

COAGULASE-NEGATIVE STAPHYLOCOCCI
MASTITIS MANAGEMENT

By

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Abstract

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Coagulase-negative staphylococci (CNS) are the most common pathogens associated with intramammary infections (IMI) in dairy cows. We hypothesize that post-milking teat disinfection, teat dip, would reduce the microbial colonization of the streak canal and thus reduce the prevalence of IMI caused by CNS species. The efficacy of iodine post-milking teat dip was tested against CNS colonization of the streak canal, and incidence of IMI was measured. Using an udder-half model, 43 Holstein cows at the Washington State University Dairy were enrolled in the trial; teat dip was only applied after milking to one udder-half. Streak canal swab solutions and mammary quarter milk samples were taken in duplicate once a week for 16 weeks for microbial culture. A CNS IMI was identified when two of three consecutive duplicate quarter milk samples were identified with the same CNS species containing >120 cfu/ml. The isolates were speciated using PCR-RFLP and gel electrophoresis. Colonization of the streak canal and IMI by CNS were assessed. Twenty-five CNS IMI were diagnosed; IMI were significantly reduced by 61.1% ($P<0.05$) in the treated quarters. The majority of CNS IMI were caused by *Staphylococcus chromogenes* (44%) and appeared to be linked to streak canal colonization. Conversely, the second most prevalent cause of CNS IMI was by *Staphylococcus*

xylosum (36%), but did not appear to be linked to colonization of the streak canal. The IMI by *S. xylosum* and *S. chromogenes* were reduced by 71.4% ($P < 0.05$) and 16.6% ($P > 0.05$) in the treated quarters. In conclusion, the iodophor post-milking teat disinfectant was efficacious for reducing IMI caused by CNS, but CNS IMI was not necessarily linked to streak canal colonization.

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CHAPTER 1

Introduction

1.1 Bovine Mastitis

Mastitis is defined as an inflammation of the mammary gland, and is currently a major health and economic problem for dairies. It is estimated that 38% of all cows in the United States are affected by mastitis (NMC b) and that mastitis costs \$2 billion to the United States dairy industry annually (Harmon, 1994). These losses include: treatment costs, lost milk production, discarded milk, lowered milk quality, extra labor, veterinary costs, premature culling, and death. Mastitis can be caused by injury, chemicals or other irritants, and by invading pathogens; the latter is the most common cause of mastitis (NMC a). Mastitis may be subclinical, acute, gangrenous, or chronic, but most commonly categorized as either subclinical (SCM) or clinical (CM) (NMC a). Healthy, non-mastitic cows usually have a somatic cell count (SCC) < 200,000 cells/ml and some have a SCC < 100,000 cells/ml (Harmon, 1994). The SCC primarily consists of leukocytes, mostly neutrophils (PMNs), and some epithelial cells (Akers, 2002). Usually SCM is identified when the SCC > 200,000 cells/ml of milk. Subclinical mastitis is more prevalent than CM in dairy cows with potentially 15 to 40 times more cases of SCM than CM (Akers, 2002). The increase of SCC during SCM and CM reduce milk production; for every doubling of SCC cows may produce between 135 kg and 270 kg less of milk per lactation (Raubertas and Shook, 1982). The loss in milk production is caused by migrating-leukocytes from the blood to the milk; the leukocytes pass through tight-junctions, between the mammary epithelial cells, and damage the secretory tissue and structure resulting in reduced milk production (Harmon, 1994). Milk SCC are an indicator of udder health and are a measure of inflammation which is a useful tool for dairy operators to detect cows with SCM.

1.2 Mastitis Pathogens

Mastitis is most frequently caused by invading pathogens in the mammary gland. The three categories of mastitis pathogens are contagious, environmental, and opportunistic. The contagious pathogens, like *Staphylococcus aureus* and *Streptococcus agalactiae*, are normally transmitted from cow to cow, often in the milking parlor (Oura et al., 2002). Environmental pathogens, like *Escherichia coli* and *Streptococcus uberis*, infect the mammary gland from the environment, with sources such as soil and bedding (Hogan et al., 1988a). Finally, opportunistic pathogens, coagulase-negative staphylococci (CNS) and *Corynebacterium bovis*, naturally reside on the teat skin and can be transferred into the mammary gland during milking or injury (Hassan et al., 2009). An opportunistic pathogen resides on the teat skin and may infect the mammary gland during milking or injury. Major mastitis pathogens, like *Staphylococcus aureus*, *Streptococcus agalactiae*, and the environmental pathogens are capable of causing considerable histological damage and reduced milk production. Coagulase-negative staphylococci are minor mastitis pathogens, meaning they infrequently cause CM and their signs are generally mild.

CHAPTER 2

Literature Review

2.1 Introduction

Coagulase-negative staphylococci are defined as non-*Staphylococcus aureus* staphylococci that are also known as a group of opportunistic mastitis pathogens. Coagulase-negative staphylococci are gram-positive, catalase-positive, cocci-shaped bacteria and mostly coagulase-negative. The presence of coagulase production differentiates coagulase-positive staphylococci from CNS. Coagulase-positive staphylococci are identified as *Staphylococcus aureus*, whereas CNS is a heterogeneous group comprised of 39 species and subspecies (Taponen and Pyorala, 2009). Although *S. hyicus* may be misidentified as *S. aureus* because some strains are coagulase positive, they are still considered a CNS. Specific antigen-agglutination tests have been designed to correctly identify *S. aureus* from potential coagulase-positive CNS (Sampimon et al., 2009).

Coagulase-negative staphylococci predominantly cause SCM, hence their classification as minor mastitis pathogens. Usually SCM is identified when the SCC >200,000 cells/ml of milk and is more prevalent than CM in dairy cows. Although SCM is less severe than CM, there are still losses in milk production related to the increase of SCC. The most frequently isolated pathogen from mastitic milk is CNS, which caused 25% of all SCM and up to 10% of CM cases (Thorberg et al., 2009). Not only does CNS affect a large number of cows, it also infects a larger proportion of clinically infected high-producing cows, signifying the importance of reducing IMI by this group of mastitis pathogens (Grohn et al., 2004).

2.2 Coagulase-negative Staphylococci

Before genotypic speciation all Staphylococci other than *S. aureus* were commonly referred to as *S. epidermidis* and sometimes misidentified as *Micrococci sp.* (Klastrop and Madsen, 1974; Kloos and Schleifer, 1975). Baird-Parker (1963) provided the methods of differentiation of *Staphylococci* from *Micrococci* and provided some of the first physiological and biochemical tests for their identification. The most notable difference between the two genera was the ability of *Staphylococci* to produce acid from glucose under anaerobic conditions. Development of tests to differentiate between the *Staphylococci* was refined by the development of additional methods like guanidine and cytosine content of the genome and lysostaphin susceptibility testing (Lachica et al., 1971). By 1974, three species of *Staphylococci* were recognized, *S. epidermidis*, *S. aureus*, and *S. Saprophyticus* (Langlois et al., 1983). Only *S. aureus* was considered pathogenic, and CNS were considered non-pathogenic (Watts et al., 1984).

Kloos and Schleifer (1975a) developed a simplified system for identifying *Staphylococcus sp.* using 13 key traits, which included coagulase activity, hemolysis, nitrate reduction, and fermentation of fructose, xylose, arabinose, ribose, maltose, lactose, sucrose, trehalose, mannitol, and xylitol. These fermentation reactions along with ability to reduce nitrate and produce hemolysins became the basis of the phenotypic traits used to further differentiate between *Staphylococci sp.* previously broadly categorized as *S. epidermidis*. These 13 key traits had the ability to differentiate between *S. epidermidis*, *S. aureus*, and *S. Saprophyticus*. Kloos and Schleifer (1975b) continued to develop phenotypic tests and were able to classify three new species using conventional methods: *S. haemolyticus* previously classified as *S. aureus*, and *S. xylosus* and *S. cohnii* previously classified as *S. saprophyticus*. Later, Kloos and Schleifer

(1975c) identified four new species, *S. warneri*, *S. capitis*, *S. hominis*, and *S. simulans* from human skin. With the advancement of biochemical testing and the interest in CNS, many more species were identified. By 1976, ten species of *Staphylococci* were recognized: *S. aureus*, *S. simulans*, *S. xylosus*, *S. cohnii*, *S. saprophyticus*, *S. haemolyticus*, *S. warneri*, *S. hominis*, *S. epidermidis*, and *S. capitis* (Kloos et al., 1976). Kloos et al. (1976) identified yet another species and two subspecies, *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *lentus*. Over time the microbiological techniques expanded and current conventional microbiological methods to identify *Staphylococcus sp.* include growth of cultures on different types of media, colony morphology, hemolysis patterns, gram stain, agglutination tests, and biochemical profiles (Ruegg, 2009). By 1995, more than 30 species of *Staphylococci* had been identified and currently there are over 50 species and subspecies of *Staphylococci*, 39 of which are CNS (Zadoks and Watts, 2009; Taponen and Pyorala, 2009).

2.21 Coagulase-negative Staphylococci Identification

Since the development of the simplified scheme of *Staphylococcus sp.* by Kloos and Schleifer (1975) many commercial biochemical test kits have been manufactured taking advantage of these key phenotypic reactions and some are commonly used today to speciate the *Staphylococci sp.* The biochemical test kits differ in the number of biochemical tests, the method of identification, and the time of the assay. Crouch et al. (1987) compared the accuracy of two biochemical kits, Minitek (BBL Microbiology Systems, Cockeysville, MD), a 24-hour assay, and the API Staph-Ident system (Analytab Products, Plainview, NY), a rapid 5-hour assay, to speciate human isolates of *Staphylococci sp.* They reported an 88% accuracy of Minitek and 100% accuracy of the Staph-Ident compared to the American Type Culture Collection (ATCC)

strains. The Minitex system and conventional methods agreed on 88% of the species. A comparison between the API Staph ID 32 (Biomerieux, Inc., France) and conventional methods were made by Layer et al. (2006) for human isolates. The Staph ID 32 system identified 85% and 98% of the ATCC and human isolates correctly. The largest source of error for the Staph ID 32 system was the lack of information in the database (Layer et al., 2006). Three reference strains used with the Staph ID 32 were not recognized as species in that database so they could not be identified properly. The database should have resulted in no match, instead the database provided an incorrect match. De Paulis et al. (2003) compared the API Staph ID 32 system with the Staph-Zym (Rosco, Tastrup, Denmark) and correctly identified 95.5% and 85.5% of human clinical CNS strains. The most frequently isolated strains were *S. epidermidis* and *S. haemolyticus*. Overall, these biochemical kits identified *Staphylococcus sp.* with accuracy and efficiency from clinical human samples.

Coagulase-negative staphylococci cause the majority of IMI in dairy cows, thus proper speciation would enable researchers to advance epidemiological studies of mastitis. Many studies indicated that the CNS were the most prevalent pathogens found in milk and causing IMI in dairy cows (Jones et al., 1982; Oliver and Mitchell, 1983a; Oliver and Mitchell, 1983b; Watts et al., 1984). In addition, many cows with elevated SCC had CNS isolated from their quarters (Langlois et al., 1983). The biochemical kits developed for human isolates of CNS were recognized as a potential means to identify *Staphylococcus sp.* for use with bovine isolates.

Staphylococcus chromogenes is frequently isolated from dairy cattle (White et al., 1989). Until 1986 *S. chromogenes* was classified as a subspecies *S. hyicus* (Hajek et al., 1986 as by Zadoks and Watts, 2009). Therefore studies before 1986 that report on the prevalence and distribution of CNS species do not refer to the most predominant species found among dairy

cows. Misclassification of other species occurred as investigators were determining the phenotypic characteristics of other CNS in an effort to distinguish species in this grouping. For example, *S. equorum* was not identified in studies using the API Staph ID 20 kit (Biomérieux, Inc., France), and could not be identified with a rapid test until the API Staph ID 32 kit was created where additional biochemical tests refined the ability to distinguish between *Staphylococcus sp.* (Zadoks and Watts, 2009).

Langlois et al. (1983) compared the accuracy of the API Staph-Ident system to conventional methods of identification for bovine samples and found that the API Staph-Ident system identified *S. aureus* more accurately (94%) than CNS (42%). However, the Staph-Ident system did identify all *S. hyicus* and *S. simulans* correctly; at that time, those species were believed to be the most common bovine *Staphylococcus sp.*, as *S. chromogenes* was still considered a subspecies of *S. hyicus*. Later, Langlois et al. (1984) compared the Staph-Ident system and the DMS Staph-Trac system (Analytab Products, Plainview, NY). They identified 45% and 81% of bovine isolates correctly as compared to conventional phenotypic culturing methods. Another study reported 80% accuracy of the DMS Staph-Trac system for identification of bovine samples (Matthew et al., 1990).

The API Staph ID 32 system identified 41% and 77% of CNS accurately with sensitivity for *S. chromogenes* of 37% from bovine samples (Sampimon et al., 2009a; Thorberg and Brandstrom, 2000). Park and coworkers (in press 2010) compared the API Staph ID 20 kit to a genetic sequence analysis in which only 57% of the isolates from bovine milk were speciated correctly. For the identification of bovine *Staphylococcus sp.* the Staph-Zym system has identified 92%, 72%, and 94% of CNS isolates correctly with 0% sensitivity for *S. chromogenes* (Sampimon et al., 2009a; Capurro et al., 2009; Thorberg and Brandstrom, 2000). Sampimon et

al. (2009a) also reported that the Staph-Zym system misidentified 23% of *S. chromogenes* strains and was unable to match 9% of all the CNS strains. Conversely Watts and Washburn (1991) found the Staph-Zym system correctly identified 80% of *S. chromogenes* as compared to conventional methods.

Ruegg (2009) compared multiple studies that reported the accuracy of biochemical tests for *Staphylococcus sp.* identification and concluded that the biochemical assays were more accurate for human samples, with 82% and 98% accuracy as compared to conventional microbiology methods for both API Staph ID 32 and Staph-Zym systems. Accuracy of identification from bovine isolates ranged from 45% to 94% as compared to conventional microbiology methods with the best accuracy from the Staph-Zym system (Matthews et al., 1990; Thorberg and Brandstom, 2000).

The biochemical test kits for *Staphylococcus sp.* identification were primarily developed for human diagnostics and have been adapted over time for veterinary use. The utility of these test kits as diagnostic aids and species identification of bovine mastitis isolates has been questioned (Ruegg, 2009). These tests are based on qualitative examination of phenotypic reactions that can lead to variable and sometimes ambiguous results. Yet their ease of use with no new equipment needed in laboratories using traditional microbial culture methods makes them appealing. The genomic data base of bacteria is expanding and the cost of equipment and supplies needed to identify and amplify genetic components is decreasing. Thus use of techniques to distinguish bacteria based on their genotypes is thus made less expensive, easier, and more accurate.

2.22 Genotypic Speciation Methods

The genotypic methods of identification for the speciation of CNS have been referenced as more accurate than phenotypic biochemical tests given that they are quantitative and not qualitative in nature. This is important because many species of *Staphylococcus* have variations among the species and do not always express the same phenotypic traits (i.e. coagulase-negative *S. aureus*) (Fox et al., 1996). Some methods of genotypic identification include 16S rDNA gene sequencing, Amplified Fragment Length Polymorphism (AFLP), transfer RNA-intergenic spacer polymerase chain reaction (tDNA-PCR), and the genetic sequencing and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of various housekeeping genes (i.e. *rpoB*, *hsp60*, *tuf*, *sodA*, *gap*).

Many more species of *Staphylococci* have been identified using genotypic methods in recent years. From 1995 to 2007, the number of identified *Staphylococci sp.* increased from 31 to over 50 species and subspecies (Zadoks and Watts, 2009; Taponen and Pyorala, 2009). Since 2009, four new species of *Staphylococci* have been classified, among them *S. devriesei* from dairy cows (Riesen and Perreten, in press 2010; Masalma et al., 2009; Novakova et al., 2010; Supre et al., in press 2010).

The 16S rDNA genetic sequencing of *Staphylococcus sp.* has yielded an accuracy of 97.4% for isolates of multiple origins (Joussan et al., 2007) and some consider the 16S rDNA to be a gold standard of speciation methods for *Staphylococcus sp.* (Joussan et al., 2007; Park et al., in press 2010). Supre et al. (2009) identified CNS species from bovine samples with accuracy of 94.1% to 99.2% using tDNA-PCR. Generally genotypic methods are more expensive than biochemical kits, but Supre and coworkers (2009) calculated the cost was \$3 per sample. Genetic sequencing of housekeeping genes is another method of species identification, but can

cost from \$15-27 per sample (Supre et al., 2009). Capurro et al. (2009) used the partial *tuf* gene, which codes for the elongation factor EF-Tu, sequencing to identify CNS from bovine samples and identified all of the strains correctly; the most common species identified using the *tuf* gene sequencing was *S. chromogenes* (Capurro et al., 2009). Poyart et al. (2001) sequenced the superoxide dismutase A (*sodA*) gene from human samples, and correctly identified all of CNS the strains. Layer et al. (2006) sequenced the glyceraldehydes-3-phosphate dehydrogenase-encoding (*gap*) gene from human samples and identified all of the isolates correctly as well.

A more cost effective method, with similar accuracy was identified by Park et al. (in press 2010). The method, originally defined by Yugueros et al. (2000), uses PCR-RFLP of the *gap* gene. Ghebremedhin et al. (2008) identified the *gap* gene as the most conserved gene, with 96% interspecies homology, compared to other housekeeping genes (*hsp60*, *rpoB*, *sodA*). They suggested that the use of the *gap* gene would decrease misclassifications among *Staphylococci sp.* Park and coworkers (in press 2010) correctly identified 97% of the CNS isolates with the *gap* gene method as compared to 16S rDNA analysis. In addition to the high accuracy of this method, Park and coworkers (in press 2010) estimated a low cost of \$2.50 per sample.

Many *Staphylococcus sp.* have been identified with genotypic methods and with the identification of new species, biochemical methods of species identification may become obsolete. Genetic testing is a quantitative analysis and can be updated and integrated more quickly and easily and account for species diversity. Although genotypic methods are more accurate than biochemical kits for CNS speciation, many studies used the biochemical kits for identification. This creates confusion when comparing species characteristics and their role as disease agents as described over time. With respect to mastitis, many of the studies have described IMI by *Staphylococcus sp.* as a group, referring to CNS mastitis in general. This lack

of true differentiation between *Staphylococcus sp.* may have created inconsistent descriptions of most attributes of CNS mastitis; especially as this pathogen group has been related to effects on SCC, milk production, response to other pathogens, and duration of infection.

2.23 Coagulase-negative Staphylococci Characteristics

Among the numerous species of CNS many variations exist, which may include virulence factors, antimicrobial susceptibility, and host-interaction (Devriese et al., 2002). Virulence factors for CNS may consist of their ability to adhere to mammary epithelial cells, invade mammary epithelial cells, and evade the immune response. Almeida and Oliver (2001) reported three species of CNS (*S. xylosus*, *S. epidermidis*, and *S. hyicus*) were capable of adhering and invading mammary alveolar cell line (MAC-T) cells. Another study reported that *S. epidermidis* isolated from SCM milk was more invasive as compared to nine other strains (four species) of CNS (Anaya-Lopez et al., 2006). Although most CNS lack virulence factors associated with *S. aureus*, like protein A, coagulase, and hemolysins, some strains do have adherence factors and enterotoxins. Some strains of *S. epidermidis* have adherence factors, such as fibrinogen binding protein (Fbe) and extracellular matrix binding protein (Embp) (von Eiff et al., 2002). Kuroishi et al. (2003) identified toxins produced by CNS isolated from clinically mastitic udders. They found Staphylococcal Enterotoxin-C (SEC) to be the most prevalent toxin among clinically and acutely infected udders and found a significant correlation between concentration of the toxin and the severity of infection. In addition to SEC, five other toxins were identified accompanying CNS mastitis, including TSST-1. These studies demonstrated that some species of CNS possess virulence factors that are normally associated with *S. aureus*. In contrast Jarp (1991) documented the frequency of isolation of CNS species between CM and SCM. Jarp found little difference in pathogenicity among the CNS species as measured by acute signs of the affected

quarter, body temperature, and abnormal clinical condition and concluded that the species did not affect severity of mastitis. Despite the genetic potential of some species to be pathogenic, CNS as a group are still considered minor mastitis pathogens that cause a small proportion of CM.

Some of the variation among CNS species may be a function of their antimicrobial susceptibility characteristics. Cephapirin, a first generation cephalosporin, is commonly used intramammarily to treat IMI caused by staphylococci, and is effective against a wide range of gram-positive organisms, but not all strains of *Staphylococcus sp.* are susceptible to cephalosporins (Donowitz and Mandell, 1988). Costa et al. (2000) compared the susceptibility of 33 CNS strains, consisting of four CNS species (mostly *S. epidermidis*) to many antimicrobials and reported that the CNS were 84% resistant to ampicillin and 86% resistant to penicillin. The CNS strains were also more resistant to antimicrobials than *S. aureus* strains ($P < 0.05$) and Costa and coworkers (2000) documented that 84% of the *Staphylococci* strains were susceptible to cephalothin. Matthews et al. (1992) reported that 31% of *S. chromogenes* and 44% of *S. hyicus* isolates were resistant to tetracycline. In addition, all *S. xylosus* were resistant to novobiocin and all *S. hominis* isolates were resistant to at least one antibiotic. Devriese et al. (2002) documented *S. chromogenes* as normally susceptible to cephalosporins and many others, while *S. sciuri* showed a low-level resistance to lincomycin, cloxacillin, and were resistant to tetracycline. Not only are their differences found between species for antimicrobial susceptibility, there are differences among the CNS species. Myllys et al. (1998) compared CNS isolates from dairy cows from 1988 to 1995 and found a 23% increase in the proportion of CNS that were resistant to at least one antibacterial drug. They also reported that much of the differences in resistance were due to the strains' capabilities of producing beta-lactamase, which

inhibits some antimicrobials. Capurro et al. (2009) reported 18% of bovine milk samples contained CNS with resistance to antimicrobials and that four species produced beta-lactamase (*S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, and *S. xylosus*). Rajala-Schultz et al. (2009) tested the susceptibility of CNS against ten antimicrobials. The group of CNS tested consisted of five species, 71% *S. chromogenes*, 13% *S. xylosus*, 5% *S. hyicus*, 5% *S. warneri*, and 2% *S. epidermidis*. Twenty-two percent of the CNS were resistant to penicillin, 14% to oxacillin, 29% to sulfadimethoxine, 11% to cephalothin, and 36% to more than one antimicrobial.

Variation in the mammary immune response to infection by *S. aureus* was measured by Zecconi et al. (2006) by comparing virulence factors of *S. aureus* to mammary inflammatory responses by measuring SCC. They reported a correlated increase of SCC associated with strains with more virulence factors, such as the coagulase gene, protein A gene, fibrinogen-binding protein gene, collagen-binding protein gene, and various enterotoxin genes. In many studies, CNS was the most commonly isolated udder pathogen from quarters with high SCC (Sampimon et al., 2009c). Coagulase-negative staphylococci infections can contribute as much as 17.9% of the cells in a herd with bulk tank SCC (BTSCC) <200,000 cells/ml (Grohn et al., 2004). Nickerson et al. (1995) found that *S. hyicus* and *S. chromogenes* were the most frequently isolated CNS species causing IMI with SCC of 193,000 cells/ml and 168,000 cells/ml, respectively. More recently, Taponen et al. (2007) reported an average SCC of 657,000 cells/ml with a median of 355,400 cells/ml for CNS IMI in persistently infected mammary quarters. Usually the greater the SCC increase, the more milk production will decline. Thorberg et al. (2009) reported that transient infections by CNS species were more detrimental to milk production than persistent infections that were primarily caused by *S. chromogenes*, *S.*

epidermidis, and *S. simulans*. These three CNS species, in addition to *S. hyicus*, cause the majority of CNS mastitis in dairy cows (Park et al., in press 2010; Pyorala and Taponen, 2009).

2.3 Colonization by Coagulase-negative Staphylococci

Coagulase-negative staphylococci are opportunistic pathogens that reside on the teat skin. The CNS have been isolated from the teat skin and teat ends as early as the first days of life and may serve as a reservoir for IMI (White et al., 1989). Many studies have indicated that CNS are the most common colonizers of the teat skin and teat ends (Edwards et al., 1966; Trinidad et al., 1990; White et al., 1989; Taponen et al., 2006; Taponen et al., 2008; Kromker and Friedrich, 2009). Just inside the teat end is the streak canal, which protects the mammary gland by restricting the passage of pathogens into the udder. Pathogens may gain entrance to the streak canal by teat injury, leakage of milk, or other physical action like back-jetting during milking. Additionally the streak canal is lined by a keratin layer containing lipids and proteins that have bacteriostatic properties (Nickerson et al., 1995; Hibbitt and Cole, 1969; Hogan et al., 1986). Bacteria that are able to survive in the keratin are better equipped to invade the mammary gland, and subsequently may serve as a reservoir for IMI (Nickerson et al., 1995; Trinidad et al., 1990). Some studies reported that the colonization of the streak canal by a pathogen increases the risk of IMI by the same pathogen (Hogan et al., 1986; Zecconi et al., 2000). The colonization of the streak canal triggers an inflammatory response from the mammary gland as evidenced by increasing SCC (Trinidad et al., 1990).

The effectiveness of the streak canal to protect the mammary gland is very important for non-lactating cows and heifers. During the dry period, the streak canal should form a keratin plug to protect the mammary gland. Day (1990) reported that teats without sufficient keratin

plug formation in the dry period experienced more IMI. In prepartum heifers, 60% of the streak canals were open two months before calving, and the majority of IMI in the open streak canals were caused by CNS (Kromker and Friedrich, 2009). To reduce this risk, teat sealants have been designed to form a barrier and protect the teat during the dry period. Berry and Hillerton (2002) suggested that the colonization of the streak canal by CNS increased the risk of IMI in the dry period because colonization prevented the streak canal from effectively closing. When they used teat sealants in the colonized quarters, IMI were reduced in the dry period and in the following lactation (Berry and Hillerton, 2002).

The most frequently isolated CNS species found on the teat skin and teat apices is *S. chromogenes* (White et al., 1989; Matthews et al., 1992; Thorberg et al., 2009; Taponen et al., 2008). In addition, frequently isolated CNS species from the teat skin are *S. xylosum*, *S. epidermidis*, *S. simulans*, and *S. hyicus*. *Staphylococcus chromogenes* most frequently colonizes the streak canal as well (Trinidad et al., 1990; White et al., 1989; Taponen et al., 2008; Matthews et al., 1992). Other species of CNS often colonizing the streak canal include *S. hyicus*, *S. xylosum*, *S. warneri*, *S. sciuri*, and *S. simulans*. A recent study contrasted bacterial colonization of the streak canal between dairy and beef cows (Gill et al., 2006). Overall the beef cow streak canals had more colonizing species. *Clostridia sp.* and *Bacillus sp.* dominated both beef and dairy cows although the beef cows were predominately colonized by *Clostridia sp.* In addition to *Clostridia sp.* and *Bacillus sp.*, the dairy cows were colonized by many species of *Staphylococci*, including seven species of CNS, mostly *S. xylosum* (Gill et al., 2006). In contrast, Trinidad et al. (1990) reported that 42.9% and 25.2% of heifers' streak canals were colonized by *S. chromogenes* and *S. hyicus*. They also noted that 5.7% of the mammary quarters of

primigravid and unbred heifers had mixed species colonizing the streak canal. These species included *Staphylococci* and *Streptococci* but they did not reveal which species were colonizing in concert. In addition, the majority of CNS colonization were persistent (>1 month) and were associated with increased SCC. Trinidad and colleagues (1990) concluded that CNS colonization is prevalent in heifers and the majority of colonization coincides with high SCC. Likewise, White et al. (1989) isolated *S. chromogenes* (34.1%), *S. warneri* (19.7%), and *S. xylosus* (14.2%) most frequently from the streak canals of nulliparous heifers. The distribution of species colonizing body sites, the vagina, haircoat, and nares, consisted of predominantly these three species, but the relative species proportions changed between body sites. White and coworkers (1989) suggested that the difference in the distribution for these species may rely on the species' ability to colonize different areas of the dairy cow.

Recently Thorberg et al. (2009) reported colonization of the teat canal by CNS species differed with different parities. *Staphylococcus epidermidis* were isolated mainly in multiparous cows and *S. chromogenes* predominantly in heifers. In addition they diagnosed IMI when a milk sample had at least 500 cfu/ml of a pure CNS growth on aerobic culture and recorded the severity of infection by SCC. Thorberg and coworkers (2009) proposed that there may be virulence factors that affect host-specificity at different ages or immune factors that affect the ability of the pathogen to colonize the mammary gland. Earlier *S. epidermidis* had been documented to be transmitted to cows mainly from humans in the milking parlor (Thorberg et al., 2006). This supported subsequent findings (Thorberg et al., 2009), given that heifers are not yet in the milking string, therefore would not be exposed to human *S. epidermidis*.

The correlation between colonization of the teat end and IMI has been demonstrated by a number of studies and colonization of *Staphylococcus sp.* at the teat orifice might be a primary predisposing factor for IMI. Roberson et al. (1994) demonstrated that colonization at the teat orifice by *S. aureus* of heifers lead to a 3.3-fold increased risk of developing IMI caused by *S. aureus* at parturition. Other studies examined the relationship between colonization of the teat canal and the teat skin with CNS. It was demonstrated that the most prevalent species colonizing both the teat skin and streak canal was *S. chromogenes*, which also caused the majority of IMI by CNS (White et al., 1989; Trinidad et al., 1990; Matthews et al., 1992). These studies suggest that extramammary colonization by specific pathogens, including CNS, appears to be related to IMI because of the similar distribution (Taponen et al., 2008).

2.4 Intramammary Infections by Coagulase-negative Staphylococci

Reports on the prevalence of CNS IMI are quite varied which in part maybe a function of the multitude of definitions for diagnosing IMI. Intramammary infections are primarily diagnosed when a pathogen is isolated from a milk sample, suggesting an infection. When multiple pathogens are in the milk, most often contamination is assumed. In many studies, the researchers look for the presence of specific pathogens of interest. Suggested methods of sampling and microbiological methods of bovine milk samples are described in laboratory handbooks (NMC, 1981; NMC, 1999; NMC, 1987). In general, 0.010 ml of a milk sample is inoculated on a blood agar plate and incubated. A larger volume of milk may aid in identification of pathogens causing infection in low amounts, like some environmental pathogens (Timms and Schultz, 1987). Microbiological methods are used to identify the pathogens so an IMI may be diagnosed (NMC, 1987).

Depending on the study and sampling schedule, the frequency of isolation required to diagnose an IMI may differ. Criteria may require the isolation of a pathogen more than once to diagnose an IMI, either in duplicate or consecutive samples, for better reliability (NMC, 1981; Erskine and Eberhart, 1988). In addition, isolating a pure culture with several colonies is more significant of an infectious pathogen (NMC, 1987). The presence of CNS in the milk is most significant when in a pure culture of several colonies. As for major pathogens like *S. agalactiae* and *S. aureus*, the presence of one colony among any number of pathogens is considered significant for infection (NMC, 1987).

The use of threshold values to determine IMI will always misclassify some samples and will not always be sensitive enough to diagnose all IMI (Pitkala et al., 2004). In research and in general, thresholds for the evaluation of IMI need to be established to simplify the evaluation of samples. Sampimon et al. (2009d) diagnosed IMI for major pathogens when a single sample yielded ≥ 100 cfu/ml of the pathogen. However minor pathogen IMI were diagnosed when ≥ 500 cfu/ml were isolated from a single sample. In general the threshold value for IMI should represent the potential of the pathogen to cause infection.

There are many variables that contribute to the lack of uniformity in studies that discuss and report CNS IMI and IMI in general. There is a lack of a uniform method used to diagnose IMI. The general convention is to diagnose an IMI when the same pathogen is isolated from two of three consecutively collected milk samples (Hogan et al., 1990). What is not defined is the interval between samplings. This could have considerable impact on the classification of infections as those of short duration would be misclassified when the time interval is long. Alternatively, if the sampling interval is short then it may be difficult to distinguish between true IMI and short term colonization of the gland. Some researchers report on duplicate mammary

quarter milk samples taken at the same milking (Boddie and Nickerson, 1994; Foret et al., 2006), samples have been taken at weekly (Aarestrup et al., 1999), bimonthly (Foret et al., 2006), and monthly (Goldberg et al., 1994) intervals; or at specified stages of lactation (Peters et al., 2000).

The method of identification of the pathogen is also important for the interpretation of IMI. The methods of identification have changed over time, especially for the CNS. Many studies still report CNS as a group (Foret et al., 2006), while other studies speciate *Staphylococci*. Clearly using the criteria that the same pathogen must be isolated from two consecutive samples would suggest that speciation would lead to a more accurate diagnosis. The method of speciation, phenotypic or genotypic, can influence the diagnosis. Many studies use biochemical kits for CNS identification (Sampimon et al., 2009a; Thorberg and Brandstrom, 2000), so the results must be interpreted carefully when comparing such results to other studies where genotypic speciation was done. The variety in the approaches to diagnose IMI makes it difficult to make comparisons between studies.

The majority of studies reported that CNS are the most prevalent cause of IMI, in heifers, lactating, and dry cows (Boddie et al., 1987; Schultze, 1983; Todhunter et al., 1993; Sampimon et al., 2009d; Oliver and Mitchell, 1983b). The majority of IMI in the prepartum and postpartum periods are caused by CNS, 74% and 55%, respectively (Oliver and Mitchell, 1983a). Others reported the range of IMI caused by CNS in prepartum heifers from 22% to 71% (De Vliegher et al., 2004). Djabri et al. (2002) conducted a meta-analysis which included 12 studies that reported CNS were responsible for 5.5% to 27.1% of IMI. The thresholds for CNS diagnoses ranged from 20 cfu/ml to 500 cfu/ml of CNS among the studies. The most common definition for an IMI was ≥ 100 cfu/ml in more than one consecutively collected sample (Foret et al., 2006). Only five of those articles identified the CNS by species using commercial

biochemical kits (Chaffer et al., 1999; Davidson et al., 1992; Hogan et al., 1987; Leitner et al., 2000; Woolford et al., 1998). The remaining studies used culture methods to identify CNS as a pathogen group, and none used genotypic methods.

Sampimon and coworkers (2009d) reported a prevalence of CNS IMI in 11% of quarters and 34% of cows which they defined an IMI as recovery of ≥ 500 cfu/ml of CNS from one sample. A study in Finland also required ≥ 500 cfu/ml as a threshold value for diagnosing IMI by CNS and they reported a prevalence of 49% to 53% (Pitkala et al., 2004). Unlike these studies, in Norway a study diagnosed IMI with one milk sample $\geq 4,000$ cfu/ml of CNS. The high threshold may have contributed to the low prevalence (3.3%) of CNS IMI in Norway and the authors suggested that it may be an underestimation (Osteras et al., 2007).

Specifically the majority of CNS IMI in heifers and cows are caused by *S. chromogenes* (Todhunter et al., 1993; Taponen et al., 2008). Taponen et al. (2007) reported that 50% of CNS IMI in lactating cows were caused by *S. chromogenes* and 31% by *S. simulans*. Intramammary infections by CNS can be persistent and the duration of infection may be species specific. In one study, *S. chromogenes* is described as a transient pathogen and *S. simulans* as persistent; persistent infections were at least two months in duration with monthly sampling (Taponen et al., 2007). Taponen et al. (2008) also reported that *S. chromogenes* was most likely an opportunistic pathogen because it was found on all extramammary sites, as well as in the mammary gland. But *S. simulans* was most likely an obligate udder pathogen as it was not found on extramammary sites but did cause mastitis.

Most CNS IMI cause an increase in SCC. Some studies have suggested that the increase in SCC caused by CNS IMI may have a protective effect for the mammary gland against major pathogens (Nickerson and Boddie, 1994; Lam et al., 1997). Mammary quarters infected with

CNS prior to challenge with a major pathogen were less susceptible to infection by *S. aureus* (Nickerson and Boddie, 1994; Lam et al., 1997). Lam and coworkers (1997) reported a higher SCC in CNS IMI quarters compared to controls. The protection against new IMI in CNS infected quarters might have been a result of the higher concentration of leukocytes. Others (De Vliegher et al., 2004) suggest a bacteriocin produced by the bacteria causing the infection may be responsible for the inhibition of infection by a second agent. Yet others have found no protective effect by CNS IMI in the establishment of a second infection by major pathogens (De Vliegher et al., 2004; Hogan et al., 1988; Spencer et al., 1968 as by Matthews et al., 1991).

The diversity of study sampling methods, identification methods, and threshold values to deem the presence of the pathogen to be an infection makes comparisons among studies difficult. The large range of CNS IMI (5.5% to 27.1%) reported by Djabri et al. (2002) is an example of the diversity found in the literature. The studies compared used different sampling methods, threshold values and identification methods; therefore, the range of prevalence reported by the meta-analysis is difficult to interpret. Overall most studies determined that CNS caused the majority of IMI in dairy cows, especially heifers. The most common species of CNS isolated was *S. chromogenes*, which is also more prevalent among heifers. The IMI caused by CNS were also noted to cause increases in SCC which reduced milk quality. In conclusion CNS IMI are prevalent among dairy cows which negatively impacts the dairy industry. Our understanding of CNS IMI could be increased with the establishment of standardized speciation techniques and standard definitions of infection.

2.5 Control Strategies

With the large effort to be rid of contagious pathogens, like *S. aureus*, the prevalence of CNS isolated from mastitic cows has increased (Myllys et al., 1998). Subclinical mastitis caused

by CNS are largely untreated in dairy cows during lactation, but have a natural cure rate between 15-40% (Schukken et al., 2009). Coagulase-negative staphylococci have become more common as a cause of SCM and CM while contagious pathogens have been largely controlled with milking time hygiene (MTH) procedures (Neave et al., 1969; Schukken et al., 2009). Many studies have reported dramatic increases in the prevalence of CNS among mastitis pathogens, inverse to the decline of major pathogens in recent years (Pyorala and Taponen, 2009; Sampimon et al., 2009d). Some studies have reported CNS to be the most common mastitis-causing pathogen as well as the most isolated pathogen from SCM in a number of countries (Pitkala et al., 2004; Myllys et al., 1998). Tenhagen et al. (2006) isolated CNS from 9.1% of cows, and of milk subclinically infected, 34.5% were CNS. In high SCC herds, 28% of cows had CNS infections on average and one herd reported a prevalence of 41% (Roberson et al., 2006 as by Pyorala and Taponen, 2009). For heifers, the prevalence of CNS IMI at calving from ranged from 12.3–45.5% and up to 74% in the prepartum period (Fox, 2009). Haltia et al. (2006) found 15.8% of pathogens isolated from milk were CNS and noted an increased SCC of 508,000 cells/ml. A national survey of dairy herds in Finland, reported an increase in prevalence of CNS in quarter milk samples in 1995 and 2001 from 11% to 16% with 49.6% of pathogens isolated as CNS (Pitkala et al., 2004). In Wisconsin, milk samples were compared from 1994 to 2001, both clinical and subclinical samples (Makovec and Ruegg, 2003). The prevalence of *S. aureus* and *S. agalactiae* isolated from those samples decreased in prevalence by 8% and 5%, respectively, whereas CNS increased in isolation from 12.7% to 17.5%. Relative to time in lactation, Davidson et al. (1992) reported an increase in prevalence of CNS SCM from about 5% to 15% from the beginning of lactation to the last few months of lactation.

The increase of prevalence in CNS IMI must be counteracted with more control strategies. Control strategies are very important for reducing the incidence of CNS mastitis. The main control strategies of mastitis are dry cow therapy (DCT), lactating cow therapy (LCT), and milking-time hygiene (MTH). Management methods may differ between farms in their use of these control strategies, but most mastitis management protocols include these practices. Both MTH and DCT are meant to protect the mammary gland when it is most vulnerable: during milking time, at the cessation of lactation, and during the periparturient period.

2.51 Therapy

Therapies are used at various times throughout the productive life of dairy cows to treat or prevent bacterial infections in the mammary gland. With non-lactating cow therapy, dry cow therapy, the dairy operator can instill intramammarily a higher dose of antibiotic in a slower release vehicle than with intramammary therapy during lactation. During the dry period milk antibiotic induced withholding periods are not of concern. Dry cow therapy has been documented to reduce IMI by curing existing infections and preventing new infections by major and minor pathogens by major and minor pathogens (Dingwell et al., 2002). Rajala-Schultz et al. (2009) reported a cure rate of 82% for CNS IMI with DCT. Because heifers do not receive DCT, they are potentially at a higher risk for CNS infections prior to parturition. Heifers have a higher infection rate by CNS than multiparous cows and are found to have CNS IMI prior to parturition (De Vliegher et al., 2005). Heifers could have a significant reduction of IMI by CNS after calving with the additional use of preparturient teat sealants (Parker et al., 2007).

The majority of CNS IMI are predominantly subclinical and are left untreated by LCT which is used chiefly to treat CM. Thus CNS IMI may persist throughout the entire lactation.

The use of LCT is effective against CNS and could increase milk production and reduce SCC for the remainder of lactation (Oliver et al., 2004). The drawbacks of LCT are the loss of milk during the withhold period, treatment costs, and extra labor. Using LCT in early lactation may be more cost-effective than using it later in lactation, as early efficacy of treatment may translate into more milk production output over the remainder of lactation than delaying therapy.

Although mastitis caused by CNS is not always treated, if the infection progresses to CM, will most likely be used. Commonly used antibiotics to treat CM are Penicillin G/Dihydrostreptomycin (36.9% usage) and Cephapirin (31% usage) (USDA, 2008). Both of these are efficacious against CNS, with a cure rate of 85.9% (Taponen et al., 2006).

Subclinical cases of mastitis have also been treated with LCT. The treatment of SCM (SCC>300,000 cells/ml) using ceftiofur hydrochloride as an intramammary treatment, resulted in cure of 86% for quarters with CNS (Oliver et al., 2004). Salat et al. (2008) reported a cure rate of 63% for SCM by CNS using a systemic treatment of penethamate hydriodide. Deluyker et al. (2005) reported a non-significant cure of CNS SCM with Pirlimycin, even with longer and stronger treatments, but recommended that this could be caused by re-infection. They suggested that CNS species identification may aid in understanding these results. Although some of these studies showed significant cure rates, none of these studies speciated CNS to determine which species were present and how efficacious the treatments were to those specific treatments.

2.52 Milking Time Hygiene

Proper MTH is an effective management practice to reduce the prevalence of contagious mastitis in a herd. Milking-time hygiene includes single-use towels, pre-milking teat dips, post-milking teat dips, use of latex or nitrile gloves by milkers to reduce contamination from human to cow epidermis, and general cleanliness and maintenance of milking equipment. In addition, a

backflush system may be implemented to reduce spread of contagious pathogens by sanitization of milking units between each use.

The first trials to show the effectiveness of MTH were in the 1960s by British scientists. They showed that the use of milking time hygiene procedures reduced IMI by about 50% (Neave et al., 1969). The MTH procedures included teat dips which are an effective method to reduce IMI and reduce SCC (Moxley et al., 1978). Generally, teat dips reduce the pathogen load on the teat skin, post-milking teat dips for contagious pathogens and pre-milking teat dips for environmental pathogens. Pankey (1989) reported a 51% reduction in new IMI caused by environmental pathogens with the use of iodine pre-milking teat dip. In addition to pre-milking teat dips for the control of environmental pathogens, cleanliness is important as well. Better udder-hygiene scores were correlated with lower prevalence of IMI by environmental pathogens, and cows with the worst udder hygiene scores were 1.5 times more likely to have major environmental pathogens in their milk (Schreiner and Ruegg, 2003).

In addition to MTH, many other herd-level factors may be controlled to lower the incidence of mastitis. Specifically for CNS mastitis, some factors identified by Sampimon et al. (2009b) that were correlated with a decrease of CNS IMI included: drinking water from tap-water source, housing dry cows in more than one group, not pasturing during the outdoor season, a low percentage of stalls contaminated with leaking milk, and a BTSCC <250,000 cells/ml. Contrary to what might be considered logical, Sampimon and coworkers (2009b) found a higher percentage of CNS IMI were correlated with the usage of a mastitis treatment protocol versus no protocol and more visits by the veterinarian. They suggested that these unlikely correlations could be explained by dairy managers employing a recommended mastitis control protocol only recently because they were responding to an existing problem with mastitis.

2.53 Teat Disinfection

Teat dipping has been identified as an effective management strategy to reduce new IMI by contagious and environmental pathogens and post-milking teat disinfection has been the most important aspect in preventing IMI by contagious pathogens in dairy cows (Pankey et al., 1984). Teat dipping reduces the pathogen load on the teat skin, thus reducing the risk of infection. The first documented teat dipping in the dairy industry was in 1916; a dilute pine oil teat dip was used to reduce the spread of *S. agalactiae*, a contagious mastitis pathogen (Pankey et al., 1984). Teat dipping has reduced the incidence of mastitis and may reduce new infections by more than 50% (Pankey et al., 1984). Although teat dips effectively reduce new IMI, they only eliminate extramammary pathogens and may not eliminate existing infections.

A post-milking teat dip is effective against CNS IMI and may be the most important step in CNS IMI prevention; pre-milking teat dips have been shown to be ineffective at preventing CNS IMI (Oliver et al., 1993; Eberhart et al., 1983; Foret et al., 2006). The combination of pre- and post-milking teat dips can reduce incidence of IMI by 40.9% compared to quarters only post-dipped (Oliver et al., 2001). Oliver and coworkers also demonstrated post-milking teat dips eliminated residual CNS on the teat skin, in addition to other contagious mastitis pathogens. Bacterial populations on the teat skin by *S. aureus* have been associated with an increased risk of IMI by the same pathogen (Roberson et al., 1994). A reduction in the number of bacteria on teat skin is correlated to the reduction of IMI, so post-milking teat dipping is an effective way to reduce IMI. Hogan et al. (1987) mentioned that the use of teat dips to reduce *Staphylococcus sp.* IMI would be beneficial.

There are six classes of postmilking teat dips according to Pankey and colleagues (1984): iodophors, quaternary ammonium compounds, chlorhexidines, sodium hypochlorites, dodecyl

benzene sulfonic acid (DDBSA), and acrylic latex. Teat dips are composed of many ingredients and have three main parts: an active ingredient, to kill pathogens; an emulsifier, to keep the ingredients together; and emollients or humectants, to reduce irritation and improve the teat skin condition. There are various modes of action for these germicidal teat dips and characteristics attributed to different dips: iodine oxidizes microorganisms, quaternary ammonium compounds disrupt the permeability of the cell membrane, chlorhexidine precipitates cellular macromolecules, sodium hypochlorite oxidizes bacterial proteins, DDBSA denatures proteins or disrupts the cell membrane, and acrylic latex barriers act as a physical barrier for bacteria (Pankey et al., 1984).

Teat dips are widely used in the dairy industry, both pre- and post-milking. Teat dips are either applied to the teats via a dip cup, where the teat is immersed in the dipping agent, or applied by a spray nozzle. The dip cups typically have better coverage on the teats, but the sprayers can be used to apply disinfectant more rapidly. Sixty-nine percent of dairies in the U.S. currently use an iodine-based post-milking teat dip, 13% use a chlorhexidine-based dip, and 6% use a fatty-acid based teat dip (USDA, 2008). Only 5.2% of dairies reported not using a post-milking teat dip (USDA, 2008). Iodine-based dips contain iodine in complex with water-soluble detergents with moisturizers; the germicidal effectiveness is based on the amount of free iodine to oxidize organisms (usually .1% to 1% iodine): iodine dips are efficacious against fungi, viruses, spores, and bacteria (Pankey et al., 1984). Additionally, iodine dips are advantageous because they are effective in high-organic loads, visible on the teat skin, and generally non-irritating to skin (Pankey et al., 1984).

Efficacy of a germicide was defined by Murdough and Pankey (1993) as reducing viable bacterial counts by 23 logs when compared to a negative control, but teat dip efficacy is defined

as the ability to reduce incidence of IMI (Pankey et al., 1984;). To compare teat dips, the incidences of IMI are measured between experimental groups in either a challenge or natural occurrence study (Hogan et al., 1990). Challenge studies require the application of pathogens to the teat, or into the mammary gland; natural occurrence studies measure the number of IMI that occur naturally within experimental groups. In most teat dip studies, different types of disinfectants or the concentrations between similar disinfectants are compared. When comparing different concentrations of active ingredients, Nickerson and Boddie (1995) found that a 1% iodine dip had a 30.6% lower infection rate than the 0.3% iodine dip, thus presuming that the higher iodine content was more efficacious. Gottardi et al. (1991) explained that iodine antimicrobial properties are reliant on the amount of free iodine and not on the actual concentration in the dip. Iodine in solution is in equilibrium with free or available iodine, and unavailable iodine. The free iodine is the antimicrobial portion of the solution, so when the free iodine is greater, the antimicrobial capabilities are greater (Foret et al., 2005).

For the control of major pathogens, sodium dodecylbenzene sulfonic acid and iodine teat dips have been effective at reducing IMI by *S. aureus* (72-100% and 86-89%) and *S. agalactiae* (75-88% and 73-78%) after challenge (Oura et al., 2002; Boddie et al., 2004). In addition a quaternary ammonium teat dip was effective in reducing *S. aureus* IMI by 77%, however was not efficacious for reducing CNS IMI (Stewart and Philpot, 1982).

Post-milking teat dips are the preferred method for reducing CNS IMI because pre-milking teat dips have been reported to not reduce CNS IMI (Oliver et al., 1993). Other teat dips like linear dodecyl benzene sulfonic acid and chlorhexidine have also reduced CNS rate of IMI by 91% and new IMI by 33.3% to 38.7% (Pankey et al., 1984; Poutrel et al., 1990; Oliver et al.,

1989). In addition, the combination of phenolic pre- and post-milking teat dips reduced CNS IMI by 40.7% (Oliver et al., 2001). The use of post-milking teat dips reduces the bacterial count on the teat, presumably reducing the risk of colonization of the streak canal, and thus IMI. For the reduction of CNS colonization on the teat skin/apices and IMI, both iodine and chlorhexidine have been efficacious. Hogan et al. (1995) compared iodine to chlorhexidine dips and found no difference in the reduction of CNS IMI. In other studies, chlorhexidine post-milking teat dips reduced CNS IMI by 49% and 57.5% (Oliver et al., 1990; Hogan et al., 1995). Using various iodine post-milking teat dips, CNS IMI were reduced by 16%, 83%, and 42.5% (Nickerson et al., 1986; Eberhart et al., 1983; Foret et al., 2006). With higher concentrations of free iodine in iodine post-milking teat dips CNS IMI were reduced by 71.6% (Foret et al., 2005).

2.6 Conclusion

The current control method of post-milking teat disinfection is efficacious for the reduction of IMI by the mastitis pathogen group of *Staphylococcal sp.* (Oliver et al., 2004; Oliver et al., 2001; Hogan et al., 1995). However, what is not known is how the control measures affect individual species of CNS that cause IMI. *Staphylococcal chromogenes* is the most prevalent CNS species colonizing the teat and causing IMI in dairy cows (Taponen et al., 2008; Park et al., in press 2010). There has been renewed interest in CNS mastitis investigations noting the apparent increase in prevalence of this disease (Pyorala and Taponen, 2009). This increased prevalence among mastitis pathogens may be attributed in part to improved accuracy of CNS IMI diagnosis, improved control of other mastitis pathogens, or an increase in CNS pathogenicity.

Some studies suggested that some CNS species have genetic characteristics similar to major pathogens, like *S. aureus*, thus the potential to cause severe mastitis (Kuroishi et al., 2003). The CNS cause up to 10% and up to 12.8% of clinical cases of mastitis and remain minor pathogens (Thorberg et al., 2009; Waage et al., 1999). The control of other pathogens through increased efforts in MTH could account for the increase in proportion of CNS causing mastitis. This would mean that the number of CNS are not increasing, but the proportion of CNS among all mastitis-causing pathogens is increasing with more dairies applying more stringent MTH practices (Schukken et al., 2009).

Mastitis research on CNS has been hampered by lack of focus on species identification. For example, most studies have identified CNS as a pathogen group and if CNS is isolated at the beginning of lactation from an aseptically collected milk sample and then at the end of lactation, by extrapolation the conclusion is that the mammary quarter was persistently infected. In reality, the isolates could be different species signifying that there may have been two different IMI. Pyorala (2007) recommends that species of mastitis pathogens should not be cavalierly grouped. The addition of species identification would clarify the actual impact CNS IMI has on the dairy industry which could lead to improved methods of control. Yet speciation of the CNS has been difficult and does not always return an unambiguous result (Thorberg et al., 2009).

The predominant species of CNS IMI and colonization of the teat skin/apices is *S. chromogenes* and the correlation between its colonization, control methods, and IMI need to be understood to more efficiently control CNS IMI in dairy cows. A study of the speciation of CNS and the effects of post-milking teat dips would enhance our knowledge and elucidate the possibilities of species specific effective control of CNS mastitis. In a pilot study conducted at

the WSU Dairy (Appendix 1) CNS were isolated from teat skin, teat apices, and streak canals to understand the effects of teat dipping on their colonization. The post-milking teat dips reduced the colonization by CNS at all three samples sites and reduced the number of recovered CNS from milk samples (Appendix 1). Speciation of CNS using an accurate method would enable a better understanding of the relationship between colonization of the teat end and IMI by species. Presumably, the rate of colonization by specific CNS species affects the incidence of IMI by those same species. It could be assumed that post-milking teat dip affects CNS species colonization differently and thus would affect IMI.

CHAPTER 3

Materials and Methods

3.1 Cows

Holstein dairy cows were chosen for this trial from a closed herd at the Washington State University Knott Dairy Center in Pullman, WA. The trial lasted 16 weeks, from September 22, 2009 to January 5, 2010. All cows were required to meet the following inclusion criteria prior to enrollment: lactating with four functional teats, teat end score <3, teat skin score <4 (Zecconi et al., 2005), and no CNS IMI prior to the start of the trial (Peters et al., 2000). The teat skin and teat ends were scored visually according to a scale described by Zecconi et al. (2005). An effort was made to enroll cows more than 24 weeks before the next expected parturition. Before enrollment, a composite milk sample was examined from each cow to determine their bacteriological status and to ensure that none had a current CNS IMI (Hogan et al., 1990).

Overall 43 dairy cows were included in this trial. The sample size of at least 40 cows, was determined by sample size calculations (power=0.80, alpha=0.05). Enrolled cows were housed in the same pen in a freestall barn with composted manure solids as bedding and fed a total mixed ration.

3.2 Milking Procedures and Treatments

Standard milking time hygiene procedures of 0.1% iodine pre-milking teat disinfection, foremilk stripping, cleaning the teats with a single-use, damp, cloth towel and application of a 1% iodine teat disinfectant, were altered. During the trial pre-milking preparation included foremilk stripping and cleaning the teats of dirt and debris with a damp, single service cloth towel; extra towels and water was used if necessary. Mammary gland halves, right vs. left, were

allocated different treatments randomly by using a random number table. The treatment udder-half was dipped with the 1% iodine teat disinfectant after the automatic milking unit detached. The control udder-halves were untreated and did not receive postmilking teat disinfection. Cessation of standard pre- and post-milking teat disinfection was done prior to the start of treatment application to create a five-day washout period. The washout period was used to ensure that previous teat disinfection would not influence response to treatment.

A 1% iodine teat dip (Westfalia-Surge, WA) was selected as the treatment dip based on pilot study findings (Appendix 1). This iodine teat dip was effective in reducing colonization of the teat skin, apices and streak canal (Appendix 1) by CNS and is the most commonly used in the United States (USDA, 2008).

3.3 Sample Collection

Milk samples were collected every Tuesday at the morning and evening milkings (10am and 10pm) from all mammary quarters in duplicate. Half of the cows were sampled prior to the morning milking, and the other half were sampled at the evening milking; each quarter was sampled only once a week. In addition, streak canal samples were taken on day five of the treatment. All samples were collected in duplicate from each quarter of all selected cows prior to a regularly scheduled milking. The samples consisted of streak canal swabs and milk samples. After pre-milking preparation, the teat end was sterilized with a cotton pad moistened with 70% isopropyl alcohol from the furthest to the nearest teat and sampled in reverse. A calcium alginate fiber tipped ultrafine aluminum applicator swab (Fisher Scientific, Tustin, CA) was inserted two to three mm into the distal end of the streak canal and rotated. The swab was then placed into a tube containing 1 ml of sodium thiosulfate solution (Appendix 2). The teat ends were sanitized

again as before and the milk samples were collected in 5-ml tubes. All samples were placed on ice until transport to the lab. The swab samples were stored at 5°C and the milk samples were stored at -20°C until processed.

3.4 Culture Procedures

Fifty microliters of duplicate milk and streak canal solutions were spread evenly and cultured on one Mannitol salt agar plate (Appendix 2), a selective media for *Staphylococci sp.*, and incubated at 37°C, 5% CO₂ for 24-48 hours. A larger amount of sample, 50 µl vs. the standard 10 µl was plated to increase the sensitivity to detect CNS (NMC, 1999). Plates were considered contaminated if cultures grew three or more dissimilar colonies. All unique colony culture types were identified and a representative culture was re-cultured on blood agar plates (Hardy Diagnostics, Santa Maria, CA) with incubation as before. The cultures from blood agar were characterized by their catalase reaction, gram stain, colony morphology and the coagulase test (NMC, 1999). The Slidex Staph plus (Biomerieux, Inc., Durham, NC) was used to further differentiate coagulase-positive cultures from *S. aureus*. All presumptively identified CNS cultures were stored in sterile glycerin solution (60%) (Appendix 2) at -80°C until speciation.

3.5 Coagulase-negative Staphylococci Speciation

Seventy-six CNS isolates were speciated using the *gap* gene method to diagnose IMI (Park et al., in press 2010). Only samples in the first two to three weeks of an IMI that met the threshold value were speciated. Once confirmed as an IMI, the streak canal isolate from the week of IMI diagnosis was speciated; if no CNS was isolated the week of diagnosis, streak canal isolates were speciated from the week prior to diagnosis. Many of the streak canal samples that

corresponded to IMI diagnosis were missing due to lack of supplies, making this comparison difficult.

After DNA extraction, identification of the isolates as CNS by presence of the *gap* gene was determined using *Taq* polymerase with PCR and gel electrophoresis. Once identified as CNS, the PCR products were digested using *AluI* restriction enzyme. The fragment lengths were compared to published sequences to identify CNS by species.

The DNA extraction method was described by Pitcher et al. (1989). Briefly, about 10 μ l of the isolates were cultured on blood agar plates and incubated at 37°C for 24 hours. Several colonies were used to inoculate 5 ml of Todd Hewitt broth (Appendix 2) from pure culture on blood agar plates and incubated at 37°C for 18 hours. One milliliter of the culture was centrifuged at 12,000 g for 2 to 3 minutes. The supernatant was discarded and 1 ml of TE buffer (Appendix 2) was added to the pellet then centrifuged as before. Again the supernatant was discarded and 1 ml of TE buffer added then mixed. Ten microliters of lysostaphin (Appendix 2) and 100 μ l of lysozyme (Appendix 2) were added and incubated at 37°C for 1 hour. Next, 500 μ l of lysis buffer (Appendix 2) and 250 μ l of ammonium acetate (Appendix 2) were added, mixed, then set on ice for 10 minutes. In equal volume, chloroform:isoamyl alcohol (Invitrogen Corporation, Carlsbad, CA) was added and contents centrifuged at 13,000 g for 15 minutes. The upper aqueous phase was collected into a new tube. An equal volume of chloroform:isoamyl alcohol was added to the new tube and centrifuged at 13,000 g for 15 minutes. The process of adding chloroform:isoamyl alcohol and centrifuging was repeated twice more. After the last centrifugation, the upper aqueous fraction was collected into a new tube and .56 times the volume of 70% isopropanol was added and placed at -20°C for 2 hours. After centrifugation at

13,000 g for 5 minutes, the supernatant was extracted and 1 ml of 100% ethanol added. This was mixed then centrifuged again at 13,000 g for 5 minutes. Another 1 ml of ethanol was added, centrifuged and repeated at least five more times. After the last centrifugation, the ethanol was removed and the tube was inverted on sterile tissues until the ethanol evaporated under ambient conditions. Fifty microliters of sterile deionized water was added and the purified DNA stored at -20°C.

The PCR-RFLP methods were modified by Park et al. (in press 2010) from Yugueros et al. (2000, 2001). Briefly, a PCR master mix was made up for all samples plus one. The master mix reagents included primers GF-1 (5'-ATGGGTTTGGGTAGAATTGGTCGTTTA-3') and GR-2 (5'-GACATTTTCGTTATCATACCAAGCTG-3') (Integrated DNA Technologies, San Diego, CA), 10X PCR buffer (Invitrogen Corp, Carlsbad, CA), 50mM MgCl₂ (Invitrogen Corp, Carlsbad, CA), 10 mM dNTPs (Invitrogen Corp, Carlsbad, CA), *Taq* polymerase (Invitrogen Corp, Carlsbad, CA), and DNase-free water (ISC Bioexpress, Kaysville, UT). Aliquots were made and the templates were added. The vials were placed in the PCR machine and run for 40 cycles (Appendix 2).

A 1% agarose gel (Applied Biosystems) was made up using TE buffer (Bio-Rad, Richmond, CA) and ethidium bromide. Once the PCR was finished, the PCR product was loaded into the wells after loading dye (Bio-Rad, Richmond, CA) was added. The gel electrophoresis was run for 20 minutes at 100 V to check for the presence of the 931 bp *gap* gene amplicons.

The *gap* gene PCR products were digested with RFLP enzyme *AluI* (New England Biolabs, Ipswich, MA). In a 100 μ l conical vial, the reagents were mixed: *AluI*, reaction buffer

(New England Biolabs, Ipswich, MA), deionized water, and PCR product and incubated overnight at 37°C. Loading dye (Bio-Rad, Richmond, CA) was added to each vial 1:5 and 10 μ l were loaded with 100 bp ladders (Bio-Rad, Richmond, CA) into a 2% agarose gel. The gel electrophoresis was run for 1.5 hours at 100 V to separate chromosomal digests and to visualize their bands for speciation.

The banding patterns from the PCR-RFLP were compared with known sequences from the NCBI GenBank[®] database (NCBI). The NEBcutter version 2.0 (Vinze et al., 2003) was used to generate band patterns from the known *gap* gene sequences. The PCR-RFLP patterns were compared to the generated patterns to determine the species of CNS.

3.6 Intramammary Infection Diagnosis

Mammary quarters were identified as having an IMI when the same CNS species was isolated from two of three consecutively collected milk samples in duplicate where there was ≥ 121 cfu/ml of the same CNS species. A mammary quarter was not eligible for a new IMI until the previous IMI was cured. A cured IMI was determined when all samples were free of the pathogen from three consecutive collection periods. The threshold value (121 cfu/ml) used for IMI diagnosis was determined by the pilot study (Appendix 1). Briefly, the concentrations of CNS isolated from milk samples from the pilot study were enumerated. Milk samples with CNS concentrations in the lower 50th percentile were averaged and the resulting value was \log_{10} 1.41 cfu/ml. The threshold value for detection of IMI was established by adding three standard deviations, 0.225, of the calculated mean resulting in a value of \log_{10} 2.08 cfu/ml or 121 cfu/ml.

3.7 Data Analysis

The total number of CNS IMI and the species type of IMI between treatment and control udder-halves were contrasted using the t statistic as described by Hogan et al. (1990). The species colonizing the streak canal during IMI were compared with the species causing the IMI.

CHAPTER 4

Results

Forty-three cows participated in the trial for a total of 2228 quarter-weeks. Thirty-three cows were enrolled at the start of the trial, seven cows were enrolled on week three, and three more were enrolled on week nine. On average 34.8 cows were on the trial per week because some cows were dried off and culled during the trial. Twenty-five CNS IMI were diagnosed in 25 quarters over the 16-week trial in 20 cows (Table 1). These IMI included six species of CNS: *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, *S. cohnii*, *S. hyicus*, and *S. simulans*. A significant difference in the proportion of IMI in control and treatment quarters by CNS was found, 10.4% vs. 4.0%. *Staphylococcus chromogenes* and *S. xylosus* accounted for 44% and 36% of the IMI. There was a 71.4% reduction ($P < 0.05$, Table 1) in *S. xylosus* IMI in control versus treated mammary quarters. Overall the control quarters harbored IMI by more CNS species than the treated quarters. Only two species caused IMI in the treated quarters. *Staphylococcus chromogenes* IMI made up a larger proportion of IMI in the treated quarters than the control quarters, 71.4% vs. 33.3%.

The distributions of streak canal colonization and IMI by species are shown in Table 2. Eight of 25 streak canal samples during IMI are missing because of insufficient supplies during the trial. *Staphylococcus chromogenes* was the only species to colonize the streak canal while causing IMI. Only half of the *S. chromogenes* IMI were preceded by *S. chromogenes* streak canal colonization. Four of the *S. chromogenes* streak canal colonizations were in control quarters which corresponded to *S. chromogenes* IMI. Only four treated quarters had streak canal colonization and all of them were colonized by *S. chromogenes*. *Staphylococcus cohnii* was not found to colonize any streak canals during IMI.

The frequency of CNS shedding during and after IMI diagnosis is shown in Figure 1. All 25 IMI by cow and quarter are presented with the corresponding \log_{10} cfu/ml of CNS isolated from their milk and depicted as the mean of duplicate milk samples. During IMI diagnosis, the first two of three milk samples depicted, the concentration of CNS was above the threshold line of \log_{10} 2.08, or 121 cfu/ml by definition. The number of CNS recovered in 85% of the milk samples from mammary quarters with IMI was consistently greater than the threshold value after diagnosis was confirmed. A mammary quarter was deemed to be free of IMI if the number of CNS in milk was below the threshold level for three consecutive samplings. The shedding of CNS in the milk of the 11 IMI quarters was variable (both above and below the threshold), but never at a level where a diagnosis or misdiagnosis of a cured mammary quarter would be made.

In Table 3 all milk samples from infected quarters are separated by the amount of CNS shed into the milk during and after IMI diagnosis. The concentration of CNS shed into milk was above the threshold value in the majority of samples collected. Only 15.9% of the milk samples collected during the CNS IMI were below the threshold value.

The average days in milk (DIM) at the start of the trial for all 43 cows was 210 with a median of 175; 46.5% of the cows started the trial between 120 and 180 DIM. Most of the cows were in their second lactation (n=20), some in their first lactation (n=14), and a few ≥ 3 lactation (n=9). Cows averaged 90.6 days on the trial with a median of 105 days. The average milk production and geometric mean of SCC per cow at the beginning of the trial was $46,700 \pm 3,370$ cells/ml and 33.5 ± 6.4 kg/day.

Sixty-four percent of the first lactation cows experienced a CNS IMI and the average number of mammary quarters with IMI was 1.33. Thirty-nine percent of the multiparous cows were diagnosed with CNS IMI and the average number of mammary quarters with IMI was 1.1. The most common CNS IMI for first lactation cows was *S. chromogenes* (50%) then *S. xylosum* (25%). The most common CNS IMI for multiparous cows was *S. xylosum* (46.1%) then *S. chromogenes* (38.4%).

The geometric mean amount of CNS in streak canal and milk samples was 796 ± 5 cfu/ml and 309 ± 4 cfu/ml, respectively. Among all streak canal samples, 17 quarters had mixed colonization of CNS from their streak canal at one time. Of those, 13 were in control quarters, and only four in treated quarters.

Table 1. Number¹ of mammary quarters with intramammary infections (IMI) by treatment and species of Staphylococci.

Species	# of IMI	Treated ²	Control ³
<i>S. cohnii</i>	1	0	1
<i>S. simulans</i>	1	0	1
<i>S. hyicus</i>	1	0	1
<i>S. haemolyticus</i>	2	0	2
<i>S. xylosus</i>	9	2 ^a	7 ^b
<i>S. chromogenes</i>	11	5	6
Total	25	7 ^a	18 ^b

¹ Numbers not sharing the same superscript in the same row were significantly different ($P < 0.05$).

² Treated mammary quarters received post-milking teat disinfection with 1% iodophor solution.

³ Control mammary quarters received no post-milking teat disinfection.

Table 2. Prevalence of *Staphylococci species* colonizing the streak canal during intramammary infections (IMI).

Species Associated with Intramammary Infections	Species Associated with Streak Canals					
	<i>S. cohnii</i>	<i>S. simulans</i>	<i>S. haemolyticus</i>	<i>S. hyicus</i>	<i>S. xylosus</i>	<i>S. chromogenes</i>
<i>S. cohnii</i>	0	0	0	1	0	0
<i>S. simulans</i>	0	0	0	0	0	0
<i>S. haemolyticus</i>	0	0	0	2	0	0
<i>S. hyicus</i>	0	0	0	0	0	1 ²
<i>S. xylosus</i>	0	0	0	1	0	3 ²
<i>S. chromogenes</i>	0	1	1	2	1	4
Total ¹	0	1	1	6	1	8

¹ Total number of Staphylococci isolates colonizing the streak canal during IMI.

² Streak canal colonization in treated mammary quarters.

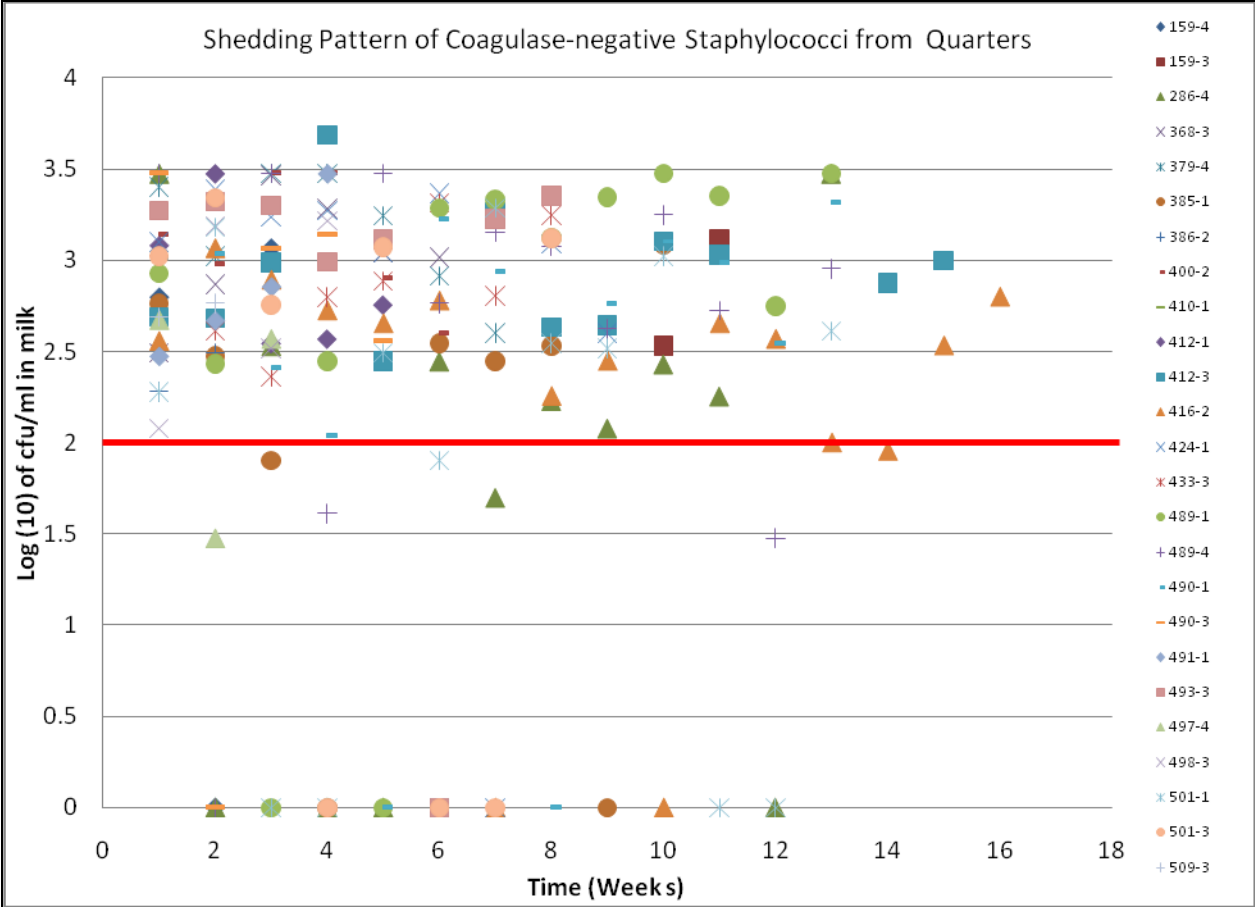


Figure 1. Concentration of Coagulase-negative Staphylococci (CNS) in milk relative to the first week that a mammary quarter was deemed infected by a CNS species. The legend includes all 25 IMI by cow and quarter with the cow identification number and mammary quarter (1=right front, 2=right rear, 3=left front, 4=left rear). The threshold for IMI diagnosis was 2.08 log₁₀ cfu/ml of milk as indicated by the red horizontal bar.

Table 3. Shedding frequency of Coagulase-negative Staphylococci (CNS) in milk by concentration (cfu/ml) after diagnosis of an intramammary infection.

Log (10) cfu/ml of CNS	% of Samples
<1.00 ¹	13.68
1.00 to 2.00	4.21
2.01 to 2.50	12.63
2.51 to 3.00	32.11
3.01 to 3.50	37.37

¹All values less than 1.00 are 0.

CHAPTER 5

Discussion

It was hypothesized that a teat dip that reduced the colonization of CNS species on the teat skin, teat apices, and streak canal would reduce CNS IMI caused by the corresponding species. In a pilot study (Appendix 1) the efficacy of three teat disinfectants with active ingredients of: an iodophor; a chlorhexidine; and a fatty acid; were documented to reduce the CNS colonization of the teat skin, teat apices and streak canal. An iodophor post-milking teat disinfectant was chosen given its efficacy noted in the pilot study and dairymen's preference for this class of disinfectant over all others (USDA, 2008).

Twenty-five CNS IMI were diagnosed among 20 cows during the trial with significantly more in the control udder-halves. The incidence of IMI for control quarters and treated quarters was 10.4% vs. 4%. These findings are consistent with other studies that diagnosed CNS IMI in 4% to 16% of quarters (Pitkala et al., 2004; Davidson et al., 1992). The iodophor teat disinfectant effectively reduced CNS IMI by 61.1% which is consistent with other studies which reported reductions of CNS IMI by 40% to 83% (Foret et al., 2006; Eberhart et al., 1983; Matthews et al., 1988; Bramley, 1978). The CNS species that caused IMI were *S. chromogenes*, *S. xylosum*, *S. haemolyticum*, *S. cohnii*, *S. hyicus*, and *S. simulans*. *Staphylococcus chromogenes* caused the majority of CNS IMI (44%) which is consistent with other studies (Taponen et al., 2007; Todhunter et al., 1993; Taponen et al., 2008; Trinidad et al., 1990; Taponen et al., 2006; Park et al., in press 2010). Iodophor post-milking teat disinfectants have been documented to be efficacious in reducing CNS IMI, but no information is available concerning the effects on specific CNS species. Since the majority of CNS IMI are caused by *S. chromogenes*, it was

assumed that a reduction in the *S. chromogenes* colonization of the streak canal would reduce IMI caused by *S. chromogenes*.

The next most-frequently isolated CNS from IMI was *S. xylosus* (36%). Other reports suggest that *S. xylosus* readily colonizes the teat skin but is not frequently isolated from IMI (Devriese and De Keyser, 1980; Harmon and Langlois, 1989; Taponen et al., 2006). The control quarters (n=7) had significantly more IMI caused by *S. xylosus* than the treated quarters (n=2) indicating that the teat disinfectant selectively reduced IMI by *S. xylosus* (Table 1). The IMI caused by *S. xylosus* were not preceded by colonization of the streak canal (Table 2). Additionally the significant difference between the treatment and control quarters for *S. xylosus* IMI suggest that the WSU herd has a higher number of IMI caused by *S. xylosus* than other herds. White et al. (1989) suggested that the colonization by CNS reflects the age and environment of the cow. They found that heifers in environments with high numbers of *S. xylosus* had more *S. xylosus* isolated from extramammary sites. We could infer that the high prevalence of *S. xylosus* IMI in the WSU herd is indicative of their environmental conditions.

The species most commonly isolated from the streak canal during IMI were *S. chromogenes* (47%) and *S. hyicus* (35%). Other studies also isolated *S. chromogenes* and *S. hyicus* most frequently from the streak canals of heifers (Boddie et al., 1992; Trinidad et al., 1990). The frequency of *S. chromogenes* isolated from the streak canal corresponds to the prevalence of IMI by *S. chromogenes*. This finding also supports the supposition that *S. chromogenes* are thought of as opportunists because they are found in equal distribution among intra- and extramammary sites (Taponen et al., 2008). Not surprisingly *S. hyicus* was the second-most frequently isolated CNS found in streak canals of quarters with IMI and are perhaps

the most closely-related species to *S. chromogenes*, as they were considered the same species before 1986 (Hajek et al., 1986 as by Zadoks and Watts, 2009). *Staphylococcus hyicus* colonized streak canals in control quarters only which corresponded to six IMI caused by four species. The *S. chromogenes* was isolated from 32% of the streak canals during IMI caused by three different species including *S. hyicus*, *S. xylosus*, and *S. chromogenes*. *Staphylococcus chromogenes* were isolated from half of the streak canals associated with IMI by *S. chromogenes*, suggesting that the streak canal is a reservoir for IMI as many studies have indicated (White et al., 1989; Trinidad et al., 1990; Matthews et al., 1992; Taponen et al., 2008). The other half of the *S. chromogenes* IMI were preceded by streak canal colonization by *S. hyicus* and *S. xylosus* (Table 2). These species have been isolated from streak canals by others (White et al., 1989; Trinidad et al., 1990). None of the streak canals were colonized by more than one CNS species during IMI.

Intramammary infections were diagnosed when two of three consecutive samples in duplicate yielded ≥ 121 cfu/ml of the same CNS species. The definition was based on the findings from a preliminary study (Appendix 1). In the preliminary study, 20 cows were treated for 21 days with three different post-milking teat disinfectants. The CNS isolated from six milk samples collected at four day intervals were enumerated. The CNS isolated from six samplings collected at four day intervals were enumerated. The concentration of CNS isolated from duplicate milk samples in the lower 50th percentile of concentration were averaged and the resulting value was \log_{10} 1.41 cfu/ml. The threshold value for detection of IMI was established by adding three standard deviations, 0.225, of the calculated mean resulting in a value of \log_{10} 2.08 cfu/ml or 121 cfu/ml. Thus CNS IMI by definition had to have more than \log_{10} 2.08 cfu/ml

of CNS recovered from duplicate milk samples at two of three consecutive samples collected in the current trial. This definition of IMI was consistent with some previous research, but differed with others. The threshold value of 121 cfu/ml for the determination of a CNS IMI was much less than many studies reporting on CNS IMI. For example, Osteras et al. (2007) used the same value of ≥ 4000 cfu/ml for all minor pathogen IMI, including CNS. The threshold value was based on the recommendations of Klastrup and Schmidt Madsen (1974). Osteras and coworkers (2007) did acknowledge that the use of this value may have resulted in an underestimation of CNS prevalence in Norway. Many studies used the value of 500 cfu/ml as the threshold of CNS IMI diagnosis (Sampimon et al., 2009d; Nickerson and Boddie, 1994; Pitkala et al., 2004). All of these studies used 10 μ l of milk on blood agar for the initial analysis. Sampimon et al. (2009d) identified a CNS IMI when only one milk sample had ≥ 500 cfu/ml. They identified the CNS from *S. aureus* by lack of hemolysis and an agglutination test specific for *S. aureus*. Pitkala and coworkers (2004) also required one milk sample to yield ≥ 500 cfu/ml of the same pathogen. Nickerson and Boddie (1994) required that two consecutive samples had ≥ 500 cfu/ml of the same pathogen or that three consecutive samples yielded between 100 and 400 cfu/ml of the same pathogen. These studies used the same threshold for CNS IMI diagnosis, but the number of samples required to meet the criteria for diagnosis was different. In addition none of these studies attempted to speciate the CNS so no information was gained for prevalence of CNS species.

Other studies used lower threshold values than 121 cfu/ml of CNS to diagnose IMI. Many studies used the threshold value ≥ 100 cfu/ml for CNS IMI diagnosis (Hogan et al., 1988b; Woolford et al., 1998; Foret et al., 2006). Hogan and coworkers reported that CNS IMI could be

diagnosed when recovered CNS were ≥ 100 cfu/ml from two of three consecutive samples taken periodically throughout lactation. Woolford et al. (1998) required two duplicate milk samples to meet or exceed the threshold value of 100 cfu/ml for IMI determination. Likewise Foret and coworkers (2006) required agreement in isolation from duplicate milk samples to meet or exceed the threshold value for diagnosis of CNS IMI. Timms and Schultz (1987) used a threshold value of 20 cfu/ml of CNS in two consecutive milk samples within one week. The CNS were identified by gram stain, catalase test, thioglycollate test, and the coagulase test. Once the first sample was positive for CNS another sample was taken within seven days to confirm the IMI. The threshold was lower than most but they used 50 μ l to inoculate the blood agar to prevent false negative results for environmental pathogens thus one colony would yield 20 cfu/ml.

The amount of CNS recovered from the majority of milk samples during and after IMI diagnosis was above the threshold value of 121 cfu/ml (Figure 1, Table 3). Even after collection of milk samples from a mammary quarter previously considered to have CNS IMI, where the pathogen concentration was below the threshold, the next consecutive samplings yielded CNS concentrations above the threshold. Thus CNS from IMI are shed into milk with sufficient consistency that misdiagnosis using the threshold of 121 cfu/ml might be rare. Moreover all CNS IMI persisted throughout the trial, no mammary quarters were deemed to have spontaneously cured. It was assumed that the CNS from IMI quarters were of the same species thereafter diagnosis thus represented isolates from persistent infections while cows were on trial. However, once a diagnosis was made, CNS isolates were not speciated thereafter. Thus firm conclusions about the persistence of the CNS species could not be made. Additional work is needed to elucidate any persistent effects of CNS species for this study.

The majority of cows on the trial were multiparous, but 32.5% were primiparous cows. The prevalence of CNS IMI in primiparous was greater than multiparous cows, 64% and 39%, respectively, thus consistent with many studies (Oliver and Mitchell, 1983a; Sampimon et al., 2009d). In addition the majority of CNS IMI in primiparous cows were caused by *S. chromogenes*, also consistent with other studies (Todhunter et al., 1993; Taponen et al., 2008).

Conclusion

The results suggest that the iodophor teat disinfectant had a species specific effect on recovery of CNS from intramammary and extramammary sites. The number of CNS species causing IMI was reduced with the use of the iodophor teat disinfectant. The species that caused the most IMI was *S. chromogenes* and the number of IMI in treated (n=5) and control (n=6) mammary quarters was almost equivalent. The quarters treated with the teat disinfectant had significantly less *S. xylosus* IMI (n=2) as compared to IMI in control mammary quarters (n=7). These results indicate that the iodophor teat disinfectant had a significantly different affect on IMI by CNS by species.

The results suggest that the threshold value used for IMI diagnosis was sufficient to diagnose CNS IMI. The stringent criteria protected against false positive diagnoses of IMI by requiring CNS to be isolated from both duplicate and consecutive samples in greater concentration than the threshold value (121 cfu/ml). The threshold value was consistently surpassed by most quarters with IMI after diagnosis. Even when concentration was below the threshold value the shedding rate was great enough that at subsequent sampling periods the concentration returned to a level above threshold. None of the IMI spontaneously cured during the trial. The results suggest that the IMI diagnosed with these criteria were indicative of actual infections.

The data support that iodophor post-milking teat disinfectant is efficacious against CNS IMI and may have a species specific effect. In addition the data suggest that the stringent criteria and threshold value for CNS IMI diagnosis is indicative of actual infection. A standard definition of CNS IMI needs to be established to facilitate a better understanding of the CNS as

mastitis pathogens. Additionally more studies are warranted to elucidate the relationship between colonization and IMI for specific CNS species so effective control measures may be utilized.

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APPENDIX 1

Pilot Study Summary

In order to test the hypothesis, that teat dips that reduce the colonization of the teat skin, teat ends and streak canal by coagulase-negative staphylococci (CNS) will reduce the prevalence of IMI caused by CNS in dairy cows, a pilot and follow-up study were designed and carried out. The first study lasted 21 days and included 20 Holstein cows from the WSU dairy. All cows were treated with three teat disinfectants: iodophor, chlorhexidine, and fatty acid-based. The three teat sites were sampled every four days and CNS presence was evaluated using standard culturing methods. The goal of the first study was to identify the most efficacious post-milking teat disinfectant against CNS colonization of the identified teat sites.

This study resulted in significant effectiveness of each teat dip to reduce the colonization of the teat skin, teat apices, and streak canal by CNS compared to the control quarters. All treatments were efficacious in reducing IMI occurrence compared to the control. The treatments were significantly different from the control, but the treatments were not significantly different from each other. The iodophor teat disinfectant was the most significantly different from the control for skin and apex samples while the chlorhexidine teat disinfectant was the most significantly different for the streak canal samples. Because the iodophor dip reduced colonization for two sample types and is comparable to the chlorhexidine teat disinfectant, the iodine teat dip has been selected as the experimental teat dip for the next trial. In addition the iodine teat disinfectant is the most frequently used post-milking teat dip used in the dairy industry, so the information gained from these trials will be more useful to more people.

For the follow-up study, only one sample type will be used in addition to milk samples.

To determine which sample type will be most correlated with teat colonization overall, a correlation coefficient analysis was run using SAS version 9.1. The streak canal samples were the most correlated with all of the samples ($P < 0.0001$) than any other sample type. This sample type will be used for the next trial to track the colonization of the teat by CNS in addition to weekly milk samples.

In the first trial, the requirements for an IMI were determined by previously described criterion (Sampimon et al., 2008). These criterion were set arbitrarily, but for the next trial the criterion for an IMI will be based on these results. The new threshold for a CNS IMI to be diagnosed will be three standard deviations above the average colony count of the lower 50th percentile cfu/ml of CNS from the positive milk samples in this study. Therefore both duplicate milk samples must have at least 121 cfu/ml of CNS to be considered an IMI.

APPENDIX 2

Methods Supplement

Sodium Thiosulfate Quench

17 g sodium chloride

2 g proteose peptone

4 g sodium thiosulfate

2000 ml deionized water

Dissolve all ingredients into the deionized water by mixing and heating with a hot plate and magnetic stir bar. Aliquot into three 1-liter bottles and autoclave for 1 hr at 121.1°C and 18 PSI. Store at room temperature.

Mannitol Salt Agar

75 g sodium chloride

10 g proteose peptone

10 g mannitol

1 g beef extract

0.025 g phenol red

15 g agar

1 L deionized water

Dissolve ingredients into 1 L deionized water using a hot plate and stir bar. Heat until all ingredients are dissolved, watch carefully so not to boil over. Aliquot into two 1-liter bottles for 1 hour at 121.1°C and 18 PSI. Use sterile technique and pipette 15 ml of autoclaved agar into empty sterile Petri dishes; cover and allow to cool overnight. Store upside down in plastic sleeves at 4°C until used.

Buffered Glycerol

6.8 g potassium phosphate, monobasic (0.1 M) in 500 ml deionized water

8.7 g potassium phosphate, dibasic (0.1 M) in 500 ml deionized water

While stirring with a stir bar, adjust pH of dibasic solution to 7.00-7.05 using monobasic solution by adding increments of 1 ml. Add glycerol to this solution in a 60:40 ratio (e.g. if there is 400 ml of the solution, add 600 ml of glycerol). Aliquot 2 ml into capped vials and autoclave for 1 hour at 121.1°C and 30 PSI.

Todd Hewitt broth

Dissolve the appropriate amount of powder in 1 L of water using a hot plate and a stir bar. Aliquot into two 1-liter bottles and autoclave at 121.1°C for 1 hour at 30 PSI.

TE Buffer

50mM Tris pH 7.5

10mM EDTA pH 7.5

If 1 L of TE buffer is needed, then calculate the required amount for each ingredient to the listed molar concentration. Before mixing, adjust the pH using hydrochloric acid or sodium hydroxide until the pH reaches 7.5. Mix together using a stir bar. Aliquot into two 1-liter bottles and autoclave for 1 hour at 121.1°C at 18 PSI. Store at room temperature.

Lysostaphin

1 mg lysostaphin

1 ml sterile deionized water

Mix together in smallest amount needed and aliquot into small tubes. Store at -20°C until needed.

Lysozyme

1 mg lysozyme

1 ml sterile deionized water

Mix together in smallest amount needed and aliquot into small tubes. Store at -4°C until needed.

Lysis Buffer

25 ml Tris (1 M, pH 8.0)

50 ml 10% Sarcosyl

50 ml EDTA (0.5 M, pH 8.0)

375 ml deionized water

Mix ingredients using a hot plate and magnetic stir bar. Once all ingredients are dissolved, pour into a 1-liter bottle and autoclave at 121.1°C for 1 hour at 18 PSI. Store in a brown bottle at room temperature.

Ammonium Acetate

Ammonium acetate (7.5 M, pH 7.5)

Mix to appropriate concentration using deionized water. Stir the solution using a magnetic stir bar and adjust the pH to 7.5 using hydrochloric acid. Autoclave for 1 hour at 121.1°C at 18 PSI. Store at 4°C until used.

PCR Conditions

95 °C 10min

95 °C 30sec

55 °C 30sec

72 °C 40sec

72 °C 10min

} 40 cycles