within species, or cow-level factors.

One limitation of this study was that it was only conducted on one farm, and hence results may not be generalizable to other farms. Further, specific risk factors for NAS IMI or body site colonization could not be assessed. Although this is a limitation, some of the results are corroborated by previous studies (White et al., 1989, De Visscher et al., 2016b). For example, the most common species identified from the teat skin samples were *S. chromogenes*, *S. haemolyticus*, and *S. devriesei*, which is similar to what has been recently reported on heifers from 13 commercial dairies in Belgium (De Visscher et al., 2016b). Furthermore, three of the more common species found associated with IMI post-calving were *S. chromogenes*, *S. xylosus* and *S. simulans*, which were also found to be among the more common species identified in quarter milk of heifers at parturition from 13 different dairy herds also in Belgium (De Visscher et al., 2016a).

Finally, it has been previously reported that the use of strain-typing to relate body site colonization, specifically teat apex colonization, to IMI may be confounded by the teat end becoming contaminated with staphylococcal laden milk prior to teat skin sample collection (Leroy et al., 2015). However, in the present study all body site samples were collected first, and the heifers had never been milked at the time of body site sample collection. Therefore, the risk of this contamination by isolates from within the mammary gland is predicted to be very low in the current study.

CONCLUSION

Staphylococcus chromogenes, *S. agnetis*, and *S. simulans* were the most common species identified in prepartum mammary secretions while *S. chromogenes*, *S. xylosus*, and *S. agnetis* were the most common species found in post-calving milk samples. The most common species identified from all the body sites were *S. chromogenes*, *S. xylosus*, and *S. haemolyticus*. Prepartum mammary secretions that were culture positive for *S. agnetis*, *S. chromogenes*, and *S. devriesei* had significantly increased odds of having an IMI with the same species post-calving and a *S. chromogenes* IMI either prepartum or post-calving was associated with higher SCS after calving when compared to culture negative quarters. Among heifers identified with a NAS IMI either prepartum or post-calving, heifers that had teat skin colonized with *S. agnetis* or *S. chromogenes* had significantly increased odds of having the same species found in the prepartum mammary secretions, heifers with the inguinal region colonized with *S. chromogenes* were at increased odds of having *S. chromogenes* isolated from the prepartum mammary secretions, and heifers with

prepartum teat skin colonized with *S. chromogenes*, *S. simulans*, and *S. xylosus* were at increased odds of having an IMI with the same species post-calving.

Based on PFGE, a high level of strain diversity was found among isolates collected from body site samples. However, 44% of all heifers with a *S. chromogenes* IMI around the time of parturition were found to have the same strain isolated from a body site, including teat skin, inguinal skin, and the perineum. Conversely, no body site isolates were found to be the same strain as isolates from with IMI caused by *S. xylosus*. When strain-typing was applied to understanding the relationship between IMI pre- and post-calving and body site colonization, the picture was less clear than when the data were evaluated at just the species-level. Study of these relationships at the subspecies-level demonstrated that only some of the heifers that had an IMI with a given species had the same exact strain of that species isolated from a body site. These findings illustrate the need for subspecies identification of bacterial isolates to truly understand the epidemiology of staphylococcal IMI and the relationship of body site colonization.

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Table 1. Performance of MALDI-TOF for the identification and discrimination of NAS isolates of bovine origin. The table includes results with both the manufacturer's database (Bruker Daltonics; column labelled MALDI ID) and the manufacturer's database plus the MU laboratory custom database (column labelled MALDI ID with custom database).

NAS species	FINAL ID Total	MALDI ID Number	CI% ^a	MALDI ID with custom database Number	CI% ^a
<i>S. agnetis/hyicus</i> ^b	19	14	73.6%	19	100%
<i>S. arlettae</i>	5	0	0.0%	0	0.0%
<i>S. auricularis</i>	6	1	16.7%	1	16.7%
<i>S. chromogenes</i>	359	359	100%	359	100%
<i>S. cohnii</i>	1	0	0.0%	0	0.0%
<i>S. devriesei</i>	122	0	0.0%	122	100%
<i>S. equorum</i>	3	3	100%	3	100%
<i>S. gallinarum</i>	1	0	0.0%	0	0.0%
<i>S. haemolyticus</i>	169	113	66.9%	161	95.3%
<i>S. kloosii</i>	1	1	100%	1	100%
<i>S. nepalensis</i>	1	1	100%	1	100%
<i>S. pulvereri</i>	9	0	0.0%	0	0.0%
<i>S. saprophyticus</i>	6	6	100%	6	100%
<i>S. sciuri</i>	7	6	85.7%	6	85.7%
<i>S. simulans</i>	15	15	100%	15	100%
<i>S. xylosus</i>	46	46	100%	46	100%
TOTAL	770	562	72.9%	740	96.1%

^aCI% = Per cent correctly identified

^bMALDI-TOF was unable to differentiate *S. agnetis* from *S. hyicus* and will give a score of ≥ 2 for both. Hence, when either species is identified by MALDI-TOF, supplemental testing will be required to differentiate the two species. Percent correctly identified in the table reflects the ability of MALDI-TOF to at least classify the isolate as *S. agnetis* or *S. hyicus*.

Table 2. Proportion of prepartum and post-calving mammary quarters and of body sites positive to various NAS species from heifers identified with a NAS IMI at least once using one prepartum mammary secretion sample and two post-calving milk samples.

NAS species	Number quarters pre-fresh (%) ^a	Number quarters post-calving (%) ^b	Number of heifers inguinal region (%)	Number of heifers teat region (%)	Number of heifers muzzle region (%)	Number of heifers perineum region (%)
<i>S.chromogenes</i>	59 (14.8)	51 (12.8)	67 (80.7)	49 (59.0)	24 (28.9)	42 (50.6)
<i>S.agnetis</i>	12 (3.0)	5 (1.3)	4 (4.8)	4 (4.8)	0 (0)	0 (0)
<i>S.simulans</i>	6 (1.5)	5 (1.3)	4 (4.8)	4 (4.8)	0 (0)	0 (0)
<i>S.devriesei</i>	6 (1.5)	2 (0.5)	22 (26.5)	35 (42.2)	3 (3.6)	6 (7.2)
<i>S.haemolyticus</i>	4 (1.0)	3 (0.8)	35 (42.2)	36 (43.4)	14 (16.9)	20 (24.1)
<i>S.xylosus</i>	2 (0.5)	6 (1.5)	28 (33.7)	25 (30.1)	39 (47.0)	17 (20.5)
<i>S.epidermidis</i>	1 (0.3)	1 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S.hominis</i>	1 (0.3)	1 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S.warneri</i>	1 (0.3)	1 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S.gallinarum</i>	1 (0.3)	1 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S.equorum</i>	0 (0)	2 (0.5)	3 (3.6)	9 (10.8)	33 (39.8)	12 (14.5)
<i>S.sciuri</i>	0 (0)	1 (0.3)	13 (15.7)	24 (28.9)	32 (38.6)	7 (8.4)
<i>S.lentus</i>	0 (0)	1 (0.3)	3 (3.6)	4 (4.8)	5 (6.0)	1 (1.2)
<i>S.vitulinus</i>	0 (0)	1 (0.3)	3 (3.6)	4 (4.8)	4 (4.8)	1 (1.2)
<i>S.auricularis</i>	0 (0)	1 (0.3)	1 (1.2)	0 (0)	2 (2.4)	1 (1.2)
<i>S.arlettae</i>	0 (0)	1 (0.3)	0 (0)	6 (7.2)	7 (8.4)	2 (2.4)
<i>S.pasteuri</i>	0 (0)	1 (0.3)	0 (0)	0 (0.0)	0 (0)	0 (0)
<i>S.saprophyticus</i>	0 (0)	0 (0)	6 (7.2)	6 (7.2)	23 (27.7)	7 (8.4)
<i>S.nepalensis</i>	0 (0)	0 (0)	3 (3.6)	1 (1.2)	9 (10.8)	2 (2.4)
<i>S.cohnii</i>	0 (0)	0 (0)	1 (1.2)	1 (1.2)	13 (15.7)	10 (12.1)
<i>S.kloosii</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.2)
<i>S.succinus</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2.4)
Unidentified NAS	0 (0)	0 (0)	4 (4.8)	6 (7.2)	5 (6.0)	3 (3.6)
No NAS	308 (77.0)	320 (80.0)	4 (4.8)	0 (0)	4 (4.8)	16 (19.3)
Total	400	400	83	83	83	83

^a Quarters were considered positive to a given NAS species before calving if a NAS

species was recovered from a single quarter mammary secretion sample at concentration ≥ 100 cfu/ml.

^b Quarters were considered positive to a given NAS species after calving if the NAS species was recovered from either or both post-calving quarter-milk samples (i.e. parallel interpretation) at concentration ≥ 100 cfu/ml.

Table 3. Effect of prepartum and post-calving quarter IMI status on natural logarithm of the quarter-milk SCS at the second post-calving milk sampling. Estimates obtained using a linear mixed model with cow random intercept.

IMI status	Effect of prepartum IMI status		Effect of post-calving IMI status	
	Median SCS LSmean estimate (range)	<i>P</i> -value ^a	Median SCS LSmean estimate (range)	<i>P</i> -value ^a
Culture negative quarters	1.16	---	1.15	---
<i>S. agnetis</i>	1.99	0.87	2.47	0.79
<i>S. arlettae</i>	---	---	1.00	1.00
<i>S. auricularis</i>	---	---	3.10	0.99
<i>S. chromogenes</i>	2.18	0.05	3.10	<0.01
<i>S. devriesei</i>	0.71	0.99	---	---
<i>S. equorum</i>	---	---	3.32	0.89
<i>S. haemolyticus</i>	2.29	0.97	1.00	1.00
<i>S. simulans</i>	3.58	0.49	3.54	0.55
<i>S. vitulinus</i>	---	---	1.70	1.0
<i>S. warneri</i>	0.90	1.0	0.70	1.0
<i>S. xylosus</i>	6.80	0.71	1.94	1.0
<i>S. gallinarum</i>	2.70	1.00	2.70	1.0
Quarter with mixed NAS IMI	1.80	1.00	1.36	1.0

^a *P*-value for comparison of SCS natural logarithm between quarters with a given

prepartum or post-calving NAS IMI status and culture negative quarters at prepartum mammary secretion or post-calving milk sampling (i.e. culture negative on both post-calving samples). *P*-values were adjusted for multiple comparisons using the Tukey-Kramer method.

Table 4. Effect of having the inguinal skin, teat skin, muzzle, or perineal region colonized with a given NAS species on the odds of finding the same NAS-species causing a prepartum IMI. Estimates obtained using a generalized mixed model with cow random intercept.

NAS species	Inguinal region		Teat region		Muzzle region		Perineum	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
<i>S. agnetis</i>	1.8 (0.19, 18.3)	0.59	12.7 (3.3, 48.3)	<0.01	---	---	---	---
<i>S. chromogenes</i>	10.1 (1.8, 55.9)	<0.01	4.9 (1.8, 13.1)	<0.01	1.2 (0.43, 3.2)	0.74	1.4 (0.56, 3.5)	0.47
<i>S. devriesei</i>	0.54 (0.06, 4.7)	0.58	1.4 (0.27, 6.8)	0.72	5.6 (0.59, 52.5)	0.13	2.6 (0.29, 23.4)	0.39
<i>S. haemolyticus</i>	4.1 (0.42, 40.2)	0.23	1.3 (0.18, 9.3)	0.81	---	---	1.0 (0.11, 10.2)	0.98
<i>S. simulans</i>	---	---	---	---	---	---	---	---
<i>S. xylosus</i>	1.9 (0.12, 31.7)	0.64	2.3 (0.14, 37.5)	0.56	---	---	3.9 (0.24, 63.5)	0.34

S. arlettae, *S. auricularis*, *S. cohnii*, *S. equorum*, *S. kloosii*, *S. lentus*, *S. nepalensis*, *S. pasteurii*, *S. saprophyticus*, *S. sciuri*, *S. succinus*, and *S. vitulinus* were not isolated from prepartum mammary secretion samples.

S. epidermidis, *S. hominis*, *S. kloosii*, *S. pasteurii*, *S. warneri*, and *S. gallinarum* were not isolated from any body sites.

--- Indicates that association could not be estimated due to complete or almost complete separation of the data.

Table 5. Effect of having the inguinal skin, teat skin, muzzle, or perineal region colonized with a given NAS species on the odds of finding the same NAS-species causing a post-calving IMI. Estimates obtained using a generalized mixed model with cow random intercept.

NAS species	Inguinal region		Teat region		Muzzle region		Perineum	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
<i>S. agnetis</i>	---	---	5.1 (0.53, 49.3)	0.16	---	---	---	---
<i>S. arlettae</i>	---	---	---	---	---	---	---	---
<i>S. auricularis</i>	---	---	---	---	---	---	---	---
<i>S. chromogenes</i>	1.9 (0.61, 6.1)	0.26	2.9 (1.2, 7.0)	0.02	1.4 (0.55, 3.4)	0.50	1.1 (0.50, 2.6)	0.77
<i>S. devriesei</i>	---	---	---	---	---	---	---	---
<i>S. equorum</i>	---	---	---	---	---	---	---	---
<i>S. haemolyticus</i>	2.7 (0.24, 30.6)	0.42	2.6 (0.23, 29.1)	0.44	2.4 (0.21, 27.5)	0.47	1.6 (0.14, 17.6)	0.72
<i>S. lentus</i>	---	---	---	---	---	---	---	---
<i>S. sciuri</i>	---	---	---	---	---	---	---	---
<i>S. simulans</i>	---	---	217.9 (4.7, >999)	0.01	---	---	---	---
<i>S. vitulinus</i>	---	---	---	---	---	---	---	---
<i>S. xylosus</i>	4.3 (0.53, 34.8)	0.17	13.4 (1.2, 147.4)	0.03	2.3 (0.25, 20.2)	0.47	1.6 (0.13, 20.4)	0.70

S. cohnii , *S. kloosii* , *S. nepalensis* , *S. pasteurii* , *S. saprophyticus* , and *S. succinus* were not isolated from post-calving milk samples.

S. epidermidis, *S. hominis*, *S. kloosii*, *S. pasteurii* , *S. warneri* , and *S. gallinarum* were not isolated from any body sites.

--- Indicate that association could not be estimated due to complete or almost complete separation of the data.

Table 6. Distribution of isolates subjected to PFGE strain-typing according to NAS species and source of isolate. Data are the number of isolates that yielded a PFGE strain-type. Parenthetic data in columns 3-7 represent the number of isolates within category that were declared untypeable based on no defined banding pattern after repeat analysis.

Species	No. of Heifers	No. of Isolates					Total PFGE (% of 511)	Total w/ No PFGE (% of 40)
		IMI	Inguinal Skin	Teat Skin	Muzzle	Perineum		
<i>S. chromogenes</i>	28	71	155 (1)	90 (2)	15	50 (1)	381 (75.0%)	4 (10.0%)
<i>S. agnetis</i>	4	6	1	16 (1)	0	0	23 (4.5%)	1 (2.5%)
<i>S. haemolyticus</i>	5	5	10	12	1	2	30 (5.9%)	0 (0.0%)
<i>S. simulans</i>	2	12 (1)	0	10	0	0	22 (4.3%)	1 (2.5%)
<i>S. xylosus</i>	5	6 (3)	13	9	7 (10)	3 (4)	38 (7.4%)	17 (42.5%)
<i>S. devriesei</i>	2	2 (1)	0 (6)	10 (10)	1	2	15 (2.9%)	17 (42.5%)
<i>S. sciuri</i>	1	1	0	0	1	0	2 (0.4%)	0 (0.0%)
Total	47*	103 (5)	179 (7)	147 (13)	25 (10)	57 (5)	511	40

*Total exceeds 44 because 3 heifers had IMIs with different species in different mammary quarters.

Table 7. PFGE strain typing data, including numbers and proportions, of quarters with prepartum and/or post-calving IMI and a body site positive with the same species.

NAS species	# of heifers	# of quarters	# of quarters with pre- and post-calving IMI with the same species	# of quarters with pre- and post-calving IMI with the same strain (%)	# of quarters from all IMI with a body site sample of the same strain (%)	# quarters with a prepartum IMI and a body site with the same strain	# of quarters with a post-calving IMI and body site with the same strain	# of quarters with an IMI and no body site with the same strain (%)	Body sites in which the same strain was identified in an IMI depicted by body site (n, %)
<i>S. chromogenes</i>	28	45	15	12 (80%)	20 (44%)	13	14	25 (56%)	Teat (5, 25%) Inguinal (7, 35%) Teat and inguinal (6, 30%) Inguinal and perineum (2, 20%)
<i>S. agnetis</i>	4	6	0	0	4 (67%)	4	0	2 (33%)	Teat (4, 100%)
<i>S. haemolyticus</i>	5	5	0	0	1 (20%)	1	0	4 (80%)	Inguinal (1, 100%)
<i>S. simulans</i>	2	5	3	3 (100%)	2 (40%)	2	1	3 (60%)	Teat (2, 100%)
<i>S. xylosus</i>	5	5	0	0	0	0	0	5 (100%)	0
<i>S. devriesei</i>	2	2	0	0	1 (50%)	1	NS	1 (50%)	Teat and perineum (1, 100%)
<i>S. sciuri</i>	1	1	0	0	0	0	0	1 (100%)	0

Figure 1. Distribution of SCS on second post-calving sampling across prepartum quarter NAS IMI status (control = mammary quarters that were culture negative on both post-calving samplings).

Figure 2. Distribution of SCS on second post-calving sampling across post-calving quarter NAS IMI status (control = mammary quarters that were culture negative on both post-calving samplings).

Figure 3. PFGE dendrogram including all *S. chromogenes* isolates (n = 26) from one heifer. Within this heifer, 13 different strain types were identified based on a 100% similarity cut point. The RRPF (right rear prepartum) sample was a unique strain, the LRPF (left rear prepartum) was 100% identical to the teat3 isolate, the RRF1 (right rear post-calving sample 1) was a unique strain, and the RFPF was 100% identical to the inguinal8 isolate. While many of the isolates within body site were replicates of the same strain, some were not, illustrating the need for extensive storage of isolates (in this case 10 isolated colonies per site), but also illustrating the issues with sensitivity and specificity when studying body site colonization.

CHAPTER SEVEN

RUNNING TITLE: STAPHYLOCOCCAL COLONIZATION IN HEIFERS

CROSS-SECTIONAL STUDY OF TEAT AND INGUINAL SKIN COLONIZATION
OF DAIRY HEIFERS WITH VARIOUS STAPHYLOCOCCAL SPECIES

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ABSTRACT

The purpose of this study was to describe the prevalence and distribution of staphylococcal species on the teat and inguinal skin of dairy heifers across the various stages of the heifer lifecycle. The cross-sectional study included 106 Holstein heifers with an age range of 0 d-27 mo that were selected from 11 different farm locations on a single dairy operation. A composite swabbing sample including all four teats and a second composite sample including both inguinal regions of each heifer were collected using gas sterilized electrostatic dusters (Swiffers®). Swabbing samples were mixed with 10 ml of sterile saline, agitated, and cultured on Mannitol Salt Agar (MSA). At 24 h, MSA plates were read and up to 10 staphylococcal colonies were saved for further analysis. Staphylococcal isolates were speciated using matrix-assisted laser desorption ionisation-time of flight mass spectrometry or PCR amplification and sequencing of the *rpoB* or *tuf* gene. To investigate whether the prevalence of staphylococci was different between the inguinal and teat region, the chi-square or Fisher's exact tests were conducted, as applicable. Logistic regression models were used to investigate the relationship between heifer's age (treated as a quantitative continuous variable) and the probability of having a given body site (inguinal region or teats) colonized with a certain staphylococcal species. Overall, the most common species identified was *S. haemolyticus*, followed by *S. chromogenes*, *S. xylosus*, *S. devriesei*, and *S. sciuri*. *Staphylococcus aureus* was more prevalent on the teat than in the inguinal region while *S. arlettae* was more prevalent in the inguinal region than on the teat. All other staphylococcal species were as likely to be found on the teat skin as inguinal region skin.

Colonization of the inguinal region and teat skin was associated with age for *S. agnetis*, *S. chromogenes*, *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, *S. vitulinus*, and *S. xylosus*. The probability of *S. chromogenes* and *S. agnetis* colonization on the teat and inguinal region increased with age while the probability of *S. devriesei* and *S. haemolyticus* decreased with age. This study provides further insight into the ecology of staphylococcal species involved in heifer mastitis.

Key words: staphylococci, body site colonization, heifer, MALDI-TOF

INTRODUCTION

Coagulase negative staphylococcal (CNS) species are the most common cause of subclinical mastitis in dairy heifers around the time of parturition (Fox, 2009, De Vliegher et al., 2012). The true impact of CNS intramammary infection (IMI) is still under debate, with descriptions ranging from a protective effect to an association with mild clinical mastitis (Taponen et al., 2006, Piepers et al., 2013). Some of the variability in the effect of CNS on the mammary gland may be associated with species differences. Recent research using molecular identification methods has demonstrated a large diversity among CNS species that can be found on the dairy farm as well as differences between species with regard to inflammation, persistence of infection, and ecological niches on the farm (Piessens et al., 2011, Supre et al., 2011, De Visscher et al., 2014, Fry et al., 2014). Different extramammary (defined as outside of the mammary gland) habitats in which CNS have been found in abundance include floor samples, air samples, used bedding samples, and bovine body sites, including the perineum, udder skin, and teat ends (Taponen et al., 2008, Piessens et al., 2011, De Visscher et al., 2016b).

With regard to body site colonization, the microbial population of the teat end has been most thoroughly investigated (Braem et al., 2012, Braem et al., 2013, De Visscher et al., 2016b). The bacterial diversity on the skin of the teat apices has been described and includes four bacterial phyla, specifically Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes (Braem et al., 2012). The most commonly present and frequently isolated microbiota on the teat apices are CNS (Braem et al., 2013). With regard to heifers

specifically, teat apices of pregnant heifers have been found to be predominately colonized by *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus devriesei*, and *Staphylococcus equorum* (De Visscher et al., 2016b). Teat apex colonization with *S. chromogenes* in late term heifers has been shown to significantly increase the odds of *S. chromogenes* IMI at parturition (De Visscher et al., 2016a). This finding has also been demonstrated for other host-adapted staphylococcal species, specifically *Staphylococcus aureus* (Roberson et al., 1994). Although *S. chromogenes* IMI has been linked to elevated SCC and persistent infections (Fry et al., 2014, Supre et al., 2011), teat apex colonization with *S. chromogenes* may also be protective, as it has been found to protect quarters against $\text{SCC} \geq 200,000 \text{ cells/ml}$ in early lactation (De Vlieghe et al., 2003).

Few studies have looked at colonization of other body sites, besides the teat end, with CNS species. One study, in which extramammary sites of lactating cows were evaluated, including udder skin, teat apices, perineum, and streak canal, found *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosus* to be the predominant extramammary species (Taponen et al., 2008). Another study specifically evaluated body sites, including nares, hair coat, vagina, teat skin, and streak canal, of heifers of different age groups (White et al., 1989). In that study, phenotypic speciation was used and found *S. xylosus*, *S. chromogenes*, *Staphylococcus warneri*, and *S. sciuri* to be the predominant species (White et al., 1989). Furthermore, that study demonstrated that as heifers increase in age, the recovery of *S. xylosus* and *S. sciuri* decreases, whereas *S. chromogenes* and *S. warneri* populations increase (White et

al., 1989). When investigating *S. chromogenes* specifically, others have also found that the chance of heifers having at least one teat apex colonized increases significantly with age (De Vlieghe et al., 2003). This shift in CNS flora identified on body sites of heifers of different age groups has not been reexamined since molecular typing strategies have improved. Phenotypic identification methods are based on bacterial metabolic activity and morphologic features, which can show variability due to environmental changes such as culture conditions, sub-culturing, and storage (Sauer and Kliem, 2010). Also, interpretation of phenotypic tests can be subjective (Carretto et al, 2005). Therefore, it has been recommended that phenotypic speciation methods be abandoned and replaced by molecular speciation methods (Vanderhaeghen et al, 2015). Additionally, since the White et al (1989) study, several new CNS species have been named, including several that have been identified on dairy farms, specifically *Staphylococcus nepalensis* (Spergser et al, 2003, Zadoks and Watts, 2009), *Staphylococcus devriesei* (Supre et al, 2010), and *Staphylococcus agnetis* (Taponen et al, 2012). Furthermore, the relationship between CNS colonization of teat skin and inguinal skin in various heifer age groups has not been fully examined. Previous research investigating body site colonization with *S. aureus* has specifically considered teat skin separately from the sides of the udder, and no *S. aureus* was found on udder sides (Matos et al., 1991). However, others have found that 47% of the *S. aureus* strains found on the teat and udder skin were the same strains isolated from heifer mammary secretions (Middleton et al, 2002). The colonization of the inguinal region with other staphylococcal species has not been evaluated. Therefore, the purpose of this study was to describe the prevalence and distribution of

staphylococcal species on the teat and inguinal skin of dairy heifers by examining a cross section of animals in various stages of the heifer lifecycle on one farm.

MATERIALS AND METHODS

Herd and heifer selection

A cross-sectional study including Holstein heifers was conducted at University of Missouri dairy research farm between March and June 2015. At the time of sampling, the farm milked a total of 160 Holstein and 24 Guernsey cows. During the sampling months, the Holstein herd had a geometric mean SCC of 211,000 cell/ml and an average 305-day milk production of 11,767 kg. All heifers (n = 229) are raised on the farm. Calving occurs year round with the exception of a planned reduction in calving from July to September. This study was approved by the University of Missouri Animal Care and Use Committee.

Heifers ranging in age from 0 days to 821 days (27 months) were selected from 11 different farm locations (Table 1). The sampling strategy was planned to include approximately 50% of the heifers on the farm and the number of heifers to be sampled per housing group was preselected to try to get equal representation of each housing type and age group. The planned samples included a total of 120 heifers, the median number of planned samples per group was 10 with a range of 6-15. Heifers within each group were selected for sampling using Microsoft Excel (2013) random number generator. Selected heifers were only sampled once.

Sampling procedure

The heifers were restrained in a chute for sample collection. Body site swabbing samples included a composite sample of the skin surface of all four teats and a second composite sample combining the skin surface of both inguinal regions of each heifer. Prior to sample collection, visible debris was removed. Samples were collected using gas sterilized electrostatic dusters (Swiffers®). Gloves were worn at all times and were changed between each sample collected. All body site swabbing samples were chilled on ice for transport to the laboratory and processed the same day.

Laboratory procedures

Swabbing samples were mixed with 10 ml of sterile saline, agitated, and 50 µl of the solution were plated on Mannitol Salt Agar (MSA; Remel, Lenexa, KS, USA) and incubated for at 37°C for 24 h. At 24 h, MSA plates were read and up to 10 staphylococcal colonies, including at least one of each morphologically distinct colony type, were sub-cultured onto Columbia Blood Agar (CBA; Remel, Lenexa, KS, USA), and then stored in phosphate buffered glycerol at -20°C until further processing.

Staphylococcal isolates were speciated using matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (Tomazi et al., 2014). Briefly, isolates for the MALDI-TOF target plate were prepared using the plate extraction method. One colony of each isolates was spotted on the plate in duplicate. Spots were overlaid with 1 µl of 70% formic acid. Once dried, each spot was overlaid with 1 µl of matrix solution, consisting of α -cyano-4-hydroxy-cinnamic acid diluted in a solution of 50% acetonitrile

and 2.5% trifluoroacetic acid. The Bruker bacterial test standard was used to calibrate the instrument prior to running test samples. Samples were analyzed on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using flex control software (Bruker Daltonics). Peaks produced by each isolate were compared with entries in the Biotyper database (Bruker) and the University of Missouri custom database (which included bovine reference isolates of *S. devriesei* (n = 1), *S. agnetis* (n = 1), and *S. haemolyticus* (n = 1)) using the standard parameters of the pattern-matching algorithm. The three species in the custom database were added because of low typeability relative to the reference strains in the Bruker database or absence from the Bruker database. Custom database entries were made following the manufacturer's described methods in which 100 known isolates of each species added to the custom database were run against the reference isolate in the custom database to validate that the reference isolate accurately identified each of the 100 known isolates. Species identification cut-off values were applied according to the manufacturer's instructions in which a score of ≥ 2 indicated a species-level identification. Any score < 2 was classified as inconclusive for species identification.

Any isolate that was not conclusively identified by MALDI-TOF was speciated using PCR amplification and Sanger sequencing of a portion of the *rpoB* or *tuf* gene. Briefly, isolates were sub-cultured from storage media on CBA at 37°C for 24 h and a single colony was inoculated into 100 μ l of 1X Tris-EDTA (Promega, Madison, WI, USA) for lysate preparation. Standard PCR was performed to amplify the *rpoB* (Drancourt and Raoult, 2002) or *tuf* (Hwang et al., 2011) gene. The resultant PCR products were purified

using a commercial kit (Invitrogen, Carlsbad, CA, USA) and submitted to the University of Missouri DNA Core Facility for Sanger sequencing. Sequences were compared to the GenBank database using the publicly available nucleotide-BLAST algorithm (www.ncbi.nlm.nih.gov). For *rpoB* analysis, species identity was confirmed if the sequence had $\geq 97\%$ identity with a single staphylococcal species of in the GenBank database (Heikens et al., 2005, Mellmann et al., 2006). For *tuf* gene analysis, species were confirmed if sequence identity was $\geq 98.0\%$ with $> 0.8\%$ separation between species (Hwang et al., 2011).

Statistical analyses

The prevalence of the various staphylococcal species identified was cross-tabulated by body site. To investigate whether staphylococcal prevalence was different between the inguinal and teat region, chi-square tests were conducted. Whenever a cell with an expected frequency < 5 was observed, a Fisher's exact test was used instead.

Logistic regression models were used to investigate the relationship between heifer's age (treated as a quantitative continuous variable) and the probability of having a given body site (i.e. inguinal region or teat) colonized with a certain staphylococcal species. In these models, outcome was colonization or absence of colonization of a given body site with a given staphylococcal species; one logistic model was, therefore, used for each staphylococcal species (provided that the number of isolates observed was sufficient to achieve convergence of the model) and analyses were conducted independently for the

inguinal and teat regions. To fully model the relationship between log odds of colonization and heifer's age, the relationship was always initially modelled using polynomial age terms (i.e. age, age², and age³). Whenever the coefficient of the age³ term yielded a *P*-value > 0.05, the relationship was deemed to have ≤ one inflexion point and the age³ term was removed from the model. Then, if the coefficient of the age² term yielded a *P*-value > 0.05, the relationship was deemed to be linear and the age² term was also removed. If the coefficient for age also yielded, after removal of the age³ and age² terms, a *P*-value > 0.05, then the log odds of body site colonization was deemed to be constant across heifer's age. To avoid collinearity issues, all age terms were centered around 365 days and divided by 30, so that the odds ratio (OR) reported would indicate relative increase or decrease in odds of colonization for each additional 30 days of age.

RESULTS

The total number of heifers sampled was 106, which was 46% of the total heifer population on the farm. The median number of heifers sampled per farm location was 10 (range: 3-15). Less than the planned number of animals were sampled in some groups due to fewer total numbers of heifers than expected being available in those locations at the time of sampling. Classifying the heifers based on age, 19% (20/106) were 0d-2 mo, 15% (16/106) were 2-6 mo, 24% (25/106) were 6-12 mo, 22% (23/106) were 12-18 mo, and 21% (22/106) were >18 mo.

A total of 2,044 staphylococcal isolates from 212 body sites were evaluated. At least one staphylococcal species was identified from all 212 body sites. The MALDI-TOF mass

spectrometer was able to identify 90.3% of the isolates and 9.7% could not be identified with this method. Five isolates (0.7%) could not be identified to the species level using any of the speciation methods described above. These isolates were classified as unidentified staphylococci. Due to replication of a single species on the same body site, 1,361 replicates from the original 2,044 body site isolations were eliminated from the data set for the purpose of analysis, leaving 683 occurrences of body site colonization. Of the 683 occurrences, 344 were collected from the inguinal region with the median number of species per inguinal sample being 3 (range: 1-6). A total of 339 occurrences were collected from the teat skin with the median number of species per teat skin sample also being 3 (range: 1-7). A total of 17 different staphylococcal species were identified.

Assessing species distribution based on body site location found few differences (Table 2). *Staphylococcus aureus* was more prevalent on the teats than in the inguinal region ($P < 0.01$). *Staphylococcus arlettae* was more prevalent in the inguinal region than on the teats ($P < 0.05$). All other staphylococcal species were as likely to be found on the teat skin as the inguinal region skin. *Staphylococcus cohnii*, *S. epidermidis*, and *S. succinus* were each only identified once and were only found on the teat skin.

Overall, the most common species identified was *S. haemolyticus*, followed by *S. chromogenes*, *S. xylosus*, *S. devriesei*, and *S. sciuri* (Table 2). Less commonly identified species included *S. equorum*, *Staphylococcus lentus*, *Staphylococcus vitulinus*, *Staphylococcus agnetis*, *Staphylococcus arlettae*, *S. aureus*, *S. simulans*, *Staphylococcus saprophyticus*, and *Staphylococcus nepalensis*. The proportion of heifers of a given age

colonized by a given species varied for both the inguinal region (Table 3) and teat skin (Table 4). The most commonly identified species in the inguinal region of heifers < 2 mo of age (n = 20 heifers) were *S. xylosus*, *S. lentus*, *S. sciuri*, and *S. vitulinus*. In heifers < 2 mo of age, the most commonly identified species on the teat skin were *S. xylosus*, *S. sciuri*, and *S. equorum*. In heifers 2-6 mo of age (n = 16), the most commonly identified species in the inguinal region were *S. haemolyticus*, *S. sciuri*, and *S. devriesei* while the most commonly identified species on the teat were *S. haemolyticus*, *S. sciuri*, and *S. xylosus*. In heifers 6-12 mo of age (n = 25), the most commonly identified species in the inguinal region were *S. haemolyticus*, *S. chromogenes*, and *S. xylosus* and the most commonly identified species on the teat was *S. haemolyticus*, *S. chromogenes*, and *S. devriesei*. In heifers 12-18 mo of age (n = 23), the most commonly identified species in the inguinal region were *S. haemolyticus*, *S. chromogenes*, *S. devriesei*, and *S. xylosus* and the most commonly identified species on the teat were *S. haemolyticus*, *S. chromogenes*, and *S. devriesei*. In heifers > 18 mo of age (n = 22 heifers), the most commonly identified species in the inguinal region were *S. chromogenes*, *S. haemolyticus*, *S. devriesei*, and *S. xylosus*. On the teat skin of heifers > 18 mo, the most commonly identified species were *S. chromogenes*, *S. haemolyticus*, and *S. devriesei*.

Based on the logistic regression models, for many species, colonization of the inguinal and teat region with a particular staphylococcal species was associated with age of the heifer (Figures 1 and 2). Specifically, colonization of the inguinal region and teat skin was associated with age for *S. agnetis*, *S. chromogenes*, *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, *S. vitulinus*, and *S. xylosus*. In the inguinal region, the

probability of being colonized with *S. agnetis*, *S. chromogenes*, and *S. xylosus* increased with age while the probability of colonization with *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, and *S. vitulinus* decreased with age. On the teat skin, the probability of being colonized with *S. agnetis* and *S. chromogenes* increased with age while the probability of colonization with *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, *S. vitulinus*, and *S. xylosus* decreased with age. *Staphylococcus xylosus* was the only species that differed between sites, with an increase in probability of colonization in the inguinal region as age increased and a decrease in the probability of colonization on the teat skin as age increased. The odds of having the teat or inguinal region colonized by *S. arlettae*, *S. cohnii*, *S. epidermidis*, *S. nepalensis*, *S. saprophyticus*, *S. simulans*, or by unidentified staphylococci, was not affected by age of the heifer.

DISCUSSION

A very diverse population of staphylococcal species was found on the teat and inguinal region of heifers. This is in agreement with previous publications which have found staphylococcal species to be the most commonly present and frequently isolated microbiota on the teat end of lactating cows (Braem et al., 2013) and others which identified 24 different CNS species on the teat apices of prepartum cows and heifers (De Visscher et al., 2016b). Interestingly, colonization of the teat skin was as likely as colonization of the inguinal region for most of the isolated staphylococci with the exception of *S. aureus* and *S. arlettae*. *Staphylococcus aureus* was found to be more prevalent on the teat skin than in the inguinal region. This is in agreement with one previous study, which did not identify any *S. aureus* on the udder sides of heifers, but did

find it in heifer teat skin samples (Matos et al., 1991). In contrast, *Staphylococcus arlettae* was found to be more prevalent in the inguinal region than on the teat skin in the current study. These data are similar to previous studies which found that among teat end samples *S. arlettae* was rarely identified (De Visscher et al., 2016b) and in lactating cows more *S. arlettae* isolates were found on the udder skin than the teat apex (Taponen et al., 2008).

To the best of the authors' knowledge, this is the first study to evaluate the presence of all currently known staphylococcal species on the body sites of heifers from various age groups. The results of this study show that the staphylococcal populations change with age. Host factors, such as hormone production and skin moisture content, can contribute to the variability seen in the microbial flora of the skin (Grice et al, 2011). Additionally, environmental factors, including bedding type and sun exposure, can also play a role in the changes noted within the skin bacterial populations. In the present study, some important staphylococcal species previously identified as host-adapted and frequently reported to be associated with IMI, such as *S. chromogenes*, became more prevalent as heifers age. This finding is in agreement with previous publications (White et al., 1989, De Vliegher et al., 2003). The probability of teat skin and the inguinal region being colonized with *S. agnetis* also increased with age in this study. Interestingly, this species was not identified in the inguinal region or on the teat skin of heifers < 6 mo of age. *Staphylococcus agnetis* is a newly described species and currently little is known about the ecology of this organism, however it has been previously isolated from teat apex samples (Taponen et al., 2012, De Visscher et al., 2016b). Additionally, the finding that

the probability of *S. xylosus* and *S. sciuri* colonization of the teat skin decreases with age has also been previously described (White et al., 1989). However, in this study, the opposite is true for the colonization of the inguinal region with *S. xylosus*, which was found to increase with age. A previous study also found *S. warneri* colonization increased with age and this species was the most prevalent CNS isolated from the body sites of heifers on pasture (White et al., 1989). In the present study, *S. warneri* was not identified.

Overall, *S. aureus* was not frequently identified on udder skin in the present study. *Staphylococcus aureus* only comprised 1.6% of the total isolates. Previous studies demonstrate a broad range of prevalences, ranging from 0-36.5% for teat and udder skin colonization with *S. aureus* (Matos et al., 1991, Roberson et al., 1994, Middleton et al., 2002, Anderson et al., 2012) with herd-to-herd variation being reported (Middleton et al., 2002). One reason for the low prevalence of *S. aureus* in this study may be that isolation methods were not selective for any specific staphylococcal species, whereas previous studies have used *S. aureus* selective media (Roberson et al., 1994; Middleton et al., 2002). Using methods not specifically selective for *S. aureus* may have allowed heavier growth of other staphylococci that impaired the growth of less prevalent species in the sample precluding identification of some species such as *S. aureus*. Most studies that have characterized *S. aureus* on body sites have used media such as Vogel-Johnson or Baird Parker medium (Matos et al., 1991, Roberson et al., 1994). Although these selective media should allow growth for most staphylococcal species, at this time, MSA is the only staphylococcal specific media that has been shown to be suitable for growth

and recovery of bovine associated CNS (De Visscher et al., 2013). While reported as a suitable method, the use of MSA for the isolation of CNS is not perfect for isolation of all staphylococcal species. Previous work has found that *Staphylococcus auricularis* type strain ATCC 33753, *Staphylococcus caseolyticus*, and *S. warneri* all failed to grow on MSA (White, 1988). A more recent study found that the *S. auricularis* type strain and *S. warneri* did grow on MSA, however some strains of *S. equorum*, *Staphylococcus hominis* and *Staphylococcus lugdunensis* showed delayed growth (De Visscher et al., 2013). With the exception of *S. equorum*, none of the mentioned species were found in this study. This could be due to poor growth on MSA or lack of those species on the body sites of the heifers tested in this study. No previously tested selective staphylococcal media has been found to support the growth of all *Staphylococcus* spp. (White, 1988). Columbia blood agar or other non-selective media would be the best choice to allow growth of all staphylococcal species; however, lack of selectivity allows growth of most environmental organisms and can lead to heavy overgrowth of culture plates.

One limitation of the present study is that sampling was only done on one farm. On the farm of study, heifers were kept in certain housing locations until a specific age. Therefore, it is impossible to separate the roles of changing housing environments from age and thus determine the influence of housing strategy on changes in staphylococcal body site colonization versus the influence of increasing age of the heifers. This same limitation has been noted in previous studies (White et al., 1989, Taponen et al., 2008). Although the present study only took place on one farm and therefore the generalizability of the data might be questionable, several findings corroborate previous reports from

Belgium and one from Kentucky (White et al., 1989, De Vlieghe et al., 2003, De Visscher et al., 2016b). For example, the finding that *S. chromogenes*, *S. devriesei*, and *S. haemolyticus* were the most commonly identified species on the teat skin of heifers > 18 mo of age agrees with a recent study looking at teat apex colonization in dry cows and late gestation heifers on 13 commercial dairy farms in Belgium (De Visscher et al., 2016a). Also, the finding of very few *S. epidermidis* is corroborated by others who did not find *S. epidermidis* on prepartum heifer teat apices (De Visscher et al., 2016b), and reported that subclinical mastitis associated with *S. epidermidis* was more common in older cows than in first lactation cows (Thorberg et al., 2009). Further, others have found *S. epidermidis* to be a potentially host-adapted pathogen that survives inside the udder (Piessens et al., 2011). Overall, the study conducted in Kentucky (White et al, 1989) was very similar to the present study. Similar findings between the two studies include the finding that the probability of the teat skin being colonized with *S. xylosus* and *S. sciuri* decreased as heifer age increased and the probability of teat skin colonization with *S. chromogenes* increased as heifer age increased. Although there were some similar findings, one notable difference between the two studies is the difference in the diversity of species, as White et al. (1989) found a total of 10 different species while the present study identified 17 different species. This difference is likely due to differences in speciation method, using molecular methods in the present study and phenotypic speciation in the White et al. (1989) study, and the increase in number of named staphylococcal species since 1989 (Becker et al, 2014). In 1990, 25 staphylococcal species had been named. Today, there are approximately 47 species and 23 subspecies of staphylococci that have been characterized (Becker et al, 2014). Several of the more

recently classified species were found to be prevalent in this study, including *S. agnetis*, *S. devriesei*, and *S. nepalensis*. Future work assessing staphylococcal species on heifers of different age groups on several different farms would help further the understanding of the ecology of these organisms and potentially identify specific environmental risk factors for colonization.

CONCLUSION

The most common staphylococcal species identified on teat and inguinal samples from heifers of multiple age groups were *S. haemolyticus*, *S. chromogenes*, and *S. xylosus*. Species distribution based on body site location did not differ for most species, except *S. aureus* and *S. arlettae*. The probability of *S. chromogenes* and *S. agnetis* colonization on the teat and inguinal region increased with age while the probability of *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, and *S. vitulinus* decreased with age. *Staphylococcus xylosus* was the only species identified to have different probabilities of isolation based on location, with the probability of colonization of the inguinal region increasing with age and the probability of colonization of the teat skin decreasing with age. This study provides further insight into the ecology of staphylococcal species that cause bovine mastitis. The role and impact of CNS species colonizing the teat and inguinal region of heifers of different age groups remains to be elucidated and the relationship to IMI and mastitis are the subject of other studies.

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Table 1. Housing characteristics for the different groups (n = 11) of heifers included in the study.

Group	Age	Grouping characteristics	Bedding type
New born calves	0 d – 3 d	Individually housed, within maternity barn	straw on concrete
Hutch calves	1 d -2.4 mo	Individually housed	straw covered rock
Weaned heifers	2.6 mo - 5.6 mo	5 groups, 6-7 heifers in each group	pasture and shelter bedded with straw
Pasture 1	5.6 mo - 6.5 mo	1 group	pasture and shelter with manure pack
Pasture 2	6.5 mo - 8.9 mo	1 group	pasture and shelter with manure pack
Pasture 3	9.8 mo - 13.6 mo	1 group	pasture and shelter with manure pack
Pasture 4	10.8 mo – 16 mo	1 group	pasture only
Breeding age heifers	14.9 mo – 18 mo	1 group	Free stalls with sand bedding
Early bred heifers	15.7 mo - 19.2 mo	1 group	pasture and shelter with manure pack
Confirmed pregnant heifers	15.3 mo – 28 mo	1 group	pasture and shelter with manure pack
Late term pregnant heifers	21.7 mo – 27 mo	1 group, mixed with cows	pasture and sand bedded free stalls

Table 2. Proportion of heifers with inguinal region or teat skin being colonized by various staphylococcal species on 106 heifers from one farm and aged between one day and 28 months.

Staphylococcal species	Total Number of isolates (%)	Number of heifers (%)		<i>P</i> -value ^a
		Inguinal	Teat	
<i>S. haemolyticus</i>	164 (24.0)	83 (78.3)	81 (76.4)	0.74
<i>S. chromogenes</i>	154 (22.5)	78 (73.6)	76 (71.7)	0.76
<i>S. xylosus</i>	84 (12.3)	43 (40.6)	40 (37.7)	0.57
<i>S. devriesei</i>	59 (8.6)	28 (26.4)	31 (29.3)	0.65
<i>S. sciuri</i>	57 (8.3)	28 (26.4)	29 (27.4)	0.88
<i>S. equorum</i>	33 (4.8)	14 (13.2)	19 (17.9)	0.34
<i>S. lentus</i>	32 (4.7)	17 (16.0)	15 (14.2)	0.7
<i>S. vitulinus</i>	29 (4.2)	14 (13.2)	14 (13.2)	0.84
<i>S. agnetis</i>	25 (3.7)	15 (14.2)	10 (9.4)	0.29
<i>S. arlettae</i>	16 (2.3)	11 (10.4)	4 (3.8)	0.04
<i>S. aureus</i>	11 (1.6)	1 (0.9)	10 (9.4)	<0.01
Unidentified staphylococci ^c	5 (0.7)	3 (2.8)	2 (1.9)	0.32 ^b
<i>S. saprophyticus</i>	4 (0.6)	1 (0.9)	3 (2.8)	0.25 ^b
<i>S. simulans</i>	4 (0.6)	3 (2.8)	1 (0.9)	0.25 ^b
<i>S. nepalensis</i>	3 (0.4)	2 (1.9)	1 (0.9)	0.37 ^b
<i>S. cohnii</i>	1 (0.1)	0 (0.0)	1 (0.9)	0.50 ^b
<i>S. epidermidis</i>	1 (0.1)	0 (0.0)	1 (0.9)	0.50 ^b
<i>S. succinus</i>	1 (0.1)	0 (0.0)	1 (0.9)	0.50 ^b

^a *P*-value for a chi-square test (unless otherwise specified) testing the association between prevalence of a given staphylococcal species and body site.

^b *P*-value for a Fisher's exact test testing the association between prevalence of a given staphylococcal species and body site. Fisher exact tests were used whenever expected frequency where < 5 in ≥ one cell.

^c Isolates for which staphylococcal species was still undetermined after MALDI-TOF, *rpoB* gene sequencing, and *tuf* gene sequencing.

Table 3. Proportion of heifers of a given age with inguinal region being colonized by various staphylococci species in 106 heifers from one farm and aged between one day and 28 months.

Staphylococcal species	Number of heifers (%)				
	<2 mo	2-6 mo	6-12 mo	12-18 mo	>18 mo
<i>S. agnetis</i>	0 (0.0)	0 (0.0)	5 (20.0)	5 (21.7)	5 (22.7)
<i>S. arlettae</i>	3 (15.0)	3 (18.8)	1 (4.0)	0 (0.0)	4 (18.2)
<i>S. aureus</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.6)
<i>S. chromogenes</i>	7 (35.0)	4 (25.0)	24 (91.3)	21 (91.3)	22 (100)
<i>S. cohnii</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. devriesei</i>	0 (0.0)	5 (31.3)	9 (36.0)	8 (34.8)	6 (27.8)
<i>S. epidermidis</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. equorum</i>	11 (55.0)	0 (0.0)	1 (4.0)	1 (4.4)	1 (4.6)
<i>S. haemolyticus</i>	3 (15.0)	15 (93.8)	24 (96.0)	21 (91.3)	20 (90.9)
<i>S. lentus</i>	13 (65.0)	3 (18.8)	1 (4.0)	0 (0.0)	0 (0.0)
<i>S. nepalensis</i>	1 (5.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. saprophyticus</i>	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. sciuri</i>	12 (60.0)	13 (81.3)	1 (4.0)	1 (4.4)	1 (4.6)
<i>S. simulans</i>	2 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.6)
<i>S. succinus</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. vitulinus</i>	12 (60.0)	2 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. xylosus</i>	15 (75.0)	4 (25.0)	10 (40.0)	8 (34.8)	6 (27.3)
Unidentified staphylococci ^a	0 (0.0)	1 (6.3)	0 (0.0)	2 (8.7)	0 (0.0)
Number of heifers in a given age category	20	16	25	23	22

^a Isolates for which staphylococcal species was still undetermined after MALDI-TOF,

rpoB gene sequencing, and *tuf* gene sequencing.

Table 4. Proportion of heifers of a given age with teat region being colonized by various staphylococci species in 106 heifers from one farm and aged between one day and 28 months.

Staphylococci specie	Number of heifers (%)				
	<2 mo	2-6 mo	6-12 mo	12-18 mo	>18 mo
<i>S. agnetis</i>	0 (0.0)	0 (0.0)	3 (12.0)	3 (13.0)	4 (18.2)
<i>S. arlettae</i>	1 (5.0)	0 (0.0)	1 (4.0)	1 (4.4)	1 (4.6)
<i>S. aureus</i>	1 (5.0)	0 (0.0)	1 (4.0)	5 (21.8)	3 (13.6)
<i>S. chromogenes</i>	8 (40.0)	2 (12.5)	25 (100)	21 (91.3)	20 (90.9)
<i>S. cohnii</i>	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. devriesei</i>	0 (0.0)	6 (37.5)	11 (44.0)	7 (30.4)	7 (31.8)
<i>S. epidermidis</i>	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. equorum</i>	14 (70.0)	0 (0.0)	2 (8.0)	1 (4.4)	2 (9.1)
<i>S. haemolyticus</i>	4 (20.0)	15 (93.8)	25 (100)	22 (96.7)	15 (68.2)
<i>S. lentus</i>	9 (45.0)	3 (18.8)	2 (8.0)	0 (0.0)	1 (4.6)
<i>S. nepalensis</i>	0 (0.0)	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
<i>S. saprophyticus</i>	2 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.6)
<i>S. sciuri</i>	15 (75.0)	11 (68.8)	1 (4.0)	1 (4.4)	1 (4.6)
<i>S. simulans</i>	0 (0.0)	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
<i>S. succinus</i>	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. vitulinus</i>	11 (55.0)	1 (6.3)	0 (0.0)	1 (4.4)	1 (4.6)
<i>S. xylosus</i>	16 (80.0)	7 (43.8)	5 (20.0)	6 (26.1)	6 (27.3)
Unidentified staphylococci ^a	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.4)	1 (4.6)
Number of heifers in a given age category	20	16	25	23	22

^a Isolates for which staphylococcal species was still undetermined after MALDI-TOF,

rpoB gene sequencing, and *tuf* gene sequencing.

Figure 1. Probability of colonization of the inguinal region by a given staphylococcal species as a function of age (months) of the heifer, for 106 heifers from one farm and aged between one day and 28 months.

Figure 2. Probability of colonization of the teat region by a given staphylococcal species as a function of age (months) of the heifer, for 106 heifers from one farm and aged between one day and 28 months.

CHAPTER 8

CONCLUSIONS

The all-encompassing objective of the studies included in this dissertation was to improve our understanding of the epidemiology of mastitis caused by staphylococcal species.

Staphylococci are the most common organisms isolated from cow's milk and, depending on species, can have an impact on milk quality and milk yield. Staphylococci can infect the mammary gland of not only lactating cows, but also heifers prior to parturition.

While a great deal of work has been done to understand staphylococcal mastitis in both heifers and cows, some knowledge gaps were apparent when reviewing the scientific literature.

Recent studies have illustrated the importance of defining the species of staphylococci infecting the cow's mammary gland rather than using the more simplistic historical approach of categorizing mastitis-causing staphylococci as *S. aureus* or coagulase negative *Staphylococcus* spp. (CNS). Further, with the exception of *S. aureus*, few studies have used subspecies identification methods (strain-typing) to truly understand host adaptation. The current gold standard method for species identification is some form of housekeeping gene sequence analysis, while the gold standard method for subspecies identification of staphylococci is still PFGE. These methods are relatively costly and time-consuming to perform. Hence, a portion of the dissertation explores alternative

methods to making a species and subspecies diagnosis in the context of understanding mastitis epidemiology on dairy farms.

As part of this exploration, the first study examines the whole genome sequence of *S. chromogenes*, the most prevalent non-*aureus* *Staphylococcus* isolated from cows' milk. The particular strain characterized was from a chronic IMI and several virulence genes were identified, including biofilm associated genes, which potentially helps explain the persistent nature of some *S. chromogenes* isolated from cow's milk. This whole genome sequence provided a published template for future comparative studies including some of the work herein. In collaboration with colleagues, whole genome sequences were also published for *S. agnetis* and *S. hyicus*, which facilitated an understanding of the genetic differences between these three organisms which could then be used for species identification, specifically within the *aroD* gene. Importantly, *S. agnetis* and *S. hyicus*, while closely related to *S. chromogenes*, are coagulase variable and thus may also be confused with the major coagulase positive mastitis pathogen, *S. aureus*. Further, routine housekeeping gene sequencing, such as 16S rRNA and *rpoB*, and MALDI-TOF are unable to differentiate between *S. agnetis* and *S. hyicus*. Hence, the focus of the second study was to develop a simple method to truly differentiate *S. agnetis* from *S. hyicus*. Using whole genome sequence data for *S. chromogenes*, *S. agnetis*, *S. hyicus*, and *S. aureus*, a unique multiplex PCR was developed that now provides a simple tool for differentiating *S. agnetis* from *S. hyicus* and *S. aureus* thus facilitating studies on the epidemiology of these two potential mastitis pathogens.

Recent work in Europe suggested that RS-PCR plus toxin gene identification might serve as a rapid method for strain-typing staphylococci, and the third study was aimed at using a population of well-characterized *S. aureus* isolates to not only evaluate universal applicability of the method, but also compare its discriminatory power against the gold standard of PFGE. The goal was to not only understand whether the Genotype B strain of *S. aureus* was detected among isolates from the U.S., but also determine whether RS-PCR plus toxin gene identification might be a more rapid method of subspecies identification for the longitudinal study in heifers (Chapter 6). Overall, Genotype B was not found in the U.S., and it was concluded that RS-PCR was less discriminatory than PFGE; hence, PFGE was used for subspecies identification in the remaining studies.

While a large body of work exists on heifer mastitis, two important gaps were identified. First, while studies have characterized the risk of IMI at calving relative to prepartum IMI or body site colonization, few studies, if any, have actually evaluated this relationship at the subspecies-level. Hence, the goal of the fourth study was to study the relationship between body site colonization, prepartum IMI, and post-calving IMI. A further aim was to use the isolates from this study to determine whether MALDI-TOF could accurately name staphylococci to the species-level thus providing a rapid and inexpensive method for speciation and reducing the need for more expensive and time consuming gene sequence-based methods.

Overall, MALDI-TOF proved to be accurate for the majority of staphylococcal species evaluated, including *S. chromogenes*, *S. xylosus*, *S. equorum*, *S. saprophyticus*, *S. sciruri*, and *S. simulans*. After adding *S. devriesei* and an additional *S. haemolyticus* isolate to the custom database, MALDI-TOF proved to be accurate for these species as well. After adding a *S. agnetis* isolate to the custom database, MALDI-TOF was unable to differentiate *S. hyicus* and *S. agnetis*. With all other staphylococcal species evaluated, MALDI-TOF was not found to misclassify isolates. Overall, the MALDI-TOF validation showed that it can be used as a first line screening tool for the speciation of staphylococcal isolates originating from dairy farms.

With regard to the epidemiology of peripartum IMI in heifers, *S. chromogenes*, *S. agnetis*, and *S. simulans* were the most common species identified in prepartum mammary secretions while *S. chromogenes*, *S. xylosus*, and *S. agnetis* were the most common species found in post-calving milk samples. The most common species identified from body site samples were *S. chromogenes*, *S. xylosus*, and *S. haemolyticus*. Prepartum mammary secretions that were culture positive for *S. agnetis*, *S. chromogenes*, and *S. devriesei* had significantly increased odds of having an IMI with the same species post-calving and a *S. chromogenes* IMI either prepartum or post-calving was associated with higher SCS after calving when compared to culture negative quarters. Among heifers identified with a NAS either prepartum or post calving, heifers that had teats colonized with *S. agnetis* or *S. chromogenes* had significantly increased odds of having the same species found in the prepartum mammary secretions, heifers with the inguinal region colonized with *S. chromogenes* were at increased odds of having *S. chromogenes*

isolated from the prepartum mammary sample, and heifers with prepartum teat skin colonized with *S. chromogenes*, *S. simulans*, and *S. xylosus* were at increased odds of having an IMI with the same species post-calving. Overall, 44% of all heifers with a *S. chromogenes* IMI around the time of parturition were found to be the same strain as a body site isolate. Conversely, no *S. xylosus* body site isolates were found to be the same strain as isolates associated with IMI. This study demonstrated the complexity of understanding the molecular epidemiology of the relevant NAS species, as many different strains of each species can be found on one heifer. One important finding in this study was the potential impact a prepartum IMI can have on post-calving IMI status, and in the case of *S. chromogenes*, on SCS status. However, these conclusions should be tempered by the fact that the strain of *S. chromogenes* causing the IMI may not be the same as the one found on the body site, and thus further investigation into the relationship of body site colonization with different strains and the risk of IMI is needed. If interventions or prevention strategies are pursued in the future, targeting the prepartum period is likely required.

While the relationship between body site colonization and peripartum IMI was defined in the fourth study, a second gap in our understanding of heifer mastitis is how body site colonization with staphylococci changes over the course of the heifer's life from birth to the onset of lactation. Hence, the final study was aimed at using modern speciation methods to understand heifer body site colonization with staphylococci throughout their lifecycle. In the fourth study, it was determined that teat and inguinal colonization were the most important body sites with regards to the relationship between colonization with

staphylococci and IMI. Therefore, the focus of the final study was to describe the prevalence and distribution of *Staphylococcus* spp. on the teat and inguinal skin of dairy heifers. The cross sectional study included 106 Holstein heifers with an age range of 0 d – 28 mo. The most common species identified were *S. haemolyticus*, *S. chromogenes*, *S. xylosus*, *S. devriesei*, and *S. sciuri*. *Staphylococcus aureus* was more prevalent on the teat than in the inguinal region while *S. arlettae* was more prevalent in the inguinal region than on the teat. All other staphylococcal species were equally distributed between the teat and inguinal region. Colonization of the inguinal region and teat skin was associated with age for *S. agnetis*, *S. chromogenes*, *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. lentus*, *S. sciuri*, *S. vitulinus*, and *S. xylosus*. The probability of *S. chromogenes* and *S. agnetis* colonization on the teat and inguinal region increased with age, while the probability of *S. devriesei* and *S. haemolyticus* decreased with age.

Overall, these studies demonstrate that *Staphylococcus* spp. and subspecies associated with IMI are extremely abundant and diverse on dairy farms. Among the tools studied to more quickly determine species and subspecies (strain-type) identity, MALDI-TOF was found to be a rapid, inexpensive, and accurate speciation method for many *Staphylococcus* spp. One major limitation is its inability to differentiate *S. hyicus* from *S. agnetis*. The multiplex PCR developed through analysis of whole genome sequence data for *S. chromogenes*, *S. agnetis*, *S. hyicus*, and *S. aureus* provides an accurate supplemental test for differentiating these two species. Further, some of the other species of staphylococci still require housekeeping gene sequence analysis for identification. Rapid, accurate subspecies identification of isolates remains an obstacle as, based on the

work presented herein, PFGE remains the most discriminatory tool that can be used across all staphylococcal species and a replacement is yet to be identified. Hence, further work is needed to find an easy and low-cost tool for defining subspecies, but potential targets may be found through whole genome sequencing of these organisms. As illustrated in the conclusions of the fourth study, subspecies identification is clearly important and necessary for improving our understanding of the epidemiology of staphylococcal species associated with IMI on the dairy farm. The final two studies demonstrate an association between body site colonization, prepartum IMI, and post-calving IMI for some species of staphylococci, and body site colonization with the different staphylococcal species changes over the course of the heifer's development. These studies will help inform future studies on potential intervention strategies to reduce the risk of peripartum IMI in heifers.

APPENDIX A

METHODS

A.1 Sample Collection and Storage

A.1.1 Milk/Mammary Secretion Sample Collection

All milk and mammary secretion samples were collected aseptically. For milk sample collections, samples were collected in the parlor, just prior to milking. The udder was prepared using the farm's normal procedure, which included the following: pre-dip was applied with a spray application system, each quarter was stripped, and the quarter was wiped dry with an individual damp cloth. After the milking preparation was complete, each teat end was cleaned vigorously with a separate section of a cotton pad (Webril Handi-Pads, Fiberweb, USA) soaked in 70% isopropyl alcohol. For prepartum mammary secretion sample collection, animals were restrained in a cattle chute and the teats were pre-dipped using a teat dip applicator cup, dried with a single-use towel, and scrubbed with isopropyl alcohol as described. Mammary quarter-level milk samples were each collected into separate sterile tubes (5 ml polystyrene tube; Falcon, Tamaulipas, Mexico). After milk sample collection the animal was machine milked following the farm procedure. No machine milking was performed on prepartum heifers. All teats were treated with a post-milking teat disinfectant either after milking or after sample collection (prepartum secretion sampling).

A.1.1.1 Milk Somatic Cell Count Samples

Samples for milk SCC were collected into vials provided by Mid-South Dairy Records (Springfield, MO) containing a 2-bromo-2-nitropropane-1,3-diol preservative tablet (Broad Spectrum Microtabs II, D&F Control Systems Inc., Dublin, CA). After collection, samples were stored at 4°C, and shipped at least once weekly to a commercial laboratory (Mid-South Dairy Records, Springfield, MO) for enumeration using an automated counter (Somacount 300, Bentley Instruments, Chaska MN).

A.1.2 Body Site Sample Collection

All body site samples were collected with the animal restrained in a chute or with physical restraint (animals < 2 m). Body site swabbing samples were collected from the following regions: 1) a composite sample of all four teats, 2) a composite sample of the inguinal region, 3) a muzzle sample, and 4) a perineum sample. Prior to sample collection, visible debris was removed. Samples were collected using gas sterilized electrostatic dusters that had been cut in half (Swiffers®). Latex gloves were worn at all times and were changed between each sample collected. Once the sample was collected, it was placed inside a 24 oz. Whirl-Pak™ bag (Nasco, Fort Atkinson, WI). One negative control, a gas-sterilized electrostatic duster that was carried to the farm but not used to sample an animal, was collected for each sampling visit to the farm. All body site swabbing samples were chilled on ice for transport and processed the same day.

A.1.3 Sample Storage

All milk samples and mammary secretion samples were stored at -20°C before and after processing. All body site samples were processed on the day of collection and then stored at -20°C.

A.2 Bacterial Culture

A.2.1 Milk/Mammary Secretion Cultures

All milk samples were thawed at room temperature (~22°C). Samples were vortexed and a 10 µl aliquot from each milk sample was aseptically spread across half a Columbia agar with 5% sheep blood plate (CBA; Remel, Lenexa, KS) using a flame sterilized loop. Plates were incubated at 37°C for 24 hours and then an additional 24 hours at room temperature (~22°C). All plates were read at 24 hours and 48 hours. Colonies were presumptively identified based on colony morphology including size, color, texture, and the presence of hemolysis. The total number of each colony type present was counted and recorded. All presumptively identified *Staphylococcus* spp. were subjected to the catalase test and tube coagulase test.

The catalase test was conducted by mixing a representative colony with 3% hydrogen peroxide on a slide. A positive result was recorded if effervescence was observed. The tube coagulase test was conducted by placing a representative colony of each isolate in 0.3 ml of rabbit plasma (Coagulase Plasma, Remel, Lenexa, KS). Tubes were then incubated at 37°C for approximately 24 hours. A positive result was recorded if a clot was noted in the tube post incubation. All presumptively identified coagulase negative and coagulase positive *Staphylococcus* spp. were saved for further analysis.

A.2.2 Body Site Sample Cultures

Samples were processed on the day of collection. Sterile Whirl-Pak™ bags were opened and the electrostatic duster was cut in half while still in the bag using flame sterilized scissors. One half of the duster was then moved to a new Whirl-Pak™ bag using flame sterilized forceps. One Whirl-Pak™ bag containing half of the duster was frozen dry, while the other bag containing the second half was used for same-day processing of the samples. Ten ml of sterile saline was added to the second bag and the bag was then vigorously agitated using a vortex. After agitating, 50 µl of the resultant solution was pipetted onto Mannitol Salt Agar (Remel, Lenexa, KS) and spread using a sterilized plastic spreader. The plates were then incubated at 37°C for 24 hours. After incubation, the plate was visually inspected and up to 10 presumptively identified *Staphylococcus* spp. colonies, including at least one representative of each morphologically distinct colony, were collected and subcultured onto CBA. After 24 hours of growth at 37°C, all presumptively identified *Staphylococcus* spp. colonies were stored as described (A.2.3) for further analysis.

A.2.3 Storage of *Staphylococcus* spp. Isolates

All *Staphylococcus* spp. isolates were sub-cultured on CBA by isolation streak and ~3 like colonies of each unique isolate were stored in 15 x 45 mm glass screw cap vials (Fisher Scientific, Fair Lawn, NJ) containing 2 ml phosphate buffered glycerol (A.2.3.1) at -80°C for future analysis. A purified isolation streak from CBA was used.

A.2.3.1 Preparation of phosphate buffered glycerol

Reagents

- Potassium Phosphate, Monobasic (0.1 M) 6.8 g/500 ml dH₂O (Fisher Scientific, Fair Lawn, NJ)
- Potassium Phosphate, Dibasic (0.1 M) 8.8 g/500 ml dH₂O (Fisher Scientific, Fair Lawn, NJ)
- Glycerol (Fischer Scientific, Fair Lawn, NJ)

The pH of the dibasic potassium phosphate was adjusted to 7.0-7.05 with the monobasic potassium phosphate. Once the correct pH was achieved, glycerol was mixed with buffer at a 60:40 ratio. The solution was mixed by warming and stirring. A total of 2 ml of the mixture was dispensed into 15 x 45 mm glass screw cap vials (Fisher Scientific, Fair Lawn, NJ) and autoclaved. Vials were stored at 4°C until used.

A.3. Polymerase chain reaction methods

A.3.1 Colony PCR Preparation

Isolates were plated on CBA by streaking for isolation and incubated for 24 hours at 37°C. After incubation, a single colony was picked from CBA and inoculated into 100 µl of 1X Tris- ethylenediaminetetraacetic acid (EDTA; TE) (Promega, Madison, WI, USA) in a 1.5 ml sterilized tube (Fisher Scientific, Fair Lawn, NJ).

A.3.2 DNA extraction

DNA extraction was done using a DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). The kit protocol designed for the purification of total DNA from Gram-positive bacteria was used. The manufacturer's instructions are listed below.

1. Plate isolates on CBA by streaking for isolation. Incubate for 24 hours at 37°C.
2. Add 300 µl of 1X Tris-Ethylenediaminetetraacetic acid (TE) buffer (Promega, Madison, WI, USA) to each properly labeled 1.5 ml tube (Fisher Scientific, Fair Lawn, NJ) and then add 1-3 colonies of the selected isolate using a sterile loop.
3. Centrifuge the tubes for 10 minutes at 5,400 x g and then discard the supernatant.
4. Resuspend the bacterial pellet in 180 µl of enzymatic lysis buffer.
 - a. Enzymatic lysis buffer
 - i. 20 mM Tris-Cl, pH 8.0 (1 M recipe in PFGE protocol)
 - ii. 2 mM sodium EDTA (0.05 M recipe in PFGE protocol)
 - iii. 1.2% Triton X-100 (Bio-Rad, Hercules, CA)
 - iv. Immediately before use, add 20 mg/ml lysozyme
5. Incubate for at least 20 minutes at 37°C.
6. Add 25 µl proteinase K and 200 µl of Buffer AL (included in kit). Mix by vortexing.
7. Incubate at 56°C for 30 minutes.
8. Add 200 µl of ethanol (96-100%) to the sample and mix thoroughly by vortexing.

A white precipitate may form, it is essential to collect all the precipitate and liquid when transferring the sample in the next step.

9. Pipet the mixture into the DNeasy Mini spin column (provided in kit) that is placed in a 2 ml collection tube (provided in kit). Centrifuge at $\geq 6,200 \times g$ for 1 minute. Discard the flow through in the collection tube.
10. Place the DNeasy Mini spin column in a new 2 ml collection tube and add 500 μl buffer AW1 (provided in kit). Centrifuge for 1 minute at $\geq 6,200 \times g$. Discard flow through in the collection tube.
11. Place spin column in new collection tube and add 500 μl of buffer AW2 (provided in kit) and centrifuge for 3 minutes at $17,000 \times g$. Extended time is to help dry the membrane. Discard the flow through and the collection tube.
12. Place the spin column in a clean 1.5 ml tube (Fisher Scientific, Fair Lawn, NJ) and pipet 200 μl of buffer AE (provided in kit) directly onto the spin column membrane. Incubate for 1 minute and then centrifuge for 1 minute at $\geq 6,200 \times g$ to elute.
13. The extracted DNA is in the 1.5 ml tube. Label appropriately and store at -20°C .

A.3.3 Polymerase chain reaction

Reagents:

- GoTaqGreen master mix (Promega, Madison, WI)
- Molecular grade H_2O (Nuclease-Free Water) (Sigma Aldrich, St. Louis, MO)
- PCR Primers: listed below, all reference publications can be found within the chapters of this dissertation (Integrated DNA Technologies, Coralville, IA). All primers were ordered as dehydrated oligos and rehydrated with 1 ml of nuclease free water once received.

All required reagents and DNA (colony preparation tubes or extracted DNA) were removed from the freezer and thawed at room temperature (~22°C). The GoTaqGreen was placed on ice. A quantity of PCR master mix sufficient for the number of samples being tested was prepared and aliquoted into 1.5 ml tubes as described below, and each tube was kept on ice.

1. Master mix for Colony PCR (23 µl per sample x number of samples):
 - a. 9.5 µl of nuclease free water
 - b. 12.5 µl of GoTaqGreen
 - c. 0.5 µl of forward primer
 - d. 0.5 µl of reverse primer
2. Master mix for PCR using extracted DNA (24 µl per sample x number of samples):
 - a. 10.5 µl of nuclease free water
 - b. 12.5 µl of GoTaqGreen
 - c. 0.5 µl of forward primer
 - d. 0.5 µl of reverse primer

After all reagents were added to create the master mix, the tube was inverted several times to mix the solution. A total of 23 µl (colony preparation) or 24 µl (extracted DNA) of the master mix was added to each PCR reaction tubes (Molecular Bio Products PCR tubes and tube strip; Thermo Scientific, Waltham, MA). Then, 2 µl (colony preparation) or 1 µl (extracted DNA) was added to each PCR reaction tube.

The PCR reaction was done using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). For most PCR amplifications, the thermocycler conditions were as follows:

94°C for 15 minutes followed by 35 cycles each at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 minute. The run was completed at 72°C for 5 minutes and then held at 4°C. The annealing temperature was adjusted if needed, based on the melting temperature (T_m) value of the primers.

Table 3.3.1 Polymerase chain reaction primer sets listed according to gene, primer sequence, amplicon size in base pairs (bp), staphylococcal species or virulence factor identified, and any relevant reference papers.

Gene	Primer	Sequence 5'-3'	Amplicon size (bp)	Detecton of:	Source
<i>aroD</i>	aroD-HyF	TAT GGT GTC GAC CAA TCG AAG GCT	425	<i>S. hyicus</i>	Adkins et al, submitted
<i>aroD</i>	aroD-HyR	ACC CTA TAG CCC GCT TAC TT			
<i>aroD</i>	aroD-AgF	CGC ATG AGA GAC CAA TAC GCT	293	<i>S. agnetis</i>	Adkins et al, submitted
<i>aroD</i>	aroD-AgR	TAG GAC GTA TAG AGG TGG			
<i>nuc</i>	nuc-S	CTG GCA TAT GTA TGG CAA TTG TT	664	<i>S. aureus</i>	Graber et al, 2007
<i>nuc</i>	nuc-AS	TAT TGA CCT GAA TCA GCG TTG TCT			
<i>rpoB</i>	2643F	CAA TTC ATG GAC CAA GC	598	<i>Staphylococcus</i> spp.	Drancourt et al, 2002
<i>rpoB</i>	3241R	GCI ACI TGI TCC ATA CCT GT			
<i>tuf</i>	TUF-F	GCC AGT TGA GGA CGT ATT CT	412	<i>Staphylococcus</i> spp.	Hwang et al, 2011

<i>tuf</i>	TUF-R	CCA TTT CAG TAC CTT CTG GTA A			
<i>lukE</i>	lukE-S	AAT GTT AGC TGC AAC TTT GTC A	831	Leukotoxin E	Fournier et al, 2008
<i>lukE</i>	lukE-AS	CTT TCT GCG TAA ATA CCA GTT CTA			
<i>sea</i>	Gsea-S	CTG AAT TGC AGG GAA CAG CTT TAG	512	<i>S. aureus</i> enterotoxin gene A	Boss et al, 2011
<i>sea</i>	Gsea-AS	AAA CAC GAT TAA TCC CCT CTG AAC			
<i>sed</i>	Gsed-S	AAG TAG TAC CGC GCT AAA TAA TAT GA	624	<i>S. aureus</i> enterotoxin gene D	Boss et al, 2011
<i>sed</i>	Gsed-AS	ATA TGA AGG TGC TCT GTG GAT AAT			
<i>seg</i>	Gseg-S	ACC TGA ACT ATC TAT AAG CGT GAA T	644	<i>S. aureus</i> enterotoxin gene G	Boss et al, 2011
<i>seg</i>	Gseg-AS	TCT TTA GTG AGC CAG TGT CTT G			
<i>sei</i>	Gsei-S	GCG CAA GGA GAT ATT GGT GTA G	501	<i>S. aureus</i> enterotoxin gene I	Boss et al, 2011
<i>sei</i>	Gsei-AS	AAC TTT CCC CTT ATT AAA ACC TG			
<i>sej</i>	Gsej-S	TAA CGA AAA GGG TAT CTC TGA A	332	<i>S. aureus</i> enterotoxin gene J	Boss et al, 2011
<i>sej</i>	Gsej-AS	CTA TAT CTA GCG GAA CAA CAG T			

A.3.4 Ribosomal spacer PCR (Source: Jensen et al, 1993)

Reagents:

- GoTaqGreen master mix (Promega, Madison, WI)
- Molecular grade H₂O (Nuclease-Free Water;Sigma Aldrich, St. Louis, MO)

PCR Primers:

G1: GAA GTC GTA ACA AGG

L1: CAA GGC ATC CAC CGT

All required reagents and extracted DNA were removed from the freezer and thawed to room temperature (~22°C). The GoTaqGreen was placed on ice. A quantity of PCR master mix sufficient for the number of samples being tested was prepared and aliquoted into 1.5 ml tubes as described below, and each tube was kept on ice.

1. Master mix for PCR using extracted DNA (24 µl per sample x number of samples):
 - a. 10.5 µl of nuclease free water
 - b. 12.5 µl of GoTaqGreen
 - c. 0.5 µl of forward primer
 - d. 0.5 µl of reverse primer

The PCR reaction was performed using an Eppendorf Mastercycler. The thermocycler conditions were as follows: 94°C for 5 minutes followed by 25 cycles each at 94°C for 1 minute, 2 minute ramp to 55°C, 7 minutes at 55°C, 2 minute ramp to 72°C, and 2 minutes at 72°C. The final cycle was followed by an additional 7 minutes at 72°C to complete partial polymerizations. At the end of the cycling program, the samples were held at 4°C.

A.3.5 Horizontal Gel Electrophoresis

All PCR amplicons were visualized using horizontal gel electrophoresis. Aliquots (5 µl) of each amplicon were run in a 1-2% agarose gel as below.

Materials:

- 0.5X Tris-borate- Ethylenediaminetetraacetic acid (EDTA;TBE) buffer (diluted from 10X Stock, Bio-Rad Laboratories, Hercules, CA)
- Agarose (Molecular biology grade, Gold Biotechnology, Olivette MO)
- Gel box
- Power supply
- Ethidium bromide (Bio-Rad Laboratories, Hercules, CA)
- Loading dye (10x Blue Juice Loading Buffer, Invitrogen, Carlsbad, CA)
- Molecular weight size standard (100 bp DNA ladder, Invitrogen, Carlsbad, CA)

The following instructions were used.

1. Prepare gel by mixing agarose with 0.5X TBE. To make a 1% gel, add 1 gram of agarose to 100 ml of 0.5X TBE buffer.
2. Microwave on high until the mixture is boiling and the gel is completely dissolved.
3. Cool the mixture to approximately 50°C.
4. Add 5 µl ethidium bromide (10 mg/ml) to each 100 ml of liquid agarose gel. Swirl to mix.
5. Pour the mixture into the casting tray. Immediately add the well combs to the gel and let it stand at room temperature (~22°C) to solidify.

6. Once solidified, place the gel into the gel box and add enough 0.5X TBE buffer to submerge the gel.
7. Mix 1 μ l of loading dye (10x Blue Juice Loading Buffer, Invitrogen, Carlsbad, CA) with 5 μ l of sample and then add the mixture to the corresponding gel well.
8. Add molecular weight size standard (DNA ladder) to the first and last gel wells.
9. Electrophorese the gel for approximately 45 minutes at 100 volts.
10. View the gel using UV transillumination and photographic recording.

A.3.6 PCR Purification

Post-PCR, a PCR purification kit (PureLink PCR Purification Kit; Invitrogen, Carlsbad, CA) was used to prepare the DNA for sequencing. The manufacturer's directions were followed as below.

1. Add a total of 4 volumes of Binding Buffer B2 (provided in kit) to one volume PCR product. For example, if there is 20 μ l of PCR product, 80 μ l of Binding Buffer B2 should be added and thoroughly mixed. .
2. Pipet samples into a PureLink Spin Column in a collection tube (provided in kit). Centrifuge the column at $> 10,000 \times g$ for 1 minute. Discard the flow through.
3. Place the spin column back into the collection tube. Add 650 μ l of Wash Buffer W1 (provided in kit). Centrifuge the column at $> 10,000 \times g$ for 1 minute. Discard the flow through.
4. Place the spin column in a clean 1.7 ml tube (provided in kit). Add 50 μ l of elution buffer (provided in kit) and incubate at room temperature for 1 minute.

Centrifuge at maximum speed for 2 minutes. The tube now contains the purified PCR product.

5. Store tubes at 4°C for immediate use and -20°C for long-term storage.

A.3.7 Sanger Sequencing

All Sanger sequencing was done at the University of Missouri DNA Core (216 Bond Life Sciences Center). Samples were prepared according to the DNA Core guidelines, <http://dnacore.missouri.edu/sangersequencing.html>. Directions were followed as listed below.

1. Single Sample Submission

- a. Submit samples in 1.5 ml tubes. The core specifically requires Fisher Scientific (Cat. #05408129) 1.5 ml tubes.
- b. Mix a total volume of 16 µl, including template and primer, in a 1.5 ml tube.
 - i. PCR product: 50-275 ng
 - ii. Primer: 15-26 pmol of a single primer
 - iii. Molecular grade water to complete 16 µl

2. 96-well plate submission

- a. Obtain plates from the DNA core.
- b. Mix a total volume of 6 µl, including template and primer, in each well.
 - i. PCR product: 10-50 ng
 - ii. Primer: 5-10 pmol of a single primer
 - iii. Molecular grade water to complete 6 µl

3. Place sequence orders online using the DNA Core's dnaLIMS system
(<http://128.206.82.15/cgi-bin/dna/password.cgi>)
4. Label samples according to DNA Core instructions and place in the white refrigeration unit located across from room 211 Bond Life Sciences Center.
5. View sequence results at <http://128.206.82.15/cgi-bin/dna/password.cgi> .
6. Blast sequences using NCBI nucleotide BLAST
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

A.4 Matrix-assisted laser desorption/ionization time of flight (MALI-TOF)

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was used to speciate *Staphylococcus* spp. isolates. Samples were analyzed in a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with flex control software. Each plate used at least 2 spots of bacterial test standard (BTS) for calibration and control. All tested isolates were run in duplicate. The MALDI-TOF manufacturer's identification cut-off value for species-level identification was a score ≥ 2 on at least one of the duplicate samples and was used to make a diagnosis. Any score < 2 was classified as inconclusive for species identification.

A.4.1 Preparing BTS aliquots

Bacterial test standard was used as a calibration standard. The BTS was solubilized using the following procedure provided by Bruker.

1. Add 50 μ l of standard solvent (acetonitrile 50%, water 47.5%, and trifluoroacetic acid 2.5%) (Sigma-Aldrich, St. Louis, MO) to the BTS pellet and dissolve by pipetting up and down at least 20 times at room temperature. Avoid foaming the solution.
2. Incubate the BTS solution for 5 minutes at room temperature and then mix by pipetting up and down at least 20 times.
3. Centrifuge at 16,200 x g for 2 minutes at room temperature.
4. Pipette 2 μ l aliquots of the supernatant into separate 1.5 ml tubes.
5. Store aliquots at -18°C or below.

A.4.2 Plate Extraction Method for Test Isolates

Chemicals and materials required:

- HCAA matrix solution from Bruker Daltonics (add 250 μ l of standard solvent to the screw cap tube containing the HCAA, mix until completely dissolved)
- Standard solvent (acetonitrile 50%, water 47.5%, and trifluoroacetic acid 2.5%) (Sigma-Aldrich, St. Louis, MO)
- 70% formic acid
- Sterilized tooth picks
- MALDI target plate (MSP 96 target polished steel, Part NO. 8280800, Bruker Daltonics, Billerica, MA)
- Pipettes (0.5-10 μ l)
- Pipette tips (1-10 μ l)

Manufacturer's instructions:

1. Smear a thin film of organism from a single colony directly onto a cleaned MALDI target using a sterile tooth pick.
2. Overlay each sample with 1 μ l of 70% formic acid. This step is optional, however adding formic acid improves the disruption of the cells, especially in the case of Gram-positive bacteria.
3. Allow samples to dry.
4. Carefully overlay each sample position (spot on MALDI-TOF target plate) with 1 μ l of HCAA matrix solution.
5. Allow the sample positions to dry at room temperature.
6. Samples are ready for classification process and can be put into the MALDI-TOF mass spectrometer.

A.4.3 Tube Extraction Method

Chemicals and materials needed:

- HPLC-grade water (Sigma-Aldrich, St. Louis, MO)
- Absolute ethanol
- 70% formic acid (Sigma-Aldrich, St. Louis, MO)
- HPLC-grade acetonitrile (Sigma-Aldrich, St. Louis, MO)
- HCAA matrix solution (Bruker Daltonics, Billerica, MA)
- 1.5 ml microcentrifuge tubes
- MALDI target plate (MSP 96 target polished steel, Part NO. 8280800, Bruker Daltonics, Billerica, MA)

- Vortex mixer
- Pipettes (0.5-10 μ l and 100-1000 μ l)
- Pipette tips (1-10 μ l and 100-1000 μ l)

Manufacturer's instructions:

1. Dispense 300 μ l HPLC water into a sterile 1.5 ml microcentrifuge tube.
2. Transfer biological material (from a single colony) into the tube.
3. Generate a homogenous suspension of cells by pipetting or by vortexing the mixture for at least one minute.
4. Dispense 900 μ l of pure ethanol into the tube and mix the suspension for at least one minute using a vortex.
5. Centrifuge the tube for 2 minutes at 16,200 x g and remove the supernatant using a pipette, without disturbing the pellet.
6. Repeat step 5 and then let the pellet dry for a few minutes at room temperature (all residual ethanol needs to be removed).
7. If one large colony of *Staphylococcus* spp. is used, add 10 μ l of 70% formic acid to the pellet and mix thoroughly by pipetting up and down followed by mixing with a vortex.
8. Add 10 μ l of 100% acetonitrile to the tube and mix carefully.
9. Centrifuge the tube for 2 minutes at 16,200 x g.
10. Pipette 1 μ l of microorganism extract supernatant onto a clean MALDI target.
11. Allow the sample to dry at room temperature.
12. Overlay each sample with 1 μ l of HCAA matrix solution.

13. Allow the sample to dry at room temperature.
14. The samples are now ready for the classification process and can be inserted into the MALDI-TOF mass spectrometer.

A.4.4 Running the MALDI-TOF

The following instructions were used to run the MALDI-TOF. The instructions were provided by the manufacturer.

1. Start the 'FlexControl' and 'Maldi Biotyper RTC'
2. In the FlexControl window, click on 'status', then 'details' to verify that the geometry is set at 96 well target.
3. In the MALDI Biotyper RTC window, click 'file', 'new classification'. Enter the project name and/or date, click 'new', click 'next'.
4. Click and drag to select the wells used on the plate (including the BTS) then press insert on the keyboard.
5. In the ID column, enter each isolate's ID according to location on the target.
6. On the MALDI machine base unit, press the in/out button to remove the old plate, if applicable.
7. On the computer, in the FlexControl window, the status will say 'out' when it is ready to open.
8. With gloves on, open the lid, remove the old plate and put in the new plate without touching its surface.
9. Wipe around the gasket with your finger to remove any debris, then gently close the lid and push the in/out button.

10. When the FlexControl window in the right lower corner indicates 'in', on the 96-well map, click on the BTS spot.
11. In the FlexControl window, click 'status', 'details', and 'vacuum'. When the vacuum is ready, click on 'calibrate' to calibrate with the BTS spot.
12. Once calibration is successful, go to the MALDI Biotyper RTC window, click 'next', and 'finish'. The machine will then start to run the selected spots on the plate.
13. The plate can be left inside the machine at the end of the run.
14. To view results, click 'view', 'results', and 'save'.

A.4.5 Building a custom library and adding reference isolates

Database creation was done to add isolates to a custom library. This was done to add species not included in the Bruker library, such as *Staphylococcus devriesei* and *Staphylococcus agnetis*, or to add reference isolates to improve the identification of a bacterial species where the isolates in the manufacturer's library consistently yield a less than optimal identification score, such as for *S. haemolyticus*. Only strains which had been previously been speciated with some other form of molecular identification, such as housekeeping gene sequencing, were used. After adding the isolate to the custom library, all custom library entries were validated by running them against 100 known isolates of that specific staphylococcal species. The following manufacturer's instructions were used to add an isolate to the custom library.

Manufacturer's Instructions:

1. Sample and target preparation

- a. Apply 1 μ l of freshly made BTS to at least one BTS target position, overlay with HCAA matrix.
 - b. Apply 1 μ l of tube extraction supernatant from the isolate of interest to 8-10 target spots and allow to dry. Overlay all spots with 1 μ l of freshly prepared HCAA matrix.
2. Within FlexControl, perform auto calibration on the BTS spot.
3. An AutoXecute Run is created within FlexControl for automated collection of the spectra to be used for database creation. Move to the AutoXecute tab and select 'new' on the run line.
4. Confirm that the correct target geometry is selected. Click 'next'.
5. Skip the laser power tuning page by clicking 'next'.
6. Select 'no calibration spots'. Calibration was conducted using BTS prior to the AutoXecute run.
7. Use the mouse to highlight the spots to be analyzed. Click 'next'.
8. From the pull down menus, select MBT_AutoX as the AutoXecute method and MBT_Standard.FAMSMETHOD as the FlexAnalysis method.
9. Designate a location for the spectra to be saved. If desired, create a new directory within the D: Data drive. Do not add spectra to the RealTimeClassification folder.
10. Provide an appropriate unique sample name, identifying the strain with detail. Click 'next'.
11. A finish page will appear, click 'finish'.

12. Spots to be run are highlighted in yellow as prepared. Confirm the data destination is correct. Confirm AutoXecute Methods is MBT_AutoX and FlexAnalysis Method is MBT_Standard.
13. Highlight the spots to be run with the mouse and click the green '+' twice to add second and third runs of the same spot. The total sample spot count will triple, from 8 to 24.
14. It is recommended to copy the sample name used previously and save the run with the same nomenclature. Click 'save as' to save the run.
15. After saving, press 'OK' in the lower right of the AutoXecute Run Editor window.
You will be returned to FlexControl. The saved run should appear in the run field and the spots to be run highlighted in yellow as prepared.
16. Press 'start automatic run' to begin automated collection.
17. Following the automated collection, open FlexAnalysis.
18. Locate the folder containing the spectra collected via the AutoXecute run and open via 'file', 'open'.
19. Open/load all spectra.
20. Highlight/select all spectra in the Analysis list and apply smoothing and baseline subtraction by clicking on 'process', 'smooth mass spectrum' and 'process', 'subtract mass spectrum baseline'.
21. After smoothing and baseline subtraction, use the magnifying glass in the tool bar to zoom and search the spectra set for flat line spectra, outliers, or dramatic mass shifts.
22. Click the check box to the left of the name of the outlier spectra in the Analysis list to remove them from the view. Repeat for any remaining outliers.

23. Write down the spectra to be excluded from the main spectrum profile (MSP) creation.
24. Close FlexAnalysis, selecting 'yes to all' at the save prompt.
25. Open MALDI Biotyper 3.0.
26. Click on 'file', 'add spectra' to browse and select the created spectrum folder.
27. Open all spectra. Delete the outliers noted in FlexAnalysis by right mouse-clicking on the spectra to be removed and selecting 'remove selected spectra'.
28. Following deletion of all outliers, select/highlight all spectra and right click to select 'Create MSP'.
29. Provide a name for the new MSP. The default name will be the name of your created data folder.
30. Click 'OK'. MSP creation includes automatic smoothing, baseline subtraction, peak detection, normalization and evaluation, and therefore may take a few minutes.
31. The new MSP will appear in the Taxonomy Tree Node as 'unassigned'.
32. Perform the following checks on the new MSP before incorporating it into a library.
 - a. Highlight 'unassigned MSP' within the taxonomy tree and right click to select 'Add to MSP selection'.
 - b. Select several or all of the spectra used for MSP creation to search against the new entry. Highlight the spectra and click the start button.
 - c. The match score should be 2.7 or higher on all spectra run against the new MSP entry. If some scores are lower than 2.7, start over with fresh formic acid tube extractions.

- d. Right click the new MSP and select 'View MSP Peak List' to confirm that 70 peaks were found and each occurs with high frequency (greater than 75%). If this is not the case, repeat the formic acid tube extractions and start over.
 - e. It is also recommended to match the new MSP against all existing entries. Highlight the new MSP within the taxonomy tree and right click to select 'Add MSP for identification'.
 - f. Load all other MSPs from libraries using the 'Load MSP Library'.
 - g. Add the new MSP to the 'selected MSP' list. You are now searching the new MSP against itself and all other library MSPs. Start identification by clicking the play button.
33. After the new MSP has passed the QC tests, details about the new entry can be noted and saved within the MSP Metadata.

A.5 Pulsed Field Gel Electrophoresis (PFGE)

A. 5.1 Gram-positive bacteria protocol

A. 5.1.1 Preparation of bacterial plus

1. Bacterial colonies that incubated overnight at 37°C on blood agar plates are suspended in 2-3 ml of Cell Suspension TE buffer (100 mM Tris and 100 mM EDTA. pH 7.5) using sterile cotton swabs. Swab the colonies gently without disturbing the agar surface.

2. Adjust the turbidity of bacterial cell suspension to an optical density of 1.4 using a spectrophotometer at a wavelength of 610 nm. Zero the machine using a tube of TE cell suspension buffer as a blank.
3. Place 1.5 ml of each isolate from step 3 into a labeled sterile 1.5 ml microcentrifuge tube and centrifuge in a micro-centrifuge for 2 minutes. Aspirate off the supernatant, being careful not to lose the pellet.
4. Completely re-suspend the pellet in 0.5 ml EET (EDTA, EGTA, TRIS). Once EET has been added use pipette to mix the solution and then vortex to fully re-suspend the mixture.
5. Melt previously prepared or prepare 1% Chromosomal Grade Agarose and keep in a 65°C water bath.
6. Warm the cell suspension in a 65°C water bath for a few seconds, then mix with 0.6 ml of the 1% Chromosomal Grade Agarose. Pipette the suspension several times (5-10 X) and vortex at low speed to thoroughly mix the agar and cells. Be careful not to create bubbles. Fill desired number of wells in the plug molds (2-4 plugs/isolate are usually made in disposable Bio-Rad molds, they hold ~100 µl/well). Repeat with remaining isolates.
7. Cool the plug molds for ~15 minutes at room temperature or ~5-10 minutes at 4°C to solidify the agarose before removing the plugs.
8. While wearing gloves, remove the plugs by peeling off the sealing tape on the bottom of the plug molds and pushing the plugs out with a flame sterilized spatula into a sterile 50 ml centrifuge tube (1 tube for the 2-4 plugs from each respective isolate) containing ~1 ml freshly made EET-LL buffer (EDTA, EGTA, TRIS, Lysozyme,

- Lysostaphin). Make sure all plugs are covered by EET-LL. Incubate at 30°C for 4 hours.
9. Use a sterile pipette to remove the EET-LL buffer, then add ~1 ml freshly made EET-SP buffer (EDTA, EGTA, TRIS, SDS, Proteinase K) (warmed to 50°C to dissolve the SDS) to cover the plugs. Incubate overnight at 50°C overnight.
 10. Use a sterile pipette to remove the EET-SP buffer, wash by adding 40 ml sterile TE wash buffer and agitating constantly over a 30 minute period at room temperature using the MaxQ Shaker (Thermo Scientific, Waltham, MA). Pour off the TE buffer, taking care not to lose the plugs (pour the solution through a tea strainer). Repeat wash 3 times. Store plugs at 4°C in TE wash buffer (~1 ml in 1.5 ml tubes). For 24 samples, a total of 4 L sterile TE will be needed.

A. 5.1.2 Digestion with Restriction Endonuclease *SmaI*

1. Prepare 1X CutSmart Buffer (New England Biolabs, Ipswich, MA) from the 10X stock CutSmart Buffer. You will require 300µl/isolate (150µl for soaking, 150 µl to which *SmaI* is added for digestion).
2. Soak about one quarter of a plug in 150 µl 1X CutSmart buffer in a sterile 1.5 ml tube for ~30 minutes at room temperature. Wear gloves when handling plugs.
3. Remove and discard the buffer. Add 1 µl/isolate of *SmaI* to the remaining 1X CutSmart Buffer and vortex. Then, aliquot 150 µl 1X CutSmart Buffer + *SmaI* to each isolate.
4. Incubate the plugs with *SmaI* for 4 hours at 25°C in a water bath. Use a foam microcentrifuge tube float to suspend the tubes in the water bath.

A.5.1.3 Preparation of the gel

1. Make 0.5X TBE buffer by diluting the 10X TBE stock solution with dH₂O. Make a total of 2 L 0.5X TBE buffer for the CHEF PFGE unit. After removing the necessary volumes to make the PFGE gel (see step 2) place the remainder of the buffer in the fridge to cool.
2. CHEF UNIT Gel: Aliquot 150 ml of the 0.5X TBE buffer into a flask and add 1.5 g PFGE agarose (This is for the large Bio Rad gel casting unit; the small gel uses 100 ml 1% agarose). Microwave on high until the solution comes to a rolling boil and the agarose is completely dissolved. Once the agarose has dissolved, place 3 ml in a 10 ml test tube and save for sealing the plugs to the comb. Cool the agarose to 50°C stirring constantly (in the MaxQ Shaker) to prevent a film forming on top.
3. CHEF UNIT: Pour the remaining 1.85 L of 0.5X TBE into the CHEF unit and turn on the cooling unit. Cool the buffer to 15°C.
4. Remove the *Sma*I digest tubes from the 25°C water bath and decant off the digestion liquid from each tube leaving the plug. Soak each plug in 150 µl of 0.5X TBE for 30 minutes. After 30 minutes remove the 0.5X TBE and place each plug onto the comb provided with the unit in the following order: *Salmonella* BAA-664 Standard plugs are placed in the first and last lanes and the unknowns are placed in the remaining lanes (see instructions for *Salmonella* plug preparation in section A.5.2). The isolate identification and its associated PFGE lane number are recorded for future reference. Place the comb in the gel mold and pour a small amount of 50°C PFGE agarose

- around the base of each plug using a pipette. Also seal the edges of the mold using a 50°C PFGE agarose and a pipette. Allow this agarose to set. Once the initial agarose applications have set pour the rest of the 50°C agarose into the mold and allow it to set. Placing the gel in the refrigerator will accelerate the setting time. Once the gel has set remove the comb. Remove the gel from mold and mount in the machine as described by the manufacturer.
5. Machine Settings: CHEF UNIT = 6 volts/cm, initial pulse time = 5 seconds, final pulse time = 50 seconds, run time = 20 hours. Once the program is entered or a saved program has been selected, press Start. Let gel run for required time.

A.5.1.4 Staining and Photographing the Gel

1. Turn off all equipment and remove the gel. Place the gel in ethidium bromide staining solution and leave in the MaxQ Shaker, set at a low shaker speed, in the dark for 15 min. While the gel is staining, drain the TBE buffer from the PFGE unit and fill the unit with distilled water. Turn on the circulating pump and circulate the distilled water for ~ 15 minutes. Drain the unit and blot dry carefully avoiding damage to the electrodes.
2. Remove the EtBr from gel container and cover the gel with distilled water and let it soak for ~ 20-60 minutes in the MaxQ Shaker, set at a low speed.
3. Drain distilled water and remove gel from staining container. Place gel on UV light box. Photograph the gel using digital imaging software and camera with UV filter.

A.5.1.5 Materials and Equipment

- Blood Agar Plates (Remel, Lenexa, KS)
- Glass tubes, 13 x 100 mm (Kimax 51, VWR, West Chester, PA)
- 1.5 ml Microcentrifuge tubes w/snap caps
- Plugs molds – (Bio-Rad Laboratories, Hercules, CA)
- Pipette tips (200 μ l and 1000 μ l)
- Test tube racks for 13 x 100 tubes and 50 ml tubes
- Microcentrifuge tube racks
- Falcon Tube, 50 ml (Becton Dickinson and Company, Franklin Lakes, NJ)
- CHEF DRII UNIT or similar, (Bio-Rad, Laboratories, Hercules, CA)
- Water bath(s)
- Refrigerator 4°C
- Autoclave
- Microcentrifuge
- Spectrophotometer, 610 nm
- Microwave Oven
- UV transilluminator and gel imaging equipment
- TRIS-HCl (Sigma-Aldrich, St. Louis, MO)
- TRIS Base (Sigma-Aldrich, St. Louis, MO)
- EDTA, disodium dihydrate (Sigma-Aldrich, St. Louis, MO)
- EGTA (Sigma-Aldrich, St. Louis, MO)
- Chromosomal Grade Agarose (Bio-Rad Laboratories, Hercules, CA)
- SDS (Sodium dodecyl sulfate, aka-Lauryl sulfate) (Sigma-Aldrich, St. Louis, MO)

- Lysozyme (Sigma-Aldrich, St. Louis, MO)
- Lysostaphin (Sigma-Aldrich, St. Louis, MO)
- Proteinase K (Gibco BRL, Life Technologies, Gaithersburg, MD)
- Pulsed Field Certified Agarose (Bio-Rad Laboratories, Hercules, CA)
- Ethidium Bromide (Sigma-Aldrich, St. Louis, MO)
- Distilled H₂O

A.5.1.6 Recipes for the Reagents

1. TRIS Stock (1 M, pH 8)

TRIS-HCl	88.8 g
TRIS Base	53.0 g
dH ₂ O	950.0 ml

Adjust pH to 8.0 and q.s. to 1000 ml. Autoclave and store at room temperature (RT).

2. EDTA Stock (0.5 M, pH 8.0)

EDTA, disodium dihydrate	18.61 g
dH ₂ O	90.0 ml

Add 2 g NaOH and then slowly add just enough concentrated NaOH to dissolve the EDTA (EDTA will dissolve at pH 8, add the conc. NaOH 2-3 pellets at a time and let the solution stir and equilibrate between additions). Q.S. to 100 ml. Autoclave and store at RT.

3. EGTA Stock (0.1 M)

EGTA	3.8 g
dH ₂ O	90.0 ml

Add enough concentrated NaOH to dissolve the EGTA and q.s. to 100 ml. Store at RT.

4. EET Stock (100 mM EDTA, 10 mM EGTA, 10 mM TRIS, pH 8)

EDTA 0.5 M	100 ml
EGTA 0.1 M	50 ml
TRIS 1M, pH 8	5 ml
dH ₂ O	345 ml

Store at RT.

5. TE Cell Suspension Buffer, pH 7.5

10 ml Tris Stock 1 M
20 ml EDTA 0.5 M Stock pH 8.0
50 ml dH₂O
Adjust pH 7.5 qs to 100 ml

6. TE Wash Buffer (10 mM TRIS, pH 8, 1mM EDTA)

	1 L	4 L
TRIS 1 M, pH 8	10 ml	40 ml
EDTA 0.5 M	2 ml	8 ml

d H ₂ O	988 ml	3952 ml
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Autoclave and store at RT.

7. EET-LL (EET, 50 µg/ml Lysozyme, 25 units/ml Lysostaphin)

Materials	Example (30 plugs)
EET	27.0 ml
Lysozyme (1 mg/ml)	1.5 ml
Lysostaphin (500 units/ml)	1.5 ml

Because lysozyme loses significant activity during thaw cycles, make aliquots of lysozyme according to how many isolates you will make plugs from at one time. For example, 1.5 ml aliquots will provide sufficient lysozyme to digest plugs for 30 isolates. Likewise, aliquots of lysostaphin can be premade. Aliquots of both can be stored at -20°C until use. Make EET-LL fresh for each time use by thawing a sufficient quantity of premade lysozyme and lysostaphin. Only make enough EET-LL for the # of isolates that you have.

8. EET-SP (EET, 1% Sodium Dodecyl Sulfate [SDS], 1 mg/ml Proteinase K)

Material	Example (30 plugs)
EET	30.0 ml
SDS (Lauryl sulfate, sodium)	0.30 g
Proteinase K	0.03 g

Dissolve the SDS and Proteinase K by warming to 50-60°C in a water bath, then slowly inverting several times to avoid making bubbles. Make EET-SP fresh for each use, only make enough for the # of isolates that you have.

9. Agarose Buffer (10 mM TRIS, pH 7.4, 0.1 mM EDTA)

1 M TRIS pH 7.4	1 ml
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0.5 M EDTA, pH 8	20 µl
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Q.S. to 100 ml with dH₂O. Autoclave and store at RT.

10. 1% Chromosomal Grade Agarose

Chromosomal Grade Agarose	0.2 g
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Agarose Buffer	20 ml
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11. TBE (TRIS, Boric Acid, EDTA) Electrophoresis Buffer

Purchase 10X Stock Solution from Bio-Rad Laboratories, Hercules, CA.

12. Ethidium Bromide Staining Solution

Ethidium Bromide (10 mg/ml)	50 µl
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0.5X TBE	500 ml
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Keep in a dark container. Can be reused.

A.5.2 Gram negative plug preparation and digestion

A.5.2.1 Preparing plugs

Salmonella Braenderup (ATCC BAA-664) was used as the PFGE molecular weight size standard. The plugs were made using the PulseNet PFGE protocol for *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*. PulseNet instructions are as follows:

1. Streak an isolated colony onto a blood agar plate for confluent growth, incubate overnight at 37°C.
2. Add colonies to 2 ml of cell suspension buffer until an absorbance of 1.00 (range 0.8-1.0) is reached at a spectrophotometry wavelength of 610nm.
3. Transfer 400 µl of adjusted cell suspension to a labeled 1.5 ml tube.
4. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently by pipetting the mixture up and down.
5. Add 400 µl melted 1% agarose to the 420 µl suspension, mix by gently pipetting mixture.
6. Immediately, dispense into plug mold wells. Do not allow bubbles to form.
7. Allow plugs to solidify at room temperature for 0-15 minutes. Plugs can be refrigerated to accelerate solidification.
8. Add 5 ml of proteinase K/cell lysis buffer to a labeled 50 ml tube and place plugs in the appropriately labeled tube.
9. Place tube in rack and incubate in a 54-55°C shaker for 1.5-2.0 hours with constant and vigorous agitation.
10. Remove tube from shaker and pour off lysis buffer.

11. Add 10-15 ml of ultrapure water that has been pre-heated to 54-55°C to the tube and shake the tube in a 54-55°C shaker for 10-15 minutes.
12. Pour off the water and repeat wash step with preheated water one more time.
13. Pour off water, add 10-15 ml of preheated sterile TE buffer and shake the tube in a 54-55°C shaker for 10-15 minutes
14. Pour off TE and repeat wash step with preheated TE three more times.
15. Decant last wash and add 5-10ml sterile TE and store at 4°C until needed.

A.5.2.2 Restriction Digestion of plugs

1. Prepare 1X dilution of the restriction buffer (CutSmart)

Reagent	μl/sample
Water	180 μl
10X restriction buffer	20 μl
Total volume	200 μl

Add 200 μl of diluted restriction buffer (1X) to each 1.5 ml labeled tube

2. Cut a 2.0-2.5 mm wide slice of each plug with a blade and transfer to the 1.5 ml tube containing diluted restriction buffer. Be sure the plug slice is under the buffer.
3. Incubate plug slice in a 37°C water bath for 5-10 minutes or at room temperature for 10-15 minutes.
4. After incubation, remove the buffer from the plug slice using a pipette.
5. Prepare the restriction enzyme master mix according to the following:

Reagent	μl/sample
Water	177.5 μl
10X buffer	20.0 μl
<i>XbaI</i> (20U/ul)	2.5 μl
Total volume	200.0 μl

6. Add 200 μl restriction enzyme master mix to each plug slice tube. Be sure the plug slices are under enzyme mixture.
7. Incubate tubes containing the plug slice in a 37°C water bath for 1.5-2 hours.

A.5.2.3 Recipes for the Reagents

1. Cell suspension buffer: use recipe in Gram positive PFGE protocol.
2. TE buffer: use recipe in Gram positive PFGE protocol.
3. Cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sacrosyl):

50 ml of 1 M Tris, pH 8.0

100 ml of 0.5 M EDTA, pH 8.0

100 ml of 10% Sacrosyl (N-Lauroylsarcosine, sodium salt – solution made by adding 100 mg/ml of dH₂O) (Sigma-Aldrich, St. Louis Mo)

Dilute to 1000 ml with sterile water. Store at RT.

4. Total volume of cell lysis/proteinase K buffer needed:

5 ml of cell lysis buffer is needed per tube

25 μl of proteinase K solution (20 mg/ml) is needed per tube. Proteinase K solution is made fresh before each use.

VITA

Pamela (Fry) Adkins was born in Columbus, Nebraska. After graduating from high school, she moved to Lincoln, Nebraska and attended the University of Nebraska-Lincoln. She received a Bachelor of Science in Veterinary and Biomedical Science in 2006. She then moved to Columbus, Ohio to attend veterinary school at The Ohio State University (OSU). While at OSU, she received her Doctorate of Veterinary Medicine in 2010 and her Master of Science in Veterinary Preventive Medicine in 2011. Her master's thesis was focused on the molecular characterization of *Clostridium difficile* of swine and human origin. She moved to Columbia, Missouri in 2011 to pursue a residency in large animal internal medicine and to start a doctoral program. She became board certified in large animal internal medicine in 2014. Pamela currently works at the University of Missouri, College of Veterinary Medicine as a Clinical Instructor.