IDENTIFICATION AND ANTIMICROBIAL RESISTANCE OF

*STAPHYLOCOCCUS AUREUS* FROM BOVINE MILK

THESIS

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**ABSTRACT**

*Staphylococcus aureus* (*S. aureus*) is a major cause of mastitis and it causes huge economic losses in dairy herds and to the dairy industry (Kossaibati, 1997; Stergaard et al., 2005; Artursson, 2010). According to Wells et al. (1998), losses due to mastitis worldwide have been estimated to be approximately $35 billion and $1.5–2 in the US annually.

Identification of pathogens in milk is considered as the definitive diagnosis of intramammary infections and it is important for disease prevention and control. Conventional identification methods of *S. aureus* in milk based on bacterial culture include examination of Gram-stain, colony morphology and hemolysis type on blood agar, and coagulase reaction. Coagulase positive *Staphylococci* (CPS) isolated from cow's milk are presumed to be *S. aureus* in routine mastitis diagnosis; however, other CPS species also have been associated with bovine mastitis. While the prevalence of non-aureus CPS is typically reported to be between 3 and 5% (Capuro et al., 1999; Rysanek et al., 2009), Roberson et al. (1996) reported that of 487 CPS isolated from bovine mastitis cases, 82.1% were *S. aureus*, 17.7% were coagulase-positive *S. hyicus*, and 0.2% were *S. intermedius*.

There are several tools for controlling staphylococcal mastitis, one of which is antibiotic therapy of dry cow (Watts, 1997; De Oliveira, 2000). However, studies carried
out in different countries have reported antimicrobial resistance among *S. aureus* isolates (Aarestrup et al., 1999; de Oliveira et al., 2000; Shi et al., 2010)

The objectives of this study were to determine if presence of CPS in bovine milk is indicative of presence of *S.aureus*, and to determine the prevalence of antimicrobial resistance phenotypically and genotypically among these isolates. We hypothesized that coagulase positive staphylococci from bulk tank milk and milk of subclinically and clinically infected cows are *S. aureus*, and therefore the coagulase tube test is sufficient for the identification of *S. aureus* in milk samples, and no further tests are needed for this purpose.

A total of 100 CPS strains isolated from milk samples of subclinically infected mammary gland of individual cows, and from bulk tank milk samples from Ohio dairy herds, were used for this study. Microbiological examinations including bacterial morphology, hemolysis after incubation on blood agar for 24 hours at 37 C, coagulase tube test, as well as mannitol fermentation, growth on CHROMagar Staph aureus, and latex agglutination for the identification of *S.aureus* (PASTOREX™STAPH-PLUS, BIO-RAD, Redmond, WA) were examined for all isolates. Amplification of the *S. aureus nuc* gene was considered a final confirmation of the identity of *S. aureus*. Multiplex PCR amplification of the *S. aureus* nuc gene, and *meca* gene was performed to confirm both presence of *S. aureus* and methicillin resistance among tested samples. Antimicrobial susceptibility testing of the isolates against ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, streptomycin, and tetracycline was performed using Kirby-Bauer disc diffusion method.
All presumptive *S. aureus* isolates were confirmed as *S.aureus* using PCR amplification of the *S. aureus nuc* gene. With the latex agglutination test (PASTOREX™STAPH-PLUS, BIO-RAD, Redmond, WA); only 32% of tested isolates were positive. Not all *S. aureus* isolates produced hemolysis, of all tested isolates, 26% exhibited complete hemolysis, 7% demonstrated incomplete hemolysis (“water mark”), 42% exhibited double hemolysis (both complete, and incomplete), and 25% did not exhibit any hemolysis.

Phenotypically, the majority of the isolates were pansusceptible (79%), only twenty-one (21%) isolates were resistant to one or more antibiotics. The isolates were most frequently resistant to penicillin (9%), and streptomycin (8%). Five isolates (5%) were resistant to oxacillin. Three isolates (3%) were resistant to tetracycline, and only one isolate (1%) was resistant to ampicillin. Genotypically, none of the tested isolates carried the *mecA* gene. Interestingly, the five isolates that were phenotypically resistant to oxacillin did not carry *mecA* gene.

The study suggests CPS other than *S. aureus* are rare in bovine milk and therefore the coagulase tube test is sufficient for the identification of *S. aureus* in milk samples. More investigations about genes other than *mecA* that are responsible for methicillin resistance are needed.
This thesis is dedicated first and foremost to my parents’ spirit. I will never forget your care, encouragement, and supporting me wherever it may take me, even if it is far from home. You have provided great mentorship and friendship.

To my Husband, Mohammad: I wish you all the best in your future life. I am very proud of you. You have my respect for being a great friend and husband.

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To all my sisters and brothers: for your support and for praying for me to be happy and healthy.

To my daughter, Jumanah: if you are reading this now, stop here you have already read the most interesting parts.

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CHAPTER 1
LITERATURE REVIEW

1-1. EPIDEMIOLOGY OF STAPHYLOCOCCS AUREUS MASTITIS

Mastitis is one of the most common diseases among dairy cattle, causing economic losses to the dairy industry worldwide (Kossaibati, 1997; Østergaard et al., 2005; Hillerton et al., 2005; Artursson, 2010). The majority of the losses are due to subclinical mastitis because of yield milk reduction (Hortet et al., 1999). *Staphylococcus aureus* (*S. aureus*) is one of the most important and frequent causes of subclinical bovine mastitis (Wilson et al., 1997). Identification of pathogens in milk is considered as the definitive diagnosis of intramammary infections, and it is important for disease prevention and control. Conventional identification methods of *S. aureus* in milk based on bacterial culture include examination of colony morphology and hemolysis type on blood agar after incubation for 24 hrs at 37°C, and coagulase test. In routine mastitis diagnosis, coagulase positive staphylococci isolated from milk are presumed to be *S. aureus*; however, other coagulase positive staphylococci (CPS) species also have been associated with bovine mastitis (Roberson et al., 1996; Capuro et al., 1999; Rysanek et al., 2009). Typical prevalence of non-aureus CPS in milk is low, and between 3-5% (Capuro et al., 1999; Rysanek et al., 2009). However, Roberson et al. (1996) reported that...
of 487 CPS isolated from bovine mastitis cases, 82.1% were *S. aureus*, 17.7% were coagulase-positive *S. hyicus*, and 0.2% were *S. intermedius*.

Current mastitis control practices include good milking hygiene practices, regular maintenance of milking machines, culling of chronically infected animals, segregation of infected animals, and antimicrobial therapy. Implementation of good control practices reduces the transmission of contagious mastitis pathogens such as *S. aureus*. Antimicrobial therapy is also one of the primary tools for controlling staphylococcal mastitis. However, studies carried out in different countries have reported antimicrobial resistance among *S. aureus* isolates (Aarestrup et al., 1999; de Oliveira et al., 2000; Shi et al., 2010), which may lead to treatment failures. Thus, antimicrobial resistance monitoring among mastitis isolates might enable the establishment of a more effective therapy for staphylococcal infections of the udder.

### 1.1.1 Prevalence of Staphylococcus aureus mastitis

The prevalence of *S. aureus* mastitis in 2007 in the top 17 dairy producing states which accounted for 79.5 percent of dairy operations and 82.5 percent of U.S milk cows was 43% at the herd level (Lombard et al. 2008). In Mexico, prevalence of *S. aureus* was 30% in 112 Holstein herds (Morales et al., 2008). *S. aureus* was isolated from at least 52% of samples in a study estimating herd prevalence of contagious mastitis pathogens in bulk tank milk on Prince Edward Island (Riekerink et al., 2006). The prevalence of *S. aureus* in Finland decreased from 11.1% in 1995 to 10.1% in 2001 (Pitkälä et al., 2001). Mastitis and teat canal infections in South African dairy herds increased from 8.1% and 24.1 in 2002, to 15.4 and 30.0 % in 2006, respectively (Petazer et al., 2009).
1.1.2-The economic impact of *Staphylococcus aureus* mastitis

According to Wells et al. (1998), losses due to mastitis worldwide were estimated to be approximately $35 billion annually. In the US, overall costs of mastitis were estimated to be $1.5–2.0 billion per year, while losses from reduced milk production, and higher replacements costs associated with high somatic cell counts (SCC), due to subclinical mastitis were estimated at $960 million (Wells et al., 1998). In 2007, the USDA reported that mastitis was considered to be one of the most costly diseases of the dairy industry (USDA, 2007). The average cost of generic clinical mastitis (CM) per cow and year in high-yielding dairy cows, based on data from 5 large herds from 2004 to 2006 in New York State, was $71, with an incidence of 39.7 CM cases per 100 cow years and the average cost of a CM case was $179. This cost was composed of $115 due to milk yield losses, $14 due to increased mortality, and $50 due to treatment-associated costs (Bar et al., 2008). The economic losses of mastitis (subclinical and clinical) per cow on an average Dutch dairy farm have been estimated to vary between €65 and €182 per cow per year (Huijps et al., 2008).

In Africa, according to Mungube et al. (2005), production losses associated with subclinical mastitis were estimated at 5.6% for the Addis Ababa Milk Shed. Losses were highest (9.3%) for urban dairy farms and small-scale farms (6.3%). The estimates of the financial losses ranged from $29.1, for dairy herds in secondary towns, to $66.6 for urban dairy farms. A total loss of $38 was estimated for each cow per lactation (Mungube et al., 2005). Additional losses occurred due to culling of animals in cases of gangrenous or chronic mastitis.
The occurrence of intramammary infection (IMI) increases somatic cell count (SCC), leads to milk production losses, and changes in milk composition, for example, decreases in fat and lactose. Mammary tissue damage due to mastitis causes increases in enzymes such as lipase that can increase the content of free fatty acids, which produce off-flavors in milk and contribute to inhibition of bacterial culture used in manufacturing fermented dairy products (Azzara et al., 1985; Oliver et al., 1995). Mastitis can lead to decreased nutritional value due to decreased casein content. Casein is very important for cheese manufacturing, and lower casein levels in milk from cows with mastitis results in reduced cheese yield (Oliver et al., 1995).

1.1.3 - Virulence factors of *S. aureus*  

*S. aureus* strains have special virulence factors such as leukocidin, Alpha toxin, protein A, and extracellular protein. According to Vestweber et al. (1993) protein A has a negative impact on the immune system by binding FC fragment of immunoglobin G (neutrophil attachment region) instead of the Fab region (specific antigen attachment region). Opsonization is required to attract neutrophils to the bacteria and to initiate phagocytosis and killing of the bacteria (Sears et al., 2003). Coagulase, as an extracellular protein (exoenzyme), binds to protein to form a molecular complex with thrombin-like activity which converts fibrinogen to fibrin around the infection site. Hemolysis toxins, which lead to lysis of erythrocytes and results in cell death, are additional virulence factors of *S. aureus* (Vestweber et al., 1994).
1.1.4- Transmission of *Staphylococcus aureus*

*S. aureus* is transmitted from an infected to an uninfected mammary gland during the milking process. Shared equipment, udder cloths, and even milkers’ hands can transmit *S. aureus* between cows, if good hygienic practices are not followed. Environmental factors, such as bedding, housing, and feedstuffs can also be contaminated and play a role in spreading *S. aureus* infections. Thorne et al. (1960) isolated *S. aureus* from the floor of dairy barns. Also, Spencer et al. (1952) were able to maintain *S. aureus* in vitro on sterile straw for at least 49 days, which raises the possibility that bedding may be a source of the organisms. However, in low *S. aureus* prevalence herds, 0 of 183 bedding samples were positive for *S. aureus*, and 4 of 208 bedding samples from high *S. aureus* prevalence herds were positive for *S. aureus* (Roberson et al., 1994). These findings suggest that bedding may play a limited role in spreading infection (Roberson et al., 1999). To cause mastitis, *S. aureus* must enter the mammary gland through the teat sphincter. Contaminated wash cloths or milking equipment may bring the bacteria to the teat canal. After bacteria gain access to teat ducts and grow in milk producing tissue, mastitis occurs. After *S. aureus* enters the teat canal, neutrophils respond to the infection. If the leukocytes succeed, there will only be mild signs. If *S. aureus* growth continues and leukocytes are not able to prevent bacterial growth, swelling of the mammary gland and changes in the milk will occur resulting in clinical mastitis. Infections caused by *S. aureus* strains that produce α-toxin can cause gangrenous mastitis, which can lead to death due to toxemia or culling of the infected cow.

In addition to mammary glands, *S. aureus* has been found on other body sites of animals and humans. Davidson et al. (1961) isolated *S. aureus* from teat skin, udder skin,
nose, lips, vagina, and rectum of cows. Haveri et al. (2008) reported that the S. aureus strains causing bovine intramammary infection (IMI) were not different from those isolated from extramammary sites. Teat skin and especially teat canals were important potential reservoirs of S. aureus causing IMI. Heifers that had S. aureus on their skin were at a higher risk to develop S. aureus mastitis than heifers without skin colonization (Sears et al., 2003). Roberson et al. (1994) reported that milk from heifers that have S. aureus on their teat skin could be another source of S. aureus. Although some authors (Haveri et al. 2008; Thorne et al. 1960) have reported that S. aureus on the udder skin were genetically similar to S. aureus isolates from milk samples, it has also been reported that teat skin is not an important reservoir for S. aureus mastitis because less than 4% of cows had the same type of S. aureus on teat skin as the type that causes S. aureus mastitis (Fox et al., 1992).

1.1.5 - Control of Staphylococcus aureus

Correct milking procedures play a significant role in reducing new intramammary infections. Washing the udder with water or sanitizing solution and drying with individual towels, preferably disposable towels are recommended for preventing the spread of infection between cows (Vestweber et al., 1994; Roberson et al., 1999; National Mastitis Council, 2003; Sears et al., 2003). To prevent spread, milkers should wear disposable gloves and sanitize the equipment for each use (Roberson et al., 1999).

Germicidal teat dip after each milking is an effective method in preventing spread of infection from cow to cow (National Mastitis Council, 2003). Both pre and post-milking teat disinfection have significant impacts on reducing new infections by S.aureus.
and Streptococci (Vestweber et al., 1994). Quarters which were dipped before and after milking showed a lower rate of new infections than quarters which were dipped only post-milking (Vestweber et al., 1994). Backflushing, which is an automated way of washing milking clusters with water and rinsing them with disinfectant after the removal of milking units from teats, may also decrease the spread of infection. However, backflushing may be less beneficial in herds that follow effective postmilking teat dipping (National Mastitis Council, 2003).

Intramammary dry cow antibiotic therapy is one component of mastitis control programs. One of the goals of dry cow therapy is to prevent new mastitis infections from occurring during the dry period, as cows are susceptible to infection during this time and it may also cure existing infections. Intramammary antibiotic dry cow therapy cure rates are higher than those achieved during lactation because udders have less volume and antibiotic is present for a greater length of time. In some countries, including the USA, it is recommended that all quarters should be routinely treated at drying off (National Mastitis Council, 2003; Robert et al., 2006), while other countries, such as Finland, Norway, and Switzerland typically use selective treatment based on identification of udder infection (Pitkälä et al. 2004; Østerås et al., 2006).

Cows with *S. aureus* should be segregated and milked last to prevent uninfected cows from getting infected (Vestweber et al., 1994; Roberson et al., 1994). Cows infected with chronic mastitis, especially those with additional problems, should be culled as recommended by NIRD (National Institute for Research in Dairying) and NMC (National Mastitis Council). Culling is considered the most effective way to lower the prevalence of *S. aureus* in dairy herds (Radostits et al., 1994). *S. aureus* may reside inside the udder and
on teat skin, and they provide the most important source of infection to other cows in the herd. With the limited success of dry cow treatment of chronic infections, culling is the most reliable way to remove the organism and reduce exposure to other cows and heifers. However, culling is primarily recommended for chronically infected cases.
1.2- IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* FROM MILK SAMPLES

1.2.1- *Identification of S. aureus based on colony morphology and hemolysis*

Detection and identification of bacterial pathogens in milk is considered the definitive diagnosis of intramammary infections; it also provides crucial information for prevention and control of this disease. Conventional identification of *S. aureus* from intramammary infection is based on microbial culture of milk samples, including examination of colony morphology and hemolysis type on blood agar after incubation for 24 hrs at 37°C, Gram stain morphology, catalase reaction, and coagulase reaction (Hogan et al., 1999). Hemolysis is considered an important feature for rapid identification of *S. aureus* in cultures, and three types of hemolysis (complete, incomplete, and both of them together which is known as double hemolysis) can be seen among *S. aureus* mastitis isolates (Hogan et al., 1999). As described by NMC (National Mastitis Council, 1999, in *Laboratory Handbook on Bovine Mastitis*) complete hemolysis is characterized by a clear zone of complete hemolysis extending 2 mm or more from the edge of the colony while incomplete hemolysis is characterized by broad distinct zone around the colony. However, Younis et al. (2000) reported that out of 400 *S. aureus* isolates, 62.7% were found to be non-hemolytic. Similar results were reported in other studies. For example, Lam et al. (1995) reported that hemolysis had been found to be less effective in identifying *S. aureus* than a combination of other tests. On the other hand, Boerlin et al.
(2003) reported that all 159 S. aureus isolates from bovine mastitis demonstrated hemolysis. Due to the variations seen in the results of culture methods, such as different colony morphology and hemolysis patterns of the same bacterial pathogen, we cannot rely only on those criteria when identifying S. aureus mastitis.

1.2.2-Identification of S. aureus using Tube Coagulase Test

S. aureus strains have many virulence factors including leukocidin, alpha toxin, protein A, and extracellular proteins. Coagulase, an extracellular protein (exoenzyme), binds to protein to form a molecular complex with thrombin-like activity which converts fibrinogen to fibrin around the infection site (Hendrix et al., 1983; Vestweber et al., 1994). Coagulase production is an important feature used by clinical microbiology laboratories for the identification of S. aureus isolated from infections (Goh et al., 1992).

1.2.3-Latex Agglutination Tests for Identification of Staphylococcus aureus

Despite the fact that the tube coagulase test for the detection of free coagulase is considered the standard method for differentiating S. aureus from coagulase-negative Staphylococcus (CoNS), rapid tests to identify S. aureus are needed since the tube coagulase test is time consuming, requiring incubation for 4 to 24 h (van Griethuysen et al., 2001). Agglutination reagents have been developed for rapid detection of S. aureus (Essers et al., 1980). The first generation of agglutination tests used sheep erythrocytes sensitized with fibrinogen to detect clumping factor (Flandoris et al., 1981). Second generation tests appeared as latex particles coated with human plasma sensitized with fibrinogen and IgG to detect the clumping factor and protein A (Essers et al., 1980;
Personne et al., 1997). Third generation tests were designed to allow the simultaneous detection of clumping factor, protein A, and capsular polysaccharides. These tests utilize latex particles sensitized by fibrinogen and IgG as well as monoclonal antibodies against capsular polysaccharides of \textit{S. aureus} (Fournier et al. 1989; Personne, 1997).

Clumping factor is the most important of \textit{S. aureus} surface proteins that are called adhesins, and it is known to facilitate the adherence of \textit{S. aureus} to gland tissue during the first steps of \textit{S. aureus} infection. Protein A is a cell wall component of \textit{S. aureus} that prevents the correct attachment of IgG to \textit{S. aureus} cell wall, which is required to attract neutrophils to the bacteria, trigger phagocytosis and bacterial killing (Sears et al., 2003). Capsular polysaccharides form a pseudodocapsule that surrounds \textit{S. aureus} and makes it inaccessible to neutrophils and inhibits phagocytosis. The most common serotypes of \textit{S. aureus} associated with bovine mastitis are the type 5 or 8 polysaccharide capsules (Poutrel et al., 1988).

For human \textit{S. aureus} isolates, a latex slide agglutination test that detects clumping factor and protein A simultaneously has been recommended for rapid identification of \textit{S. aureus} (Essers et al., 1998). Essers et al. (1980) reported that all \textit{S. aureus} strains identified by conventional methods were correctly differentiated by the latex test (Slide agglutination test) and no false-positive results occurred with other staphylococci. Although the slide agglutination test for clumping factor is very rapid, another study reported that more than 10% of \textit{S. aureus} strains demonstrated false negative results (Kloos et al., 1999). Some \textit{S. aureus} strains (oxacillin-resistant strains) that possess capsular polysaccharide are not agglutinated by the agglutination tests (Ruane et al., 1986; Fournier et al., 1989). Therefore, negative isolates in slide tests which target bound
coagulase (clumping factor) should be confirmed with a tube coagulase test that targets the free (extracellular) coagulase (Gupta et al., 1998; Brown, 2005).

*S. aureus* strains from bovine mastitis are different than those isolated from human populations (Kapur et al., 1995). Fifty to sixty percent of the *S. aureus* strains in bovine mastitis demonstrated the presence of protein A, as compared to 90 to 99% of human strains (Kronvall et al., 1972). Hummel et al. (1992) also reported that more than 50% of *S. aureus* bovine mastitis strains investigated in the field lacked clumping factor activity. Moreover, Laevens et al. (1996) isolated an atypical clumping factor negative *S. aureus* strain as a cause of mastitis in a dairy herd. Zschöck et al. (2005) reported that agglutination tests showed lower sensitivity and specificity for identification of bovine *S. aureus* isolates than for human isolates.

1.2.4-Identification of *S. aureus* based on selective and differential media

Mannitol salt agar is a selective and differential media used to identify *Staphylococcus spp*. It is important in diagnostic laboratories due to its ability to distinguish between pathogenic microbes in a short period of time (Bachoon et al., 2008). It is selective due to the high salt concentration (~7.5%-10%NaCl) which inhibits the growth of all bacteria except halophiles. The media is differential due to the presence of mannitol and the pH indicator phenol red. *S. aureus* can ferment mannitol and produce lactic acid as a result. The phenol red turns the media yellow in low pH environments. As a result, *S. aureus* produces yellow colonies with yellow zones, whereas coagulase-negative staphylococci will produce small colorless to pink colonies with no color change to the medium.
Capuro et al. (1999) reported that all *S. aureus* isolates in their study were able to ferment mannitol, while neither *S. hyicus* nor *S. intermedius* were able to ferment mannitol. Arshad et al. (2006) reported that out of 33 staphylococcal isolates, all *S. aureus* isolates were able to ferment mannitol, whereas all *S. epidermidis* isolates were negative for this test. Brayshaw (1999) reported that mannitol salt agar recovered all 50 MRSA isolates tested. However, Oyekunle et al. (1988) reported that a higher percentage (100%) of coagulase-negative isolates than coagulase-positive isolates (88.2%) of bovine staphylococci fermented mannitol. Although it can be seen from these studies (Capuro et al., 1999; Arshad et al., 2006) that mannitol-salt agar is capable of identifying *S. aureus* correctly, poor sensitivity and specificity prevent early isolation and presumptive identification of *S. aureus* in all clinical samples (Merlino et al., 1996; Gaillot et al., 2000).

CHROMagar Staph aureus (CHROMagar, Paris, France) is a selective medium for the isolation and identification of *S. aureus* from clinical and food sources based on the formation of mauve-colored colonies. The chromogenic substrates in the medium make the differentiation of *S. aureus* from other organisms easy by the development of positive mauve, or negative blue or white colonies. These results make this test simple to read compared to blood agar. CHROMagar Staph aureus (CHROMagar, Paris, France) is also fast as results are available after 24 hours.

Gaillot et al. (2000) reported that all *S. aureus* strains isolated from clinical human samples in their study yielded mauve colonies after 24 h of incubation at 37°C, while coagulase negative staphylococci (CoNS) isolates grew as blue, white, or beige colonies. A similar result was reported by Samra et al. (2004) who concluded that CHROMagar
Staph aureus (CHROMagar, Paris, France) is a reliable media for rapid detection of *S. aureus* and reported that its sensitivity after 24 hours was 93.5% and increased after 48 hours to 99%. All isolates used in their study were obtained from human clinical specimens. El Rahmab et al. (2009) supported the use of CHROMagar Staph aureus (CHROMagar, Paris, France) for *S. aureus* detection; they reported that CHROMagar Staph aureus (CHROMagar, Paris, France) demonstrated better sensitivity, and specificity for *S. aureus* detection than the conventional culture media (chocolate agar and blood agar).

1.2.6- *Identification of S. aureus using polymerase chain reaction (PCR):*

Accurate and rapid identification of microbial pathogens such as *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), in clinical specimens is very important for timely decisions on appropriate antimicrobial therapy (Jonas et al., 2002). There are a number of methods such as PCR that have been used for this purpose. PCR has proven to be a powerful research tool, and its use for the sensitive and specific detection of microorganisms and antibiotic resistance genes is increasing in clinical microbiology laboratories. The *nuc* gene encodes for the thermostable nuclease of *Staphylococcus spp.* PCR has been used to amplify a segment of the *nuc* gene that is specific for *S. aureus* (Barkstad et al., 1992; Khan et al., 2007). PCR has also been used in many studies to confirm absence or presence of the *meca* gene which encodes PBP2a which is responsible for methicillin resistance (Zahan et al., 2009; Khan, 2007). A multiplex polymerase chain reaction has been used for simultaneous amplification of the staphylococcal *nuc* and *meca* genes (Barkstad et al., 1993).
1.3- ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM BOVINE MILK

Antimicrobial therapy is one basis of *S. aureus* mastitis control programs; however, there are multiple factors that affect the likelihood of *S. aureus* mastitis cure. Some of these factors are related to the cow, such as age, number of infected quarters, SCC, and whether infected quarters are hind or front: Cure rate decreases with increasing cow age (Barkema et al., 2006), number of infected quarters (Janso et al., 2001), and SCC (Owens et al., 1988). In addition, some investigators have found that infections in hind quarters are less responsive to antimicrobial therapy than front quarters (Sol et al., 2000). Other factors that may affect cure rates are related to pathogen factors and antibiotic treatment characteristics such as efficacy of the drug selected, treatment duration, and route of administration (Barkema et al., 2006).

Although some authors (Taponen et al., 2003; Pyörälä, 2005) recommended that treatment of subclinical mastitis should be based on susceptibility testing, the importance of susceptibility testing for clinical mastitis treatment has been debated (Constable et al., 2003). Although *S. aureus* may be susceptible to different antibiotics under laboratory conditions, farmers often report low cure rates (Barkema et al., 2006). However, antimicrobial resistance to a variety of antibiotics has been reported for *S. aureus* in a number of countries (De Oliveira et al., 2000; Abera et al., 2010; Shi et al., 2010). There
are numerous methods of antibiotic susceptibility testing; the most commonly used will be explained in detail.

1.3.1-Methods of antibiotic susceptibility testing:
The first antibiotic (penicillin) was discovered in 1928 when Alexander Fleming noticed growth inhibition of *S. aureus* in specific area of a Petri plate that was contaminated with *Penicillinum notatum* (Fleming, 1980; Sykes, 2001). Since that time, many antibiotics have been discovered and widely used.

Antibiotics are regarded as either bactericidal or bacteriostatic, depending on their mode of action. Antimicrobial agents may be categorized by mechanism of action: inhibition of cell wall synthesis, protein synthesis, nucleic acids, synthesis of important metabolites, and plasma membrane damage (Tortora et.al, 2010). However, different microbial species, and strains within the same species, may vary in susceptibility to antibiotics. Antimicrobial susceptibility test interpretive categories reported by clinical microbiology laboratories help health care providers to choose appropriate antimicrobial treatment regimens. Standards for antimicrobial susceptibility test interpretive categories have been established by organizations such as Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) in the U.S since 1975 (Wikler et al., 2005), British Society for Antimicrobial Chemotherapy (BSAC), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe.

Antimicrobial susceptibility testing can be performed either on solid media (agar) or in liquid media (broth). There are three methods of testing using solid media: agar dilution, disc diffusion, and gradient diffusion (Turnidge et al., 2005), and two
procedures are routinely performed using liquid media: broth microdilution and macrodilution (Amsterdam, 2005).

1.3.1.1 Antimicrobial susceptibility testing on solid media (agar):

A Agar dilution susceptibility testing:

With agar dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is defined as the minimum inhibitory concentration (MIC). Both agar and broth dilution methods are used to determine the MICs of antimicrobial agents. However, as reported by Turnidge et al. (2005), the agar dilution method has the advantage over broth dilution methods of testing large numbers of isolates on a single agar plate. For example, 32 isolates on a single 9 cm plate may be tested using an antibiotic sensitivity replicator.

Diluted bacterial suspensions can be transferred to the wells of the replicator. Next the inoculum is transferred to a series of agar plates prepared by mixing a series of antimicrobial agents in molten agar, including a control plate without antimicrobial agent. Plates are then incubated at 35°C. Incubation duration depends on the bacterial species being tested.

As reported by Turnidge et al. (2005), there are certain important steps that should be considered and performed as described for achieving accurate results, including: dilution range, preparation of stock solutions, and preparation of inoculum, plate inoculation, incubation, plate reading, and use of quality control.
The disk diffusion test, which is also known as the Kirby-Bauer test, is the most commonly used method for susceptibility testing (Reese et al., 1996). The entire surface of agar media, usually Mueller Hinton agar, is inoculated with a standardized amount of bacteria. Next, paper disks impregnated with standardized quantities of antimicrobial agents are placed on the agar surface and the plates are incubated for 24 hours at 35°C. During the incubation time, the antimicrobial agents diffuse from the disk into the agar. If the organism is susceptible (killed or growth inhibited) to the antimicrobial agent, a clear zone (inhibited growth of tested bacteria) around the antimicrobial disk will appear. The diameters of the zones of inhibition are measured and compared to interpretive breakpoint values published by CLSI or EUCAST which assign a value of “susceptible”, “intermediate” or “resistant” to the isolate.

There are a number of critical steps in this method, such as which medium is used, depth and moisture content of the agar in the plate, and incubation conditions. Disks also must be placed firmly in contact with the agar surface; otherwise the diffusion rate will not be correct.

Gradient diffusion was introduced as the Epsilometer test (E-test), in 1991 by AB Biodisk (Solan, Sweden). It can be performed by placing a strip that has been impregnated with a gradient of antibiotic concentrations on an agar plate that had been inoculated with bacteria being tested. The antibiotic then diffuses out into the agar, and
after 24 hours of incubation, an oval zone of inhibition is produced and the point at which the oval meets the strip provides an indication of the minimum inhibitory concentration (MIC) of the antibiotic through a scale printed on the upper side of the strip.

The E-test gradient diffusion test was easy and rapid to perform and required less work compared to broth or agar dilution methods (Amsterdam, 2005). Moreover, results obtained by E-test were similar to those produced by conventional MIC testing. Giovanni et al. (1998) reported that MICs obtained by the E-test correlated well with those determined by the agar dilution and interpretive categories determined by disk diffusion methods (88 and 92.5% agreement, respectively).

1.3.1.2-Antimicrobial susceptibility testing in liquid media:

Antimicrobial susceptibility testing in liquid media, referred to as broth dilution methods, which can be performed by microdilution or macrodilution (Amsterdam, 2005). Both methods are prepared similarly, except the final volume for macrodilution is 1-2 ml and the test is conducted in test tubes, whereas the microdilution volume is 50 µl/well and the test is conducted in a 96-multiwell microdilution plate (Amsterdam, 2005).

Microdilution broth test:

The microdilution broth test can be performed either using commercial or laboratory prepared test panels (Amsterdam, 2005). Commercial panels contain approximately 96 wells that contain measured concentrations of frozen or freeze dried antibiotics. The inoculum can be prepared by emulsifying a minimum of three colonies from the starting culture of the microbe being tested in sterile saline to reach standardized
turbidity (0.5 McFarland). The inoculum is then added at the same time to all wells in a row of tested antibiotics. Quality control strains should be tested in the laboratory on a regular basis to help ensure accurate results.

Prepared antimicrobial agent stock solutions, are added to Mueller-Hinton broth in microdilution trays, which are then inoculated with test bacteria, approximately $5 \times 10^4$ cfu per well. After incubation, the MIC can be determined visually by reading the incubated trays, and is defined as the lowest concentration of antibiotic at which the tested bacteria did not show visible growth.

1.3.2- Antimicrobial resistance among S. aureus causing bovine mastitis:

Shi et al. (2010), using the disc diffusion method to determine susceptibility of S. aureus found that, in general, S. aureus resistance in China was high against nine groups of antimicrobial agents tested in their study. They found that 232 out of 236 (98.3%) isolates were resistant at least to one of the antimicrobial drugs, and the isolates were most commonly resistant to penicillin (87.6%). Resistance against the following groups of antimicrobial drugs was examined: penicillins, cephalosporins, sulfamides (SMZ-TMP), aminoglycosides, quinolones, nitrofurfurans (Furaxone), macrolides, and lincosamide, and glycopeptides.

De Oliveira et al. (2000) tested more than 800 S. aureus isolates from bovine mastitis, one isolate per herd, from nine European countries, USA, and Zimbabwe. Minimum inhibitory concentrations (MIC90) were determined for the following antimicrobial agents: penicillin, oxacillin, cephalothin, ceftiofur, penicillin + novobiocin, erythromycin, pirlimycin, neomycin, premafaxacin, lincomycin, and sulfamethazine.
They reported that, overall, the MICs were in susceptible ranges for all drugs tested regardless of country.

Younis et al. (2000) using the disk diffusion method, reported that all 319 isolates from chronic mastitis cows in Israel were susceptible to methicillin and 96.6% and 52% of the isolates were resistant to penicillin and oxytetracycline, respectively. Hendriksen et al. (2008) conducted a survey from 2002 to 2004 and found that *S. aureus* isolates from more than 9 out of 13 European countries were susceptible to most of the antibiotics tested. However, they mentioned that over 10% of *S. aureus* isolates from 10 countries were resistant to penicillin. They also reported that different methods of antibiotic susceptibility testing had been used among countries. Most countries that were involved in this survey used the minimal inhibitory concentration method; some used the disk diffusion method, while some used both the disk diffusion method and the minimal inhibition concentration method. Abera et al. (2010), using the disk diffusion method, found that *S. aureus* was commonly resistant to penicillin (94.4%) and amoxicillin (36.1%), while susceptible to other antimicrobial agents such as chloramphenicol and gentamicin.

In conclusion, because of the use of different antimicrobial agents, methods, sample sources, number of isolates, as well as isolates obtained from different regions, it is difficult to determine the prevalence of antimicrobial resistance of *S. aureus* from bovine mastitis cases based on a simple comparison. Different geographical regions may have different *S. aureus* bovine mastitis strains. As a result, comparisons between studies from different countries may not be of great value. However, among most of the studies examined, *S. aureus* bovine mastitis isolates consistently show resistance against
ampicillin and penicillin, and are susceptible to the following drugs: cephalothin, ceftiofur, enrofloxacin, erythromycin, clindamycin, lincomycin, pirlimycin, neomycin, lincomycin + neomycin, and sulfamethazine.

In order to conduct meaningful comparison studies, a global database on \textit{S. aureus} bovine mastitis antimicrobial resistance could be useful, with implementation of standard methods. Information such as the country/region of origin, type of mastitis (subclinical/clinical), lactation stage, colony morphology, and following standardized standard methods for determining antimicrobial resistance would allow for comparison studies to be made.

1.3.4-\textit{Methicillin-resistant Staphylococcus aureus}:

\textit{Methicillin-resistant} \textit{S. aureus} (MRSA), which can also be called multidrug-resistant \textit{Staphylococcus aureus}, is defined as any strain of \textit{S. aureus} that has developed resistance to all beta-lactam antibiotics, including the penicillins (methicillin, dicloxacillin, oxacillin, etc.) and the cephalosporins. \textit{S. aureus} is a normal inhabitant on the skin and nose of healthy people and animals (Gorwitz et al., 2008), but can also cause severe infections or deaths in immunocompromised host (Akpaka et al., 2011).

The penicillin-binding proteins (PBPs) are required to initiate the cross-linking of peptidoglycan required to form the cell wall (Tortora et al., 2010). \(\beta\)-lactam drugs act by binding to the normal high-molecular-weight PBPs (PBP 1, 85,000; PBP 2, 81,000; and PBP 3, 75,000) to inhibit their activity, and as a result, disrupt cell wall synthesis (Waxman et al., 1983; Reynolds, 1988). MRSA has an altered penicillin-binding protein (PBP\textsubscript{a2}) that is encoded in the \textit{mecA} gene. The PBP\textsubscript{a2} binds beta-lactams with lower
affinity than normal PBPs, which results in resistance to all β-lactam antimicrobial agents. The mecA gene is located on a mobile genetic island; called the staphylococcal cassette chromosome mec (SCCmec). This element can carry different genes of antimicrobial resistance and can serve as a mechanism for multi-drug resistance (MDR) (Matthew et al., 1987). The mecA gene is the most important gene of this cassette.

In addition to mecA gene, there are other mechanisms for methicillin resistance in *staphylococcus* spp. such as hyper-production of the drug-inactivating enzyme β-lactamase (e.g. some bacteria produce enzymes to inactivate β-lactam antimicrobial agents) and selective mutations in the PBPs (Tortora et al., 2010).

MRSA infections have been identified in animals, including pigs, cats, dogs and horses (Weese et al., 2007; Huijsdens et al., 2008). Devrieze et al. (1972) reported that 18 methicillin-resistant *S. aureus* strains were isolated from mastitic milk samples from 12 of 232 farms. Devrieze et al. (1975) reported that 68 methicillin-resistant *Staphylococcus aureus* strains were isolated from mastitis milk samples originating from 20 Belgian dairy herds. Virgin et al. (2009) reported that 7 of 190 mixed *Staphylococcus* isolates from bulk tank milk samples from USA dairy herds were positive for the mecA gene. However, it was unclear whether any of these were *S.aureus*. MRSA transmission has been documented between animals, and people who were in close contact with farm animals with MRSA (van Loo et al., 2007).

MRSA strains have been identified in different food products, including meats and raw milk (Nomanno et al., 2007; De Boer et al., 2009). Transmission of foodborne pathogens from animals, including dairy cattle, has been investigated in a number of different countries (Chao et al., 2007; Nomanno et al., 2007). Although the food supply
in the USA is considered to be among the safest in the world, foodborne pathogens cause
76 million illnesses, 325,000 hospitalizations, and 5,200 deaths in the US each year
(Mead et al., 1999).
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CHAPTER 2

IDENTIFICATION OF STAPHYLOCCUS AUREUS FROM BOVINE MILK USING BIOCHEMICAL TESTS AND PCR

2.1-INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is a major cause of mastitis and it causes huge economic losses in dairy herds and to the dairy industry (Kossaibati, 1997; Østergaard, 2005; Artursson, 2010). Identification of bacterial pathogens in milk is considered the definitive diagnosis of intramammary infections; it is also important to prevent and control this disease. Conventional identification methods of *S. aureus* include colony morphology and hemolysis type on blood agar after incubation for 24 hrs at 37°C, and coagulase reaction. In routine mastitis diagnosis, coagulase positive staphylococci isolates from cow's milk are presumed to be *S. aureus*; however, other coagulase positive staphylococci (CPS) species also have been associated with bovine mastitis. Prevalence of non-aureus CPS in milk is low, typically between 3-5% (Capuro et al., 1999; Rysanek et al., 2009). However, higher number of non-aureus CPS isolates in milk was reported by Roberson et al. (1996) who stated that of 487 CPS isolated from bovine mastitis cases, 82.1% were *S aureus*, 17.7% were coagulase-positive *S. hyicus*, and 0.2% were *S. intermedius*.

PCR has proven to be a powerful research tool, and its use for the sensitive and specific detection of microorganisms and antibiotic resistance genes is increasing in clinical microbiology laboratories. The *nuc* gene encodes for the thermostable nuclease of *Staphylococcus spp*. PCR has been used to amplify a segment of the *nuc* gene that is
specific for S. aureus (Barkstad et al., 1992; Khan et al., 2007). The goal of this study was to confirm the identity of coagulase-positive staphylococci from bovine milk as S. aureus, using PCR as the gold standard.

2.2- MATERIALS AND METHODS

2.2.1- *Staphylococcus aureus* Isolation/Identification

A total of 100 CPS strains isolated from subclinically infected udders of individual cows and from bulk tank milk of Ohio dairy herds in the USA were used in this study. Fifty-one out of these 100 CPS strains were isolated from bulk tank milk of Ohio dairy herd, and forty-nine were isolated from subclinically infected udders of individual cows from ten Ohio dairy herds.

A 100 µl inoculum of bulk tank milk was plated onto Baird-Parker agar (Becton, Dickinson and Diagontics Company, Sparks, MD) supplemented with Egg Yolk Tellurite (Remel, Lenexa, KS). Black colonies surrounded by halos from Baird-Parker agar after incubation for 24 hrs at 37°C were plated onto blood agar. After incubation for 24 hrs at 37°C, conventional identification methods were used which included Gram-stain colony morphology, catalase reaction, and coagulase reaction. Coagulase-positive organisms were considered as *S. aureus* and saved for further analysis.

Forty-nine CPS strains, isolated from subclinically infected udders of individual cows from ten dairy farms around Ohio, originated from previous studies conducted on Ohio dairies. All of these CPS isolates had been identified as *S. aureus* based on Gram stain, colony morphology, catalase, and coagulase tube test. Isolates had been stored at -80°C and were revived by plating on blood agar for this study.
All CPS were considered *S. aureus* and saved to examine their reaction on certain biochemical tests and final identification by PCR.

2.2.2-Biochemical identification

2.2.2.1- Mannitol Salt agar and CHROMagar™ Staph aureus

One hundred *S.aureus* isolates were refreshed on sheep blood agar before plating onto Mannitol Salt agar (BBL Mannitol Salt Agar, Becton, Dickinson and Company, Sparks, MD), CHROMagar™ Staph *aureus* (CHROMagar, Paris, France), and incubated at 37°C for 24 hrs. Reaction on mannitol salt agar was interpreted and recorded as positive or negative based on criteria described by manufacturer: isolates that grew and fermented mannitol and as a result, produced yellow zones on mannitol salt agar after 24 hrs incubation at 37°C were considered *S.aureus*. If incubated 24 hrs on mannitol slat agar, CPS other than *S. aureus* produced small to large red zones, and coagulase-negative staphylococci will produce small colorless to pink colonies with no color change to the medium. Reaction on CHROMagar™ Staph aureus were interpreted and recorded as positive or negative based on criteria described by manufacturer: The formation of mauve-colored colonies on CHROMagar™ Staph aureus at 37°C for 24 hr were considered to be *S. aureus* while blue or white colonies were considered negative.
2.2.2.3- *Pastorex Staph plus latex agglutination*

The isolates were plated on Mueller Hinton (MH) agar, incubated at 37°C for 24 hrs, and examined for their reaction on latex agglutination test (PASTOREX™ STAPH-PLUS, BIO-RAD, Redmond, WA) according to manufacturer’s guidelines. Results were recorded within 30 seconds of beginning the card rotation, as described by manufacture.

2.2.3- *Molecular Identification:*

2.2.3.1- *DNA Extraction*

The DNA extraction protocol of *S. aureus* used by Infectious Disease Molecular Epidemiology Laboratory (IDMEL, The Ohio State University, Columbus, OH) was adapted from Qiagen DNA easy Blood and Tissue Kit. Pure colonies were cultured onto Mueller Hinton agar, and incubated overnight at 37°C. Isolates were then harvested in 1500 µl Molecular Grade Water in 1.5ml microcentrifuge tubes. The DNA extraction of isolates was done using enzymatic lysis buffer as outlined in the instruction manual. Briefly, the lysis buffer contained a mixture of 20 mM pH 8.0 Tris-HCl (Fisher BioReagents, Fairlawn, NJ), 2 mM sodium EDTA (Gibco, Grand Island, NY), 1% Triton X-100 (Sigma, St. Louis, MO), and 20 mg/ml lysozyme (Lysozyme Ultrapure, USB Corporation, Cleveland, OH). Molecular Biology Grade Water (Eppendorf, Hamburg, Germany) was used for the elution step as described in the instruction manual. The resulting elute was stored at 4°C for further genotypic analyses.
2.2.3.2- *S. aureus* nuc identification by PCR

The *S. aureus* nuc gene, encoding for the thermostable nuclease of *S. aureus*, was amplified using the PCR protocol used by Infectious Disease Molecular Epidemiology Laboratory (IDMEL, The Ohio State University, Columbus, OH). *S. aureus* nuc primer sequences were nuc-F 5′GCGATTGATGGTGATACGGTT3′, nuc-R 5′AGCCAAGGCTTGAGGAACTAA3′, with an expected size of 270 bp (Table 2.1). PCR reactions were performed using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Piscataway, NJ). A final reaction volume of 25 μl with 1 μl of template DNA was used. Amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc, Watertown, MA) under the following conditions: initial denature at 95°C for 10 min followed by 45 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min. One microliter of 5X loading dye (10X UltraClean Gel Dye, MO BIO Laboratories, Inc, Carlsbad, CA) was added to each PCR reaction. The samples were filtered through a 1% Ultrapure Agarose gel (Invitrogen, Carlsbad, CA) containing ethidium bromide (10% of the total volume) in 0.5X TAE buffer (Ultrapure DNA Typing Grade 50X TAE Buffer, Invitrogen, Carlsbad, CA). The amplicons were visualized using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA) and analyzed using Quantity One (Bio-Rad Laboratories, Hercules, CA). The size of the amplicons was measured using a 100 bp molecular marker (Simply Load DNA ladder, Lonza Rockland, Inc, Rockland, MD). ATCC strain 43300 was used as positive control.
2.3-RESULTS

All isolates were confirmed as *S. aureus* using PCR based on the presence of *S. aureus nuc* gene (Figure 2.1). Ninety percent of isolates were positive for mannitol fermentation, 6% were weak positive, and 4% were negative. A majority of the isolates (93%) exhibited typical growth on CHROMagar™ Staph aureus, 3% were weak positives, and 4% were considered negative on CHROMagar™Staph aureus. Only 32% of isolates were positive on latex agglutination test (PASTOREX™ STAPH-PLUS). A summary of biochemical test results is presented in Table 2.2. Twenty-six percent of isolates exhibited complete hemolysis, 7% demonstrated incomplete hemolysis (“water mark”), 42% exhibited double hemolysis (both complete, and incomplete), and 25% did not exhibit any hemolysis (Table 2.3).

2.4-DISCUSSION

Hemolysis is considered an important feature for rapid identification of *S. aureus* in cultures (Hogan et al., 1999; Boerlin et al., 2003). However, 25% of the *S. aureus* strains tested in this study did not produce hemolysis. Similar results were also reported in other studies. For example, Younis et al. (2000) reported that out of 400 *S. aureus* isolates, 62.7% were found to be non-haemolytic. Lam et al. (1995) reported that hemolysin production had been found to be less effective in identifying *S.aureus* among Micrococcaceae than a combination of other tests. Our results were in disagreement with Boerlin et al. (2003) who reported that all 159 *S.aureus* isolates from bovine mastitis cases exhibited hemolysis.
Coagulase production is one of the most reliable criteria for *S. aureus* identification (Kloos et al., 1986). All our CPS isolates were confirmed as *S. aureus* based on results from PCR. Therefore, we concluded that the coagulase tube test is sufficient for the identification of *S. aureus* in milk samples. Similar results have been reported by Yazdankha et al. (1998). They reported that the coagulase tube test demonstrated 88.5% sensitivity and 100% specificity for the direct detection of *S. aureus* in milk samples.

Mannitol salt agar is a selective and differential media used to identify *Staphylococcus sp.* Only *S. aureus* can ferment mannitol after 24 hours of incubation and produce lactic acid as a result. Out of our 100 isolates, 96% (90% clear positive, 6% weak positive) were positive for mannitol fermentation. Our results are in agreement with Arshad et al. (2006) who reported that out of 90 bacterial isolates from bovine milk samples including 33 of staphylococci and 57 of other bacterial species, 23 were *S. aureus*, and all of these *S. aureus* isolates were able to ferment mannitol. Capuro et al. (1999) reported that all *S. aureus* isolates in their study were able to ferment mannitol while neither *S. hyicus* nor *S. intermedius* were able to ferment mannitol. However, both Capuro et al. (1999) and Arshad et al. (2006) did not state clearly for how long tested isolates in their study were incubated and it is known that *S. hyicus* and *S. intermedius* can ferment mannitol if incubated more than 24 hrs (Schleifer et al., 1986). However, Oyekunle et al. (1988) reported that a higher percentage (100%) of coagulase-negative isolates than coagulase-positive isolates (88.2%) of bovine *staphylococci* fermented mannitol. Thus, relying only on mannitol fermentation, when identifying *S. aureus*, could increase false positive results which will decrease specificity.
CHROMagar Staph aureus is a selective medium for the isolation and identification of *S. aureus* from clinical and food sources based on the formation of mauve-colored colonies. The majority of the isolates (93%) in this study exhibited typical growth on CHROMagar™ Staph aureus, 3% were weak positive, and 4% were considered negative. Our results were in agreement with Samra et al. (2004), who reported that the sensitivity of Chromagar Staph aureus after 24 hour incubation was 93.5%. A higher percentage was reported by Gaillot et al. (2001) who reported that all *S. aureus* isolates yielded mauve colonies after 24 h of incubation at 37°C. However, *S. aureus* isolates used in their studies were obtained from human samples.

PASTOREX™ STAPH PLUS, (BIO-RAD, Redmond, WA) is one of the third generation of latex agglutination tests which is designed to detect clumping factor, protein A, and capsular polysaccharides of *S. aureus* simultaneously. It has red latex particles sensitized by fibrinogen, IgG, and specific monoclonal antibodies against capsular polysaccharides of *S. aureus* (Fournier et al., 1993). Of all the isolates in the current study, only 32% were positive. Similar results were reported by Zschöck et al. (2005). They reported that agglutination tests showed lower sensitivity and specificity for identifying *S. aureus* isolates from bovine origin than human sources. This may be because *S. aureus* strains from bovine mastitis are different from those from humans (Delgado et al., 2011). Fifty to sixty percent of the *S. aureus* strains in bovine mastitis demonstrated the presence of protein A, as compared to 90 to 99% of human strains (Kronvall et al., 1972). Hummel et al. (1992) also reported that more than 50% of *S. aureus* mastitis strains of those investigated in the field lacked clumping factor activity. We could conclude from our study that current latex agglutination tests (PASTOREX™

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STAPH PLUS, BIO-RAD, Redmond, WA) are unreliable when preformed on bovine strains of *S. aureus*. More improvements of current latex agglutination kit are needed before these tests may be used to identify bovine *S. aureus* isolates accurately.

**Figure 2.1:** PCR product obtained with SA *nuc* gene (270 bp), *mecA* gene (162bp), showing *S.aureus* nuc gene positive, *mecA* negative isolates

Kb – standard molecular weight, N = negative control; 1,2,3, and 4 are PCR products obtained with tested isolates that are positive for *S.aureus* nuc gene, and negative for *mecA* gene.
Table 2.1: Primers sequences used in PCR for identifying the S.aureus nuc gene

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence(5′-3′)</th>
<th>Amplicon Size( bp)</th>
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</thead>
<tbody>
<tr>
<td>nuc-F</td>
<td>GCGATTGATGGTGTACGGTT</td>
<td>270</td>
</tr>
<tr>
<td>nuc- R</td>
<td>AGCCAAGGCTTGAGGAATAA</td>
<td></td>
</tr>
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</table>

Table 2.2: Results of Biochemical Tests

<table>
<thead>
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<th></th>
<th>Latex test</th>
<th>Mannitol</th>
<th>Chromagar</th>
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<tbody>
<tr>
<td>Positive</td>
<td>32</td>
<td>90</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 weak</td>
<td>3 weak</td>
</tr>
<tr>
<td>Negative</td>
<td>68</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
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Table 2.3: Types of Hemolysis among *S. aureus* Isolates.

<table>
<thead>
<tr>
<th>Type of hemolysis</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>26</td>
</tr>
<tr>
<td>Incomplete</td>
<td>7</td>
</tr>
<tr>
<td>Double (complete, incomplete)</td>
<td>42</td>
</tr>
<tr>
<td>No hemolysis</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
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REFERENCES


CHAPTER 3
DETERMINATION OF ANTIMICROBIAL RESISTANCE OF
STAPHYLOCOCCUS AUREUS ISOLATES FROM BOVINE MILK

3.1-INTRODUCTION

*S. aureus* is an important and frequent cause of bovine subclinical mastitis (Wilson et al., 1997). Current control of *S. aureus* mastitis include proper milking hygiene practices, culling of chronically infected animals, segregation of infected animals, and antimicrobial therapy. Implementation of good control practices reduces the transmission of contagious mastitis pathogens such as *S. aureus*.

Antimicrobial therapy is one method in *S. aureus* mastitis control. Antimicrobial therapy of intramammary infection with penicillin-resistant *S. aureus* isolates results in a lower cure rate than those caused by penicillin-sensitive *S. aureus* with either beta-lactam or non-lactam antibiotics (Barkema et al., 2006) Antimicrobial resistance among *S. aureus* isolates has been reported in studies carried out in different countries (Shi et al., 2010; De Oliveira et al., 2000; Abera et al., 2010). The purpose of this study was to determine the level of antimicrobial resistance, especially methicillin resistance among one hundred *S. aureus* isolates that were detected from subclinically infected cows in ten Ohio dairy herds and from bulk tank milk samples from ninty-six herds.
3.2-MATERIALS AND METHODS

3.2.1.-Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of isolates was performed in the Infectious Disease Molecular Epidemiology Laboratory (IDMEL, the Ohio State University, Columbus, OH) using Kirby-Bauer disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI). Briefly, colonies were suspended in 0.7% saline and standardized to 0.5 McFarland using a Vitek Colorimeter (Hach Company, Loveland, CO). They were then cultured on Mueller Hinton (MH) agar and incubated at 37°C for 24 hrs. The following eight antimicrobial drugs commonly used in mastitis treatments were tested: ampicillin (Am) (10 μg), ceftiofur (Cf) (30 μg), cephalothin (Ch) (30 μg), erythromycin (Er) (15 μg), oxacillin (Ox) (1μg), penicillin (Pn) (10 μg), tetracycline (Te) (30 μg), and streptomycin (S) (10 μg) (Table 3.1). All disks were purchased from Becton Dickinson Microbiology Systems (Sparks, MD). Quality control strains Escherichia coli ATCC 25922, S. aureus ATCC 25923, and Pseudomonas aeruginosa ATCC 27853 were used (American Type Culture Collection, Manassas, VA).

3.2.2-Molecular identification of MRSA

3.2.2.1-DNA Extraction

The DNA extraction protocol of S. aureus used by Infectious Disease Molecular Epidemiology Laboratory (IDMEL, The Ohio State University, Columbus, OH) was adapted from Qiagen DNAeasy Blood and Tissue Kit (QIAGEN Inc.
Valencia, CA). Pure colonies were cultured onto Mueller Hinton agar and incubated overnight at 37°C. Isolates were then harvested in 1500 µl Molecular Grade Water in 1.5ml microcentrifuge tubes. The DNA extraction of the isolates was done using enzymatic lysis buffer as outlined in the instruction manual. Briefly, the lysis buffer contained a mixture of 20 mM pH 8.0 Tris-HCl (Fisher BioReagents, Fairlawn, NJ), 2 mM sodium EDTA (Gibco, Grand Island, NY), 1% Triton X-100 (Sigma, St. Louis, MO), and 20 mg/ml lysozyme (Lysozyme Ultrapure, USB Corporation, Cleveland, OH). Molecular Biology Grade Water (Eppendorf, Hamburg, Germany) was used for the elution step as described in the instruction manual. The resulting elute was stored at 4°C for further genotypic analyses.

3.2. 2.2-Identification of MRSA by PCR

The meca gene encoding for the protein PBP2A (Penicillin binding protein 2A) was amplified using a PCR protocol used by Infectious Disease Molecular Epidemiology Laboratory (IDMEL). meca primer sequences are meca-F 5'TCCAGATTACAACCTTCACCAGG'3, meca-R 5'CCACTTCATATCTTTGTAACG'3, and expected size is 162 bp (Table 3.2).

PCR reactions were performed using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Piscataway, NJ). A final reaction volume of 25 µl with 1 µl of template DNA was used. Amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc, Watertown, MA) under the following conditions: initial denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min.
One microliter of 5X loading dye (10X UltraClean Gel Dye, MO BIO Laboratories, Inc, Carlsbad, CA) was added to each PCR reaction. The samples were filtered through a 1% Ultrapure Agarose gel (Invitrogen, Carlsbad, CA) containing ethidium bromide (10% of the total volume) in 0.5X TAE buffer (Ultrapure DNA Typing Grade 50X TAE Buffer, Invitrogen, Carlsbad, CA). The amplicons were visualized using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA) and analyzed using Quantity One (Bio-Rad Laboratories, Hercules, CA). The size of the amplicons was measured using a 100 bp molecular marker (Simply Load DNA ladder, Lonza Rockland, Inc, Rockland, MD). To confirm reproducibility, a select MRSA positive isolate (ATCC 43300) was used in PCR runs as a control.

3.3. RESULTS

Phenotypically, the majority of the isolates (79 of 100) were pansusceptible, only twenty-one (21%) were resistant to one or more antibiotics. The isolates were most commonly resistant to penicillin (9%), and streptomycin (8%). Five isolates (5%) were resistant to oxacillin. Three isolates (3%) were resistant to tetracycline, and only one isolate (1%) was resistant to ampicillin (Table 3.3). Eight resistance profiles were observed among tested isolates: pansusceptible (Pattern I, 79%); ampicillin/penicillin (Pattern II,1%); streptomycin/oxacillin (PatternIII, 2%); streptomycin/oxacillin/penicillin (Pattern IV, 1%); penicillin (Pattern V, 7%); streptomycin (Pattern VI, 5%); tetracycline (Pattern VI, 3%); oxacillin (Pattern VII, 2%) (Table 3.3). Genotypically, none of the tested isolates carried the mecA gene.
3.4. DISCUSSION

Genotypically, none of the tested isolates carried the \textit{mecA} gene which encodes for the Penicillin Binding Protein 2A that indicates methicillin resistance. Our finding is in disagreement with several studies (Devriese et al., 1972; Kwon et al., 2005; Moon et al., 2007; Turutoglu et al., 2009) that have reported \textit{mecA}-positive \textit{S. aureus} from bovine mastitis. Phenotypically, twenty-one (21\%) isolates were resistant to one or more antibiotics, 79 isolates were pansusceptible. The proportion of susceptible isolates in this study was higher than that reported by Shi et al. (2010) or Rabello et al. (2005). Shi et al. (2010) reported that 98.3\% of tested isolates in their study were resistant to at least one antibiotic. In other words, only 1.7\% of tested isolates were susceptible to all tested antimicrobial groups. Rabello et al. (2005) reported that 48 out 107 (44.9\%) tested isolates were susceptible to all antimicrobials tested. In the current study, nine isolates (9\%) were resistant to penicillin and eight were resistant to streptomycin (8\%). Five isolates (5\%) were resistant to oxacillin. Three isolates (3\%) were resistant to tetracycline and one isolate (1\%) was resistant to ampicillin. Higher levels of resistance to penicillin have been reported in other studies (Younis et al., 2000; Shi et al., 2010; Abera et al., 2010). Younis et al. (2000) reported that 96.6\% of isolates were resistant to penicillin. Shi et al. (2010) reported that 87.6\% of isolates were resistant to penicillin. Abera et al. (2010) reported that 94.4\% of isolates were resistant to penicillin. Data from our study indicated that oxacillin, tetracycline, and ampicillin resistance among \textit{S.aureus} bovine isolates is low (5\%, 3\%, and 1\%, respectively). Our results are in agreement with De Oliveira et al. (2000) who reported that the overall level of resistance was generally low for all antimicrobial agents tested including ampicillin and oxacillin. In contrast to results
from the current study, Vanderhaeghen et al. (2010) reported that all isolates (118 *S. aureus* isolates from cases of subclinical or clinical mastitis) in their study were resistant to tetracycline. Rabello et al. (2005) reported that approximately 55% of *S. aureus* isolates tested were resistant to ampicillin. Although resistance level to antimicrobial agents tested in our study was in general low when compared to other studies (Younis et al., 2000; Shi et al., 2010; Abera et al., 2010), resistance to antimicrobial agents such as penicillin and streptomycin was found and could be because of their common use in dairy cattle.

Interestingly, five of the isolates were phenotypically resistant to oxacillin, but as mentioned earlier, none of them carried the *mecA* gene. Similar results were reported for other studies. For example, Tiao (2008) found that none of the 20 *S. aureus* isolates from Ohio dairies tested in her study carried the *mecA* gene and one showed methicillin resistance phenotypically. Turutoglu et al. (2009) reported that of 18 *S. aureus* isolates, 15 were phenotypically resistant to methicillin but did not carry the *mecA* gene. The lack of the *mecA* gene, which is responsible for methicillin resistance, in the presence of phenotypic oxacillin resistance indicates that the isolates may be using a different mechanism of resistance. The *mecA* gene is the most common mechanism of methicillin resistance; however, there are other mechanisms for methicillin resistance such as the *blaZ* gene, which is responsible for β-lactamase production and mutations in the PBPs. Vesterholm et al. (1999) reported that the *blaZ* gene has been found in bovine staphylococci isolates. Therefore, we believe, in addition to *mecA* gene, other mechanisms such as overproduction of β-lactamase and mutation of PBPs may be involved in the expression of oxacillin resistance.
Table 3.1: Concentration and inhibition zone diameters for antimicrobial drugs used in the study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Res</th>
<th>Int</th>
<th>Sus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin – Am (10µg)</td>
<td>≤13</td>
<td>14-16</td>
<td>≥17</td>
</tr>
<tr>
<td>Erythromycin – E (15µg)</td>
<td>≤13</td>
<td>14-22</td>
<td>≥23</td>
</tr>
<tr>
<td>Streptomycin – S (10µg)</td>
<td>≤11</td>
<td>12-14</td>
<td>≥15</td>
</tr>
<tr>
<td>Oxacillin – OX S. aureus (1µg)</td>
<td>≤10</td>
<td>11-12</td>
<td>≥13</td>
</tr>
<tr>
<td>Tetracycline – Te (30µg)</td>
<td>≤14</td>
<td>15-18</td>
<td>≥19</td>
</tr>
<tr>
<td>Penicillin – P (10µg)</td>
<td>≤28</td>
<td>-</td>
<td>≥29</td>
</tr>
<tr>
<td>Ceftiofur – XNL (30µg)</td>
<td>≤17</td>
<td>18-20</td>
<td>≥21</td>
</tr>
<tr>
<td>Cephatothin -CF (30µg)</td>
<td>≤14</td>
<td>15-17</td>
<td>≥18</td>
</tr>
</tbody>
</table>

Table 3.2: Primers sequences used in PCR for identifying the *mecA* gene

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence(5′-3′)</th>
<th>Amplicon Size( bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MecA-F</td>
<td>TCCAGATTACA ACTTCCACCAGG</td>
<td>162</td>
</tr>
<tr>
<td>mec-R</td>
<td>CCACTTCA TATCTTGAACG</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3: Frequency of resistance patterns in tested isolates. Am:ampicillin, Ox:oxacillin, Pn:penicillin, Te:tetracycline, S:streptomycin

<table>
<thead>
<tr>
<th>Isolates number</th>
<th>Resistance Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Pn</td>
</tr>
<tr>
<td>3</td>
<td>Tr</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>OX</td>
</tr>
<tr>
<td>1</td>
<td>AmPn</td>
</tr>
<tr>
<td>1</td>
<td>SOxPn</td>
</tr>
<tr>
<td>2</td>
<td>SOx</td>
</tr>
<tr>
<td>79</td>
<td>Pansusceptible</td>
</tr>
</tbody>
</table>
REFERENCE:


CONCLUSION

This study found that: (1) coagulase positive staphylococci (CPS) other than *S. aureus* are rare in bovine milk, (2) the coagulase tube test is sufficient for the identification of *S. aureus* in milk samples, (3) not all *S. aureus* produce hemolysis, (4) for bovine isolates, the latex agglutination test (PASTOREX™ STAPH-PLUS) for the identification of *S. aureus* was not reliable, (5) the *mecA* gene was not found among *S. aureus* isolates, and (6) antimicrobial resistance among *S. aureus* isolates was lower than had been reported in other studies (Younis et al., 2000; Shi et al., 2010; Abera et al., 2010). Resistance to tested antimicrobial agents such as penicillin and streptomycin likely occur because of their general use on dairy farms. Twenty-one percent of the isolates showed phenotypic resistance against five different types of antimicrobial drugs, and five of them showed resistance against oxacillin, although they all lacked *mecA*. This may be because the isolates used a different mechanism for conferring resistance to methicillin.

Data from this study indicated that hemolysis criterion alone was not adequate for identifying *S. aureus* mastitis. With regard to the use of latex agglutination test for identification of *S. aureus*, this study found that current latex agglutination (PASTOREX™STAPH-PLUS, BIO-RAD, Redmond, WA) kits did not accurately identify *S. aureus* bovine isolates. Therefore, improvements in sensitivity and specificity
of current latex agglutination kits would be beneficial for quick and reliable identification. Results from this study indicate a need for future studies to investigate mechanisms or genes other than \textit{mecA} responsible for methicillin resistance.


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