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To the Graduate Council:

I am submitting herewith a thesis written by Jacqueline M. Vaughn entitled "Virulence Characteristics and Genetic Diversity of *Staphylococcus aureus* Isolates from Cases of Bovine Mastitis in Eastern Tennessee.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Oudessa Kerro Dego, Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Virulence Characteristics and Genetic Diversity of *Staphylococcus aureus* Isolates from Cases of Bovine Mastitis in Eastern Tennessee.

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Jacqueline M. Vaughn
August 2018

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# ABSTRACT

*Staphylococcus aureus* is an important zoonotic mastitis pathogen that has significant effects on animal and human health. *S. aureus* is also a foodborne pathogen that causes food poisoning through its diverse enterotoxins. Some studies showed that *S. aureus* strains that cause infection in a particular host are genetically distinct and are host specific, although most strains are believed to be infective to a wide range of host species. However, there are no clearly defined clonal patterns of *S. aureus* that possess certain virulence factors responsible for causing a disease. The objectives of this study were: 1) evaluate clonal diversity of *S. aureus* isolates from cases of bovine mastitis 2) determine staphylococcal enterotoxin production patterns 3) evaluate *in vitro* adhesion and invasion ability of dominant strains on bovine mammary epithelial cell line (MAC-T cells). Milk samples from bovine mastitis were evaluated at Tennessee Quality Milk Laboratory (TQML) for causative agents. Overall, 111 *S. aureus* strains were isolated and evaluated for genetic diversity by pulsed field gel electrophoresis (PFGE) and for presence of toxin genes that encode for staphylococcal enterotoxins (*sea*, *seb*, *sec, see, sej)* and toxic shock syndrome toxin 1 (*tsst-1)* by PCR. The *in vitro* adhesion and invasion ability of the dominant strains were evaluated on mammary epithelial cell line (MAC-T). We found 16 PFGE types (dominant clones) ranging from A – P. The PFGE type M is the most prevalent of all 16 PFGE types. The PCR results of enterotoxins genes showed that some of these strains were positive for staphylococcal enterotoxin genes including *seb* (11.7%), *sec* (2.7%), *see* (0.9%) and toxic shock syndrome toxin 1 (*tsst-1*) (7.2%), whereas most strains



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(75.7%) were negative for enterotoxin genes. In addition, evaluation of association of PFGE types, enterotoxins and other virulence factors, that were evaluated previously, showed that PFGE types O and M tend to cluster with beta-hemolysin, absence of enterotoxins and susceptibility to antimicrobials. Analysis of *in vitro* adhesion to and invasion into MAC-T cells showed relatively higher number of O strain adhered to and invaded into MAC-T cells followed by M and I strains however were not statistically significant.



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# CHAPTER ONE

# INTRODUCTION



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One of the most common and economically important diseases in dairy production is mastitis. *Staphylococcus aureus* is the most frequent causative agent of bovine mastitis. *S. aureus* mastitis causes large economic losses to the dairy industry. Economic losses due to mastitis is estimated to be around 2 billion dollars annually in the United States (DeGraves and Fetrow, 1993) and $300 - $400 million dollars annually in Canadian dairies (Canadian bovine mastitis and milk quality research network (CBMQRN), 2006). These losses could be direct which include treatment, veterinary fees, discarded milk, labor, and death of the infected animal and/or indirect costs which include decrease in milk yield, decrease in quality and value of milk due to elevated somatic cell count, culling and replacement costs (Petrovski et al., 2006). Mastitis also affects public health directly or indirectly through several ways such as infection from consumption of unpasteurized milk, intoxication from consumption of preformed toxins in milk or milk products, spoilage and reduced shelf life of milk and milk products and transfer of antimicrobial resistant bacteria or resistance genes to human and/or human pathogens.

Some mastitis pathogens, such as *S. aureus*, can produce heat stable enterotoxins that cause food poisoning (Kadariya et al., 2014). *S. aureus* caused 241,000 foodborne illnesses every year in the US (Kadariya et al., 2014). Kadariya et al (Kadariya et al., 2014) reported *S. aureus* prevalence rates of 31% in bulk tank milk and 35% in cows. Most *S. aureus* isolates from food are not enterotoxin producers (Le Loir et al., 2003). The prevalence of *S. aureus* mastitis varies from farm to farm because of variation in hygienic milking practices and overall farm management differences on application of control measures for contagious



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mastitis pathogens like *S. aureus*. Good hygiene in the milking parlor can significantly reduce the occurrence of new mastitis in the herd but it does not remove existing cases within a herd (Neave et al., 1969). Neave et al. concluded that it is nearly impractical to keep all udder quarters of dairy cows free of all pathogens at all the times. Since this early observation by Neave et al. many studies have confirmed that management practices can reduce new cases of intramammary infection (IMI) (Hillerton and Berry, 2005, Blowey and Edmondson, 2010) but cannot eliminate existing infection. In the United States, the prevalence of clinical and subclinical *S. aureus* mastitis ranged from 10% to 45% (Sischo, 1993) and 15% to 75% respectively.

## Pathogenesis of *S. aureus* mastitis

*S. aureus* enters mammary glands through the teat opening and subsequently multiply in the mammary glands where they may form biofilms, attach to and internalize into the mammary epithelial cells causing inflammation of mammary glands characterized by swelling, degeneration of epithelial cells, and epithelial erosions and ulcers (Gudding et al., 1984, Zecconi et al., 2006). During this inflammatory process, the milk samples showed a rapid increase of somatic cells, characterized by increased number of neutrophils in the secretion (Gudding et al., 1984, Harmon, 1994). Despite increased recruitment of somatic cells into infected mammary glands characterized by increased number of neutrophils, infection usually does not clear but became subclinical infection. Intramammary infections



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during early lactation may become acute clinical mastitis characterized by gangrene development due congestion and thrombosis (blockage) of blood supply to the tissue but most new infection during late lactation or dry period become acute or chronic mastitis (Zecconi, 2010, Keefe, 2012)

**Diversity of *S. aureus***

*S. aureus* strains can be host specific, meaning that they are found more commonly in a specific species (van Leeuwen et al., 2005). Some studies showed that *S. aureus* that causes mastitis belong to certain dominant clones, which are frequently responsible for clinical and subclinical mastitis in a herd at certain geographic area indicating that the control measures may need to be directed against specific clones in a given area (Anderson and Lyman, 2006, Graber et al., 2009, Capurro et al., 2010). However, because *S. aureus* is such a big problem in human health, cross- infection has been a big research topic. Several studies have reported cases of cross-infection in several different species (Rodgers et al., 1999, Simoons-Smit et al., 2000, Zadoks et al., 2002). In the dairy industry, there have been reports of human origin methicillin resistant *S. aureus* infecting bovine mammary glands (Monecke et al., 2007, Türkyılmaz et al., 2010). These studies add to the unease that strains can gain new mutations or virulence factors and adapt to cross the interspecies boundary relatively rapidly (Pantosti et al., 2007).



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## Virulence factors of *S. aureus*

*Staphylococcus aureus* exhibits many virulence factors which can be broadly categorized into non-secretory factors and secretory extracellular factors. In addition to non-secretory surface proteins, *S. aureus* produces different extracellular products, mainly toxins and enzymes (Wadstrom, 1974) including enterotoxins (Aydin et al., 2011), non-enteric exfoliative or hemolytic/cytolytic (alpha, beta, delta and gamma) and leucocidin toxins (Rogolsky, 1979).

***Non-secretory factors***

Non-secretory cell surface factors involved in virulence include surface proteins, exopolysaccharides (capsule, slime, biofilm), teichoic acids and lipoteichoic acids. Overall, *S. aureus* has over 24 surface proteins and 13 secreted proteins that are involved in the immune evasion (McCarthy and Lindsay, 2010) and about 15 to 26 proteins for biofilm formation (Brady et al., 2006, den Reijer et al., 2017).

Surface proteins such as staphylococcal protein A (SpA) (Foster et al., 2014), clumping factors A and B (ClfA and ClfB) (Clarke and Foster, 2006, Hauck and Ohlsen, 2006, Speziale et al., 2009) , fibrinogen binding proteins (Burke et al., 2010), iron-regulated surface determinants (IsdA, IsdB and IsdH) (Clarke and Foster, 2006, Zecconi and Scali, 2013), fibronectin binding proteins A and B (Camussone and Calvinho, 2013), biofilm associated protein (BAP) play roles in *S. aureus* adhesion and invasion of host cells



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(Szweda et al., 2012). The BAP expression enhances biofilm production and the BAP gene is only found in *S. aureus* strain from bovine origin (Cucarella et al., 2001, Lasa and Penadés, 2006, Valle et al., 2012). Evaluation of BAP gene of *S. aureus* from bovine and human isolates using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) showed that bovine and human isolates are not closely related (Cucarella et al., 2004). Thus, some host specific evolutionary factors may have been developed between both strain types.

Biofilms are considered an important virulence factor in the pathogenesis of bovine *S. aureus* mastitis (Stewart and Costerton, 2001, Donlan and Costerton, 2002). Slime, an extracellular polysaccharide layer, acts as a barrier against phagocytosis and antimicrobials. It also helps with adhesion to a surface (Milanov et al., 2010). If a biofilm forms in a mammary gland, it will protect those bacteria from antimicrobials and the host's immune system (Stewart and Costerton, 2001, Donlan and Costerton, 2002). In addition, once the biofilm matures and the immune attack has subsided the biofilm can break open and re-infect the mammary gland (Melchior et al., 2006). There are many contributors to biofilm production, such as polysaccharide intercellular adhesin (PIA) also known as Poly-N-acetyl-β(1 -6)-glucosamine (PNAG), MSCRAMMS, teichoic acids and extracellular DNA (eDNA) (Gotz, 2002, Otto, 2008) that are known to help these bacteria cells to hold onto a surface (Dhanawade et al., 2010). Various proteins encoded by intercellular adhesin locus such as icaA, icaB, icaC and icaD are involved in PIA production which in turn result in biofilm formation (Gotz, 2002, Otto, 2008). Vasudevan et al. (Vasudevan et al., 2003)



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evaluated correlation of slime production and presence of the intercellular adhesin (*ica*) genes with biofilm production and found that all tested isolates were positive for *icaA* and *icaD* genes and most tested isolates produce slime but not all slime positives produced biofilms in vitro. Similarly, a study in Poland found that all isolates were positive for *icaA* and *icaD* (Szweda et al., 2012) genes. While adhesion is promoted with biofilm production, the *bap* gene prevents the invasion of host cells (Valle et al., 2012). Despite the presence of *ica* gene strongly support biofilm production the presence of *ica* gene is not mandatory for biofilm production since *S. aureus* lacking *ica* gene can still produce biofilm through other microbial surface components recognizing adhesive matrix molecules (MSCRAM) and secreted proteins (O'Gara, 2007, Otto, 2013).

***Secretory factors***

*S. aureus* also produces different extracellular products that attribute to the virulence of the bacteria. These extracellular factors are mainly toxins and enzymes (Wadstrom, 1974) including enterotoxins (Aydin et al., 2011), non-enteric exfoliative or hemolytic/cytolytic (alpha, beta, delta and gamma) and leucocidin toxins (Rogolsky, 1979). *S. aureus* isolates from bovine mastitis produce alpha (α), beta (β), gamma (γ), and delta (δ) hemolysins that cause hemolysis of red blood cells of the host (Bramley et al., 1989) All are antigenically distinct.

Alpha-hemolysin is a pore-forming toxin that binds to a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) receptor resulting in pore formation and cellular



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necrosis (Berube and Bubeck Wardenburg, 2013, Otto, 2014). It is also known to increase inflammatory response and decrease macrophage function (Songer and Post, 2004). Alpha-hemolysin damages plasma membrane of the epithelial cell resulting in leakages of low molecular weight molecules from the cytosol and death of the cell (Kerro and Nederbragt, 2002). It is produced by 20 - 50 % of strains from bovine IMI (Sutra and Poutrel, 1994). A study reported that the alpha (α), hemolysin may be required for cell to cell interaction during biofilm formation (Caiazza and O'toole, 2003). β-hemolysin hydrolyses the sphingomyelin present in the plasma membrane resulting in increased permeability with progressive loss of cell surface charge (Low and Freer, 1977). It is produced by 75-100 % of *S. aureus* strains from bovine IMI (Sutra and Poutrel, 1994). Gamma hemolysin expression requires specific growth conditions in vitro because its' growth is inhibited by agar (O'Callaghan et al., 1997).

The ability of *S. aureus* to invade mammary epithelial cells during mastitis plays a big role in the pathogenesis of *S. aureus*. Internalized bacterial cells can hide from the host's immune system inside the host cell and continue to multiply inside the host cell (Almeida et al., 1996). There may be many mechanisms that *S. aureus* uses to invade into host cells and each mechanism can be strain dependent. Mammary epithelial cells have a structure called fibrinogen underneath the epithelial layer of the cell. *S. aureus* strains have a fibronectin binding protein that can bind to the fibronectin on the mammary epithelial cell surface. Fibronectin binding protein is thought to be a common way for the bacteria cells to invade bovine mammary epithelial cells. Fibronectin binding protein deficient strains



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cannot invade host cells (Lammers et al., 1999). The presence of a capsule prevents the adherence to epithelial cells (Cifrian et al., 1995, Hensen et al., 2000).

Another virulence factor of *S. aureus* is staphylococcal enterotoxins. These toxins are heat stable and can resist pasteurization. They also have superantigenic activity that dramatically activate the host's immune system (Bergdoll et al., 1967). *S. aureus* produces staphylococcal enterotoxins A, B, C, D, E, G, H, I and J - Q as well as toxic shock syndrome toxin 1 (tsst-1) (Dinges et al., MATSUNAGA et al., 1993). Enterotoxins can get into the food chain through the consumption of contaminated food and cause food poisoning (Hennekinne et al., 2012). Some studies showed that about 20% of *S. aureus* isolates from IMI produce toxic shock syndrome toxin-1 (Kenny et al., 1993, Hennekinne et al., 2012). Toxic shock syndrome toxin causes toxic shock syndrome and can be fatal (Todd et al., 1978). Besides the superantigenic effect of enterotoxins, their role in the pathogenesis of mastitis is unknown and may be specific to each strain or area based on selective pressures in the habitat (Moon et al., 2007). Enterotoxin prevalence seems to vary between geographical areas. The strains producing enterotoxin C have been isolated relatively frequently from cases of bovine mastitis (MATSUNAGA et al., 1993, Stephan et al., 2001, Cenci-Goga et al., 2003).

Antimicrobial resistance is a growing problem in staphylococcus aureus mastitis. Antimicrobial resistance helps bacteria to stay alive after treated with antibiotics and some of the mechanisms of resistance is the presence of antimicrobial resistance genes that can



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spread by horizontal transfer from bacteria to bacteria by mobile genetic elements such as plasmids, phages and pathogenicity islands (Brussow et al., 2004). This resistance can also occur through random mutations when the bacteria is under stress (Pantosti et al., 2007). In the cases of mastitis, the prevalence of antimicrobial resistant bacteria seems to be increasing at least for some antimicrobials. Studies reported over 50% of isolates that cause mastitis were resistant to either beta lactam drugs or penicillin (De Oliveira et al., 2000). In human medicine, methicillin resistant *S. aureus* (MRSA) is a huge problem because MRSA strains are resistant to most of antibiotics making them very difficult or impossible to treat. There have also been reports of cases of bovine mastitis caused by MRSA (Jamali et al., 2014, Savic et al., 2014, Silva et al., 2014, Luini et al., 2015). Some report that these infection are due to the human strain, but others have found MRSA strains of bovine origin (Gentilini et al., 2000, Holmes and Zadoks, 2011). These authors suggested that MRSA strains isolated from bovine probably gain resistance from human MRSA strain through transfer of resistance genes (Febler et al., 2010).

## Host response to *S. aureus* infection

Host responses to IMI with *S. aureus* begins with the innate immune responses characterized by inflammation. The innate immune system uses phagocytic cells (macrophages & neutrophils) that recognizes pathogens associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs), which includes toll like



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receptors (TLR), C-type lectin receptors (CLR), nucleotide oligomerization (NOD) like receptors (NLR), Retinoic acid-inducible gene I (RIG-I) like receptors (RLR), etc. (Oviedo-Boyso et al., 2007). Upon recognition of infecting pathogen a pro-inflammatory cytokines such as TNFα, IL-1β, IL-8 are released by macrophages resulting in inflammation that recruit neutrophils to the site of infection (Bannerman et al., 2004). The neutrophils are readily recruited to the mammary glands which accounts for the increase in somatic cell count during mastitis. If the initial influx of neutrophils does not clear the pathogen, the adaptive (acquired) immune system will be initiated and the host produces specific humoral (antibodies) and/or cellular immune responses against invading pathogens. (Rainard, 2003).

## Control and prevention

Currently available *S. aureus* mastitis control measures are hygienic milking practices such as wearing gloves during milking, pre- and post-milking teat dipping in antiseptic solution; use of properly functioning milking machines; maintaining clean, dry, comfortable housing areas; good nutritional programs; segregation and culling of persistently infected animals; dry cow antibiotic therapy; and proper identification and treatment of cows with clinical and subclinical mastitis (Neave et al., 1969). These measures control new infections but will not remove the existing infection in the herd. Once *S. aureus* infection is established, it becomes persistent and difficult to clear, due to



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biofilm formation, bacterial ability to attach and internalize into the mammary epithelial cells where it may become small colony variant (L-forms) that are protected against the immune system and therapeutics (Wellnitz et al., 2012). Treatment with antibiotics is of limited success which may dictate the culling of the animal (Barkema et al., 2006, McDougall et al., 2009). Furthermore, the emergence of aggressive, antibiotic resistant *S. aureus* strains such as methicillin resistant *S. aureus* (MRSA) has become a significant problem, as these strains have spread beyond their typical hospital milieu to bovine sources (Holmes and Zadoks, 2011, Fitzgerald, 2012, Jamali et al., 2014, Savic et al., 2014, Silva et al., 2014, Luini et al., 2015). On an average, the cure rate of lactating cow therapy against *S. aureus* mastitis is about 30% or less (Mellenberger and Kirk, 2001). Currently, there is no effective vaccine against bovine *S. aureus* mastitis (Pereira et al., 2011), and since treatment is of limited efficacy, control of *S. aureus* mastitis focuses on prevention of contamination and spread, rather than treatment (Barkema et al., 2006, McDougall et al., 2009). There are some none-protective vaccines such as Lysigin® (Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) in US and Startvac® (Hipra S.A, Girona, Spain) in Europe. None of these vaccines confer protection in field trials as well as under controlled experimental studies (Middleton et al., 2006, Middleton et al., 2009, Schukken et al., 2014, Bradley et al., 2015).



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# CHAPTER TWO

# GENETIC DIVERSITY AND ENTEROTOXIN PRODUCTION PATTERNS OF *S. AUREUS* ISOLATES FROM CASES OF BOVINE MASTITIS



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# Abstract

*Staphylococcus aureus* is an important zoonotic mastitis pathogen that has significant effects on animal and human health. *S. aureus* causes food poisoning through its diverse enterotoxins. Some studies showed that *S. aureus* strains that infect different host species are genetically distinct, although most strains can infect wide range of host species. However, there are no clearly defined clonal patterns of *S. aureus*. We hypothesize that *S. aureus* strains that infect bovine mammary glands have specific sets of virulence factors that help them to resist host defense mechanisms. The objectives of this study are: 1) evaluate clonal diversity of *S. aureus* isolates from cases of bovine mastitis to determine dominant strains responsible for mastitis and, 2) determine staphylococcal enterotoxin production patterns. Milk samples from cases of bovine mastitis that were submitted from 11 farms in the Eastern Tennessee area for diagnosis were evaluated at Tennessee Quality Milk Laboratory (TQML) for causative agents. Overall, 111 *S. aureus* isolates from cases of mastitis in 11 farms were evaluated for genetic diversity by pulsed field gel electrophoresis (PFGE), for toxins genes such as staphylococcal enterotoxins (*sea*, *seb*, *sec, see, sej)* and toxic shock syndrome toxin 1 (*tsst-1)* by PCR. The PFGE results showed the presence of 16 PFGE types throughout 11 farms, of which three strains were the most frequent isolates from the farms included in this study. The PFGE type M is the most prevalent of all 16 PFGE types with 64 isolates being present among nine farms. The PCR results of enterotoxins genes showed that some of these strains were positive for staphylococcal enterotoxins including *seb*, *sec*, *see*, and toxic shock syndrome toxin 1 (*tsst-*




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*1*) whereas most strains were negative for enterotoxins. There were no statistically significant associations among PFGE types and presence of enterotoxin genes. However, the evaluation of association of PFGE types, enterotoxins and other virulence factors such as hemolysins, slime production, and resistance or susceptibility to antimicrobials showed that PFGE types O and M tend to cluster with β-hemolysin, absence of enterotoxins and susceptibility to antimicrobials.

## Introduction

*S. aureus* is one of the leading causes of contagious mastitis in the dairy industry, resulting in a huge economic loss consisting of direct and indirect costs. *S. aureus* is grouped under the family of *Staphylococcaceae* and genus *Staphylococcus*. It is gram-positive, catalase and coagulase positive, non-spore forming, oxidase negative, non-motile, cluster-forming, facultative anaerobe (Takahashi et al., 1999). The coagulase is not an absolute test for the confirmation of diagnosis of *S. aureus* from cases of mastitis but more than 95% of all coagulase positive staphylococci from bovine mastitis belong to *S. aureus* (Fox and Hancock, 1989). Other coagulase-positive species are *S. aureus subsp. anaerobius*, *S. pseudintermedius*, *S. scheferi subsp. coagulans*, *S. hyicus*, *S. intermedius* and *S. delphini*. Although the prevalence of *S. aureus* mastitis can be reduced with hygienic milking practices and good management systems, it is still a major problem for dairy farms, with a prevalence of 66% among farms tested in USA (USDA, APHIS 2014). *S. aureus* has many



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virulence factors that range from surface localized structural components to secretory factors, which help the pathogen to colonize the host and evade the host's defenses. Some of the virulence factors known to cause illness in humans are staphylococcal enterotoxins. Some of these toxins are known to act as superantigens that cause increased immune activity in the host. These toxins tend to contaminate dairy products because once these heat stable toxins are produced, they can survive pasteurization even though the bacteria itself cannot (Bergdoll et al., 1967, Kong et al., 2016). The enterotoxins produced by *S. aureus* that are known for causing food poisoning include staphylococcal enterotoxins A to E (seA - seE) and G to Q (seG-seQ) (Srinivasan et al. 2006). Staphylococal enterotoxins G to Q (seG- seQ) are prevalent among *S. aureus* isolates from cases of bovine mastitis and they are also implicated in the pathogenesis of mastitis. *S. aureus* can infect many host species and can cause a wide variety of illnesses ranging from mild skin infection to a life threatening systemic infection. It has been reported that certain strains of *S. aureus* with specific tissue-tropism can be adapted to infect specific tissues such as the mammary gland (van Leeuwen et al., 2005). Furthermore, a study by McMillan in 2016 (McMillan et al., 2016) showed distinct lineages of *S. aureus* in bovine, ovine, and caprine species. This suggests that there may be distinct strains within a species that have specific virulence mechanisms that helped them to thrive in that species in a particular geographic environment. Therefore, we hypothesize that *S. aureus* strains that infect bovine mammary glands have unique clonal diversity and sets of virulence factors that enable them to resist host defense mechanisms. To address this question, a study was conducted with the following objectives: 1) evaluate clonal diversity of *S. aureus* isolates from cases of bovine



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mastitis to determine dominant clones responsible for bovine mastitis 2) determine enterotoxin production patterns of *S. aureus* isolates from cases of bovine mastitis.

## Materials and methods

***Sample collection***

In this study, we used 111 samples that were submitted to the Tennessee Quality Milk Laboratory (TQML). The samples were brought to the lab for diagnosis of the cause of mastitis or suspected cause of mastitis; all samples were at least suspected of clinical mastitis by the owner. *S. aureus* was isolated from milk samples from 11 dairy farms in Tennessee collected during 2005 - 2012. These samples were evaluated for mastitis causing bacteria, including *S. aureus* and the diagnosed causative bacteria were then isolated and stored in a -80°C freezer by the Tennessee Quality Milk Laboratory (TQML). The stored bacteria were thawed from the -80°C freezer then plated and grown overnight on tryptic soy agar plates (TSA) with 5% sheep blood (Becton Dickinson Microbiology system, Cockeysville, MD). The identity of the isolates were re-confirmed by hemolytic characteristics such as alpha, beta, double and non-hemolytic, Gram staining, and catalase and coagulase tests.



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### *DNA extraction*

*S. aureus* genomic DNA was extracted from pure cultures by growing *S. aureus* on TSA with 5% sheep blood (Becton Dickinson Microbiology system, Cockeysville, MD) overnight in an incubator at 37°C. A single colony was suspended in 5mL of tryptic soy broth (TSB) and incubated overnight at 37°C in a shaking incubator at 220 rpm. The broth culture was harvested after incubation by centrifugation at 500xg for 10 min and re-suspended in 800 µL of lysis buffer (1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, 2% Cetyltrimethylammonium bromide (CTAB), 1% polyvinylpyrrolidone (PVP), 0.2% β-mercaptoethanol, pH 8) with 10 µL of 20mg/mL proteinase K per 1 mL of lysis buffer and transferred to a 1.5 mL Eppendorf tube. Then, samples were incubated at 65°C for 2 h with intermittent shaking manually every 10 min. Then the suspension was centrifuged at 6160xg at 4°C for 5 min. The supernatant was transferred to a new Eppendorf tube and an equal volume of chloroform: isoamyl alcohol (24:1) (Sigma Aldrich, St. Louis, MO) was added and mixed by gently flipping the tube. The solution was centrifuged at 8870.4xg at 4°C for 8 min. and the top aqueous phase, which has DNA, was transferred to a new Eppendorf tube. The DNA was precipitated by adding 100 µL of 5 M sodium acetate mixed and then equal volume of cold (-20°C) isopropyl alcohol was added slowly drop by drop and then kept at -20°C for 30 min. followed by centrifugation at 12073.6xg at 4°C for 10 min. Then, the supernatant was discarded, and the DNA pellet was washed 2X by adding 500 µL of cold (4°C) 96% ethanol for the first wash and 70% ethanol for the second wash and centrifuged at 8870.4 g at 4°C for 5 min. Finally, the supernatant was discarded, and



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the DNA pellet was air dried and re-suspended in 50 µL TE buffer (10mM Tris-HCl, 1 mM EDTA (pH 8).

***Polymerase chain reaction amplification (PCR)***

PCR was performed on 6 genes including staphylococcal enterotoxins *sea*, *seb*, *sec*, *see*, and *sej* along with toxic shock syndrome toxin 1 (*tsst-1*) using each gene specific primer pairs (Table 1). The primers were designed using clone manager professional version 9.2 (Scientific & Educational Software, Carry, NC) (Table 1). Amplification was performed in a DNA thermal cycler (Bio-Rad Laboratories, Hercules, CA). The total reaction volume of 20 µL consisted of 10 µL of 2x Phusion flash high-fidelity PCR master mix (Thermo Fisher Scientific, Pittsburg, PA), 7 µL of DNA grade water, 1.0 µL of each primer, and 1 µL of 100 ng/µL DNA template were used per recommended master mix guidelines.

The PCR conditions were as follows: 95°C for 10 sec; 35 cycles of 95°C for 1 sec, annealing temp 60°C for 5 sec, with extension at 72°C for 15 sec; and the final extension step at 72°C for 1 min. The PCR products were run in 1.5% agarose gel in a Bio-Rad electrophoresis chamber and the gel was stained with gel red (Phenix Research products, Candler, NC). Images were taken by ChemiDoc_ Touch Imaging System (Bio-Rad) and exported to PulseNet by Image Lab 5.2.1 Software (Bio-Rad). Positive controls were used for each reaction and strains used as positive control are as follows: ATCC 13565 for *sea*, ATCC 14458 for *seb*, ATCC 19095 for *sec*, ATCC 27664 for *see*, ATCC 23235 for *sej*, and ATCC 33586 for *tsst1* genes.



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***Pulsed field gel electrophoresis (PFGE)***

Molecular fingerprinting of *S. aureus* isolates was done using PFGE as described elsewhere (McDougal et al., 2003, Gillespie et al., 2009, Sawant et al., 2009) with minor modifications. Briefly, each bacterial sample was inoculated on blood agar plate and incubated overnight. A single pure colony was inoculated to 5 mL of Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h with shaking at 220 rpm. Bacterial concentration was adjusted to an $OD_{600}$ of 0.9 to 1.1 with phosphate buffered saline (PBS) using a spectrophotometer (Bio-Rad). A 200 µL aliquot of the culture was pelleted and re-suspended in 300 µL of Tris-EDTA buffer (pH 8.0). The suspension was mixed with 1.8% (w/v) InCert agarose (Lonza, Rockland, ME) in Tris-EDTA buffer (ThermoFisher Scientific), dispensed into the wells of disposable plug mold (Bio-Rad) and digested with lysostaphin (1 mg/mL in 20 mM sodium acetate, pH 4.5; Sigma Aldrich). The plugs were washed four times in 4 mL of Tris-EDTA buffer at 37°C for 20 min. Following the wash step, agar plugs were cut into a 2 x 2 mm size, equilibrated in 1x *Sma*I restriction buffer for 30 min and digested with *Sma*I (10 U/µl) of (New England BioLabs Inc., Ipswich, MA) in a total volume of 200µL (3 µL *Sma*I +197 µL of 1x buffer) at 25°C for 3 h. A single plug was loaded on to each tooth of 15 combs with the control *S. aureus* strain NCTC 8325 loaded into the 1$^{st}$, 8$^{th}$ and 15$^{th}$ combs and incubated at room temperature for 20 min. The comb was placed in the gel-casting platform and 1% SeaKem agarose was poured into it and kept at room temperature for 20 min until solidified. The gel electrophoresis was conducted using the CHEF Mapper at initial switch of 5 sec, with a final switch of 40 sec and running time for 21 h at 200 V (6 V/cm) at temperature of 14 °C using ramp angle of



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120°. The gel was stained with ethidium bromide (1.25 μg per mL of water, Invitrogen, Carlsbad, CA) for 25 min and washed twice for 30 min with fresh distilled water. The images were taken using ChemiDoc® Touch Imaging System (Bio-Rad), exported to PulseNet by Image Lab 5.2.1 Software (Bio-Rad) and saved as a TIFF file.

***Linking the current PFGE and enterotoxin presence data with previously generated virulence data on these same isolates included in this study.***

The same isolates were previously tested for different virulence factors in our lab and their data were retrieved and entered into the computer along with the current data generated by PFGE typing and PCR on enterotoxins. The aim was to evaluate association, if any, among several virulence factors of these isolates that may be correlated (associated) with each other in some strains to enable them more virulent and efficient in causing mastitis in dairy cows. One of the previously generated data is slime production patterns in which strains were thawed from -80°C and inoculated onto tryptic soy agar plates (TSA). Then, a single colony was streaked on Congo red agar plates (CRA: brain heart infusion agar with 0.08% Congo red dye (Sigma-Aldrich) and 5% sucrose (Thermo Fisher Scientific) and incubated at 37°C for 24 h. Slime production was identified by color. Strains that produced slime were black in color and non-producers were red (Knobloch et al., 2002). These colonies were further divided into weak, moderate and strong biofilm producers based on color intensity. Slime production is an indication of ability to form biofilm, but all biofilm forming strains may not produce slime in vitro (Vasudevan et al., 2003). The other previously generated data is hemolysin (alpha, beta, double hemolysis) presence.



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Hemolysis type (alpha, beta, double hemolysis) was identified by inoculating each bacterium on tryptic soy agar plates (TSA) with 5% sheep blood and incubating overnight at 37°C. Alpha (α) hemolytic strains caused complete hemolysis on blood agar plates whereas beta (β-) hemolytic strains caused partial hemolysis on blood agar plates. Beta (β-) hemolytic strains are most frequent isolates from animals (Dinges et al., 2000). Double (α- and β-) hemolytic strains caused complete hemolysis in the middle with partial hemolysis on the peripheral area around each colony (Dinges et al., 2000). Gamma-hemolysin is produced by almost every strain of *S. aureus,* but gamma-hemolysin is not identifiable on blood agar plates, due to the inhibitory effect of agar on toxin activity (Prevost et al., 1995).

The last data included from a previous study is antimicrobial resistance or susceptibility of these isolates. Antimicrobial susceptibility was analyzed by minimum inhibitory concentration (MIC) on Sensitire mastitis panel plate as described by Abdi et al . (Abdi et al., 2018).

***Statistical analysis***

Distribution of PFGE and virulence factors were summarized by farms and sampling year using a PROC FREQ procedure in SAS 9.4. Determination of the associations among virulence factors, farms, PFGE types and sampling year were used as an explanatory variable for presence of enterotoxin genes in bacterial strains and were analyzed with Logistic regression using a GLIMMIX model with binomial distribution in SAS 9.4.



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A PROC CORESP procedure was used to run a multiple correspondence analysis to determine if a specific virulence factor was only associated and clustered within a specific PFGE type or widespread. The PFGE, enterotoxin, slime production, hemolysis, and antimicrobial resistance or susceptibility data of each isolate were used to detect the underlying population structure of the isolates. In short, such analysis can determine the distance and relatedness between isolates and helps to group the isolates into the same or different cluster using the above listed virulence factors and PFGE types. Furthermore, previous data from our lab on slime production and antimicrobial resistance or susceptibility were also included in the PROC CORESP to determine if these traits and presence of enterotoxin genes are clustered/linked only to a specific farm or PFGE types. The percentages (Figure 3) with each dimension tell how much variability is explained by each dimension in the model in clustering the isolates. A significance was declared at a P value $\leq 0.05$.

## Results

### ***PFGE types and Presence of enterotoxin genes***

Overall, 111 *S. aureus* isolates from 11 farms were grouped into 16 PFGE types, ranging from A to P as determined by Gel Compar II software (Figures 4 and 5). The distribution of PFGE types by farm varied because these were submitted samples and some farms



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submitted few samples (Table 2). Of 16 PFGE types, the most common PFGE types were M, O, and I (Figures 4 and 5). PFGE type M was the most prevalent of all the types, accounting for 57.66% of all isolates followed by type O (9.91%) and I (8.11%). Farm 3 was the most represented farm contributing 19.8% to the total isolates followed by farm 6 and 11, each contributing 13.5% to the total isolates collected (Table 2).

PFGE type M was identified among 9 of the 11 farms. Type O was identified in 6 of the 11 farms. Some of the farms had only 1 PFGE type whereas others had many different PFGE types (Table 2). It is important to note that we had only one or few samples from some farms so the distribution of strains among farms may not be a true reflection of strain distribution since sample size from each farm were different.

PCR results of enterotoxin genes showed that three-fourths (75.7%) of the isolates did not have the enterotoxin genes tested. All 111 *S. aureus* were negative for *sea* and *sej*. The 11.7% and 7.2% of the isolates were positive for *seb* and *tsst-1* genes respectively (Table 3). We found no consistent pattern of enterotoxin genes presence within any of our PFGE types (Table 3). There was not a defined pattern of enterotoxin genes presence on a specific farm (Table 4). Farm, PFGE types and sampling year were not associated with each other or with virulence factors that were compared ($P>0.05$). Farm 3 had the most PFGE types out of all farms, however, that may be because of more samples from farm 3 than other farms.



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The number of PFGE types and enterotoxins identified per farm is skewed because of the few enterotoxins found overall and because of the number of submitted samples from each farm (Figure 1). The farm 3 has the most PFGE types, however, it also has the most submitted samples. Enterotoxin genes presence is not uniform among farms and no pattern was identified (Figure 1). When comparing the number of farms and enterotoxins types by PFGE type we found that PFGE type M is most prevalent in our samples and is present among 9 farms. PFGE type M also had the most enterotoxin presence which may be because it is the most prevalent type (Figure 2). We found few positive enterotoxin genes among our samples therefore; no pattern was found among them.

The distribution of enterotoxin gene positive isolates was scattered because 75.7% of isolates tested did not have enterotoxin genes present. Enterotoxin B is most prevalent in our samples with 11.7% prevalence followed by tsst-1 with 7.2%, *sec* with 2.7%, and *see* with 0.9% (Table 4). The PFGE types in relation to farms, types of enterotoxins, year collected, and frequency of isolates are all grouped together to visualize the relation between all factors. The dendrogram shows us the relatedness of our PFGE types by calculating the differences in band number between the samples. We found that PFGE typeM contained most of our samples, was among 9 farms. Some samples displayed enterotoxin genes *seb*, *sec*, and tsst-1, as well as being collected in multiple sample years (Appendix Figures 4 and 5). We used these relationships to further compare factors among them (Tables2-4 and Figures 1&2).



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***The prevalence of different virulence factors of three dominant PFGE types***

Slime production, hemolysin and enterotoxin presence, and antimicrobial resistance or susceptibility were evaluated among the three most widespread PFGE types, namely I, M, and O. Based on slime production, 55% of PFGE type I had moderate slime production, 49.15% of type M had low slime production and 45.45% of type O had both low and moderate slime production. Beta hemolysin was more prevalent than other hemolysins among the three PFGE types. Antimicrobial resistance was more prevalent in PFGE type I and followed by type M, but rare in type O. Enterotoxin genes were slightly more prevalent in type I but similar in types M and O (Table 5).

***Multiple correspondence analysis***

The multiple correspondence analysis showed that PFGE type M and O tended to cluster with (related) beta-hemolysin, no toxin production, weak slime production, and antibiotic susceptibility (Figure 3). Out of our samples, most were found to have beta hemolysis. This is also seen in most mastitis isolates. We also saw that most isolates we tested were susceptible to antibiotics and displayed no toxin genes.

## Discussion

Results of our PFGE findings were closely similar to Srinivasan et al. (Srinivasan et al., 2006) who found 15 distinct pulsotypes among the 78 *S. aureus* isolates evaluated. Our results were also in line with the findings of other studies on *S. aureus* isolates from dairy



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cows that showed presence of genetically diverse *S. aureus* isolates. The 16 PFGE types detected in this study showed different distribution patterns among the 111 *S. aureus* isolates tested. However, M, O, I, E, C and J spread among 9, 6, 5, 3, 2 and 2 farms, respectively, indicating their predominance and genetic expansion among and within the farms. These findings are in line with previous observations by others (Sakwinska et al., 2011). The distribution patterns of different PFGE types in the Farms showed that 19.8% of the isolates were found in farm 3, 13.5% in farms 6, 11, 12.6% in unknown farm, 11.7% in farms 5 and 9, 4.5% in farms 1 and 2 and 2.7 % in farms 8 and 10. The lowest prevalence of 1.8% and 0.9% of the total isolates were observed in farms 4 and 7 respectively. This observation showed that multiple lineages were found in one or multiple farms. This indicated that majority of the isolates co-existed in individual or multiple farms. Our findings are in agreement with observations from Pennsylvania and Ohio (Kapur et al., 1995) that showed diverse clonal types of *S. aureus* strains in a particular area, despite few dominant lineages. We also found that several farms had multiple PFGE types or lineage types. Results of this study showed that the types of lineages involved in co-existence varied with farm. Co-existence of the heterogeneous lineages within a specific farm have been reported from Canadian dairy herds (Sabour et al., 2004). In this study, existence of a single lineage was also observed in three farms. Single lineage existence per farm also reported in Canada (Sabour et al., 2004).

To infect the host, *S. aureus* uses several virulence factors such as toxins, enzymes, adhesins, and other surface proteins that allow the pathogen to survive under hostile



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environments including the host's body under the effect of host immune response and/or effect of antimicrobials during treatment. Of 111 *S. aureus* examined, 27 (24.3%) were positive for one or more enterotoxin genes. About three-fourths (75.7%) of the isolates did not produce any of the enterotoxins tested. The *seb* and *tsst-1* were relatively found at higher frequencies here as *sea* and *sej* were not found among tested strains. Two isolates harbored *seb* and *sec* and one isolate possesses *sec* and *tsst-1* combinations. Our findings are different from Srinivasan et al. (Srinivasan et al., 2006) who examined 78 *S. aureus* isolates from the milk of cows with mastitis for 16 enterotoxin genes and found that 73 (93.6%) of the isolates were positive for one or more enterotoxin genes from similar area. However, Srinivasan et al. (Srinivasan et al., 2006) tested for 16 enterotoxin genes whereas in our study we tested only 6 enterotoxin genes. This might be the reason for low prevalence of positive isolates in our study. Similar to Srinivasan et al. (Srinivasan et al., 2006), we also did not find isolates positive for *sea* and *sej* enterotoxins. Another study (Chao et al., 2015) also showed variation in presence of toxin genes in *S. aureus* isolates from different sources. Srinivasan et al. (Srinivasan et al., 2006) found that the majority of *S. aureus* isolated from milk of cows with mastitis carried newly described staphylococcal enterotoxin genes such as *sem*, *sen* and *sei* along with classical staphylococcal enterotoxin genes, *sed* and *tsst-1*. We did not test for *sem*, *sen*, *sei* but similar to his finding we also observed increased prevalence of *tsst-1* gene.

In our study, we also evaluated co-existence of certain PFGE type with specific toxin types. There were no significant association among farms, PFGE types and sampling year



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(data not shown). Similar to our findings Srinivasan et al. (Srinivasan et al., 2006) also observed that PFGE and enterotoxin gene profiles did not match with each other because of the fact that a single pulsotype carried different combinations of enterotoxin genes. We did not identify any pattern of toxin genes that were specific for a PFGE type, however, this may be due to our samples not being randomized from the population.

In this study, we evaluated association among different virulence factors of *S. aureus* strains from cases of bovine mastitis that may enable *S. aureus* to evade the host defense. We evaluated association among presence of enterotoxin genes, slime production and antimicrobial resistance or susceptibility and PFGE types. Our results showed no significant associations; however, a larger scale evaluation may be helpful to get enough number of isolates for statistical analysis.

## Conclusion

In conclusion, this study sheds light on genotypes of *S. aureus* isolates from cases of bovine mastitis in the east Tennessee region and showed presence of diverse genotypes that possess variable enterotoxin genes. A relatively high gene prevalence of *seb* and *tsst-1* in *S. aureus* isolates from cases of bovine mastitis showed critical food safety issue that requires control of foodborne pathogens that also cause mastitis at pre-harvest level through use of effective vaccine. A further detailed study should be conducted on a larger number of isolates from randomized dairy farms based on combined multilocus sequence



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typing (MLST), staphylococcal protein A (spa) typing and pulsed field gel electrophoresis with epidemiological studies are required to clarify dominant genotypes of *S. aureus* and associated enterotoxins genotypes.



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# CHAPTER THREE

# *S. AUREUS* ADHESION TO AND INVASION INTO BOVINE MAMMARY EPITHELIAL CELLS



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## Abstract

Many factors contribute to the pathogenesis of *S. aureus* mastitis, however, the most critical virulence factors or mechanisms responsible for the establishment of IMI are not clearly defined. In this study, we evaluated the adhesion and invasion of the dominant strains (PFGE types I, M and O) in vitro on bovine mammary epithelial cell line (MAC-T cells). The number of *S. aureus* adhered to and internalized into MAC-T cells were analyzed for each strain to evaluate pathogenicity of each strain on MAC-T cells. Our results showed that the number of Type O strain attached to and internalized into MAC-T cells were relatively different but not significantly higher than the other two strains, M and I.


## Introduction

Adhesion and invasion are virulence factors that help the bacteria to survive and thrive inside the host and can help to assess the pathogenicity of the bacteria that affects bovine mammary glands. Adhesion is the first step in the formation of biofilm or the invasion of host cells, which protect the bacteria from the host immune system and facilitate chronic infection (Josse et al., 2017). Adhesion is dependent on surface proteins called adhesins, which help the cell to recognize and attach to host cells. Staphylococci are coated with a wide variety of surface proteins that help them to adhere to host cells and other affiliated



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cells. Microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are the most common surface proteins that are involved in adhesion (Josse et al., 2017). The ability to bind to host tissue or the host's cell surface is a pivotal part of the bacteria's pathogenicity because adhesion is typically the first step in the invasion and biofilm formation (Loffler et al., 2014, Moormeier and Bayles, 2017). Adhesion to mammary epithelial cells is mainly achieved through fibronectin binding to α5β1-Integrin on epithelial cells and then forming bridge through binding to fibronectin binding proteins on the bacterial cell surface (Josse et al., 2017). This binding is required for *S. aureus* internalization into non-phagocytic host cells (Fowler et al., 2000). The other important structure of *S. aureus* that involve in adhesion is C-terminal peptidoglycan-binding motif (LPXTG) and the wall and membrane spanning domains anchor fibronectin binding proteins to the cell wall.

*S. aureus* invasion into host cells involve focal adhesion kinases (FAKs) and activated Src (Fowler et al., 2003, Agerer et al., 2005) that leads to actin polymerization and endocytic activity (Agerer et al., 2005, Selbach and Backert, 2005). The kinase-Src pathway, the activation of PI3K and Akt is also important for the internalization of *S. aureus* (Oviedo-Boyso et al., 2011, Wang et al., 2013). Internalization is inhibited by cytochalasin-D (Ellington et al., 1999, Sinha et al., 1999) and is temperature dependent where internalization is inhibited at 4°C and 14°C and facilitated at 37°C versus room temperature. Dynamic actin cytoskeleton (actin polymierization) is a prerequisite for invasion as well as having a host cell with a fluid membrane. By upregulating b1-integrin



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expression in the host cell through secreting alpha hemolysin, *S. aureus* can stimulate the host cell to uptake itself (Abel et al., 2011, Goldmann et al., 2016). We hypothesize that *S. aureus* strains that infect bovine mammary glands have specific mechanisms and pathogenicity that enable them to evade the host's immune system. In our previous study, we found 3 dominant PFGE types. We evaluated the ability of these three dominant strains to attach to and internalize into mammary epithelial cell line (MAC-T cells) in vitro.

## Materials and methods

### *Mammary epithelial cell cultures*

A bovine mammary epithelial cell line (MAC-T), (Huynh et al., 1991) was grown in 24 well-cell culture plates at 37°C in 5% $CO_2$/balanced air using Dulbecco's Modified Eagle media (DMEM) containing 10% fetal bovine serum, insulin (5 μg/ml), and hydrocortisone (1μg/ml) as cell growth media (CGM) as described (Almeida and Oliver, 1995, Almeida et al., 1996) until a confluent monolayer was formed.

### *Bacterial growth conditions*

A representative strain of I, O and M PFGE types were thawed from the -80°C freezer and plated and grown overnight on tryptic soy agar plates (TSA) with 5% sheep blood (Becton Dickinson Microbiology system, Cockeysville, MD). One colony from each strain was taken and suspended in 5 mL of tryptic soy broth (TSB) and grown overnight at 37°C. On



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the day of experiment, the overnight culture was diluted 1:50 (2 mL of overnight culture added into 98 mL of fresh TSB broth) and incubated at 37°C with shaking at 220 rpm until the $OD_{600}$ of 0.4 - 0.5 is reached. Then the bacterial culture was centrifuged at 5000xg for 10 min at 4°C and washed three times with PBS (pH7.2). The bacterial cell pellet was re-suspended in 100 mL of MAC - T medium. The number of bacteria in the inoculum was determined by a viable count from a 10-fold serially diluted ($10^0$ – $10^9$) inoculum from dilutions 6, 7, 8 and 9 plated on blood agar in triplicates of 25 µL per dilution. The plates were incubated overnight at 37°C and the number of CFU/ml of inoculum was calculated.

**Adherence assay**

Adherence assay was performed essentially as described by Almeida (Almeida et al., 1996). One ml of a suspension containing 1.0 x $10^7$ CFU/ ml of *S. aureus* strain I, M, and O in CGM was added per well. Co-cultures was incubated at 37°C in 5% $CO_2$:95% air balanced incubator for 1 h. After incubation, MAC-T cell monolayers was washed three times with PBS (PH 7.2), treated with 0.25% trypsin and lysed with Triton X-100 (Amersham Arlington Heights, IL) at a final concentration of 0.025% (vol/vol) in sterile distilled water. Cell-associated bacterial numbers from lysates were determined by standard plate dilution techniques. The number of internalized bacteria was determined from wells in which the internalization assay was conducted simultaneously. The number of adherent bacteria was determined by subtracting the number of internalized bacteria from the corresponding number of cell-associated bacteria. The strain and condition tested was evaluated in triplicates and the assay was repeated three times.



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*Bacterial internalization assay*

Bacterial internalization assay was performed as described by Almeida and Oliver (Almeida and Oliver, 1995). After removing CGM from MAC-T cell cultures, 1 ml of fresh CGM containing $1x10^7$ CFU/ml of *S. aureus* strain I, M and O was added per well using 3 wells for each strain and condition studied. Co-cultures were incubated for 60 min. After incubation, monolayers were washed three times with PBS (pH7.2) and incubated with CGM containing gentamicin (100 µg/ml; Sigma Aldrich) and penicillin G (100 IU/ml) for 2 h at 37°C in 5% CO2:95% air air balanced incubator. After 2 h incubation with antibiotics, the number of *S. aureus* in CFU/ml in supernatants was determined by standard plate dilution techniques to monitor effectiveness of gentamicin/penicillin G in killing extracellular *S. aureus.* After removal of CGM containing antibiotics, MAC-T cell monolayers were washed three times with PBS (pH 7.2) and treated with 0.25% trypsin and lysed with Triton X-100 (Amersham), at a final concentration of 0.025% (vol/vol) in sterile distilled water. Cell lysates were serially 10-fold diluted, 25 µl was plated in triplicate on blood agar, incubated overnight at 37°C, and the number of CFU/ml determined.

*Statistical analysis*

Comparison of means of number of *S. aureus* adhered to and invaded into the mammary epithelial cells during the in vitro assay was compared among three dominant PFGE types using analysis of variance (ANOVA). We blocked by day and had random effects of day by PFGE type interaction. The fixed effects were PFGE type and inoculation. The



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repeatability of the *in vitro* adhesion and invasion assay among days were compared using coefficient of variation. A statistical significance was declared with a P value $\leq 0.05$.

## Results

The number of inoculated bacteria (CFU/mL) was relatively different but not significantly different among the three PFGE types in the order of M > I > O in CFU/mL. The adhesion and invasion results showed that the number of PFGE types I, M and O attached to MAC-T cells were not significantly different (P>0.05) among the three strains. However, the number of O type that attached to and internalized into MAC- T cells are higher than that of M & I types.

## Discussion

After evaluation of adhesion and invasion (pathogenicity) of representative strains I, M, and O, we found no significant differences among them. Our PFGE analysis show that M and O types are more closely related than I, however, even though not statistically significant we saw relatively higher numbers of O type strain were attached and internalized into MAC-T cells compared to M type. This may be indication of O strain more effective in causing infection than other two strains. From PFGE results, type M was the most prevalent strain, but it was not more adhesive and invasive on MAC – T cells than the other strains. In general increase in number of *S. aureus* in the mammary gland in most



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cases followed by increased inflammatory changes but few number of *S. aureus* also can cause serious inflammatory changes in the mammary glands. So, we do not know if increase or decrease in number of *S. aureus* attached to and internalized into MAC –T cells is directly correlated to the ability of that strain to cause disease *in vivo*. Similarly, since *S. aureus* has several virulence factors or mechanisms that enable them to cause infection in the host, adhesion and invasion alone cannot define its pathogenicity to the host *in vivo*. There are many different interactions that are happening under *in vivo* conditions therefore, *in vitro* results cannot be directly extrapolated to *in vivo* situations. The adhesion to and invasion into cells are not achieved by the same mechanism and are not completely understood. If the number of bacteria adhered and invaded are similar, it does don't mean that they are of equal pathogenicity. *S. aureus* uses a different mechanism to attach to and internalize into host cells. This *in vitro* study uses only one type of epithelial cell but under *in vivo* condition adhesion and invasion may involve several different cells that have different receptors and molecules on their surface, so this may not reflect actual process *in vivo*. *In vivo* experimental intramammary infusion or teat dip-based challenge may provide their actual pathogenicity status.

## Conclusion

Results of this study showed that the *in vitro* adhesion to and invasion into mammary epithelial cell line (MAC-T cells) of dominant strains were not significantly different. However, the number of O type strains that attach to and internalize into MAC T cells were



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higher than M and I types. *In vivo* experimental challenge study may provide actual pathogenicity status of these strains.



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# CHAPTER FOUR

# CONCLUSION



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In conclusion, we evaluated the genetic diversity of *S. aureus* strains from bovine mastitis in the East Tennessee region and correlation of different virulence factors with genetic diversity that may be specific to the strains isolated from cases of bovine mastitis. We found no significant associations among the virulence factors and PFGE types. Further detailed research should be done to see if there is a common set of virulence factors in isolates from cases of mastitis.

*In vitro* adhesion to and invasion into mammary epithelial cell line (MAC-T cells) of dominant strains showed no significant difference. However, the number of O type that attaches to and internalize into MAC T cells were higher than M and I types. *In vivo* experimental challenge study using these strains may provide their actual pathogenicity status.



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## REFERENCES



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www.manaraa.com

Abdi, R. D., B. E. Gillespie, J. Vaughn, C. Merrill, S. I. Headrick, D. B. Ensermu, D. H. D'Souza, G. E. Agga, R. A. Almeida, S. P. Oliver, and O. Kerro Dego. 2018. Antimicrobial Resistance of Staphylococcus aureus Isolates from Dairy Cows and Genetic Diversity of Resistant Isolates. Foodborne Pathog Dis 15(7):449-458.

Abel, J., O. Goldmann, C. Ziegler, C. Holtje, M. S. Smeltzer, A. L. Cheung, D. Bruhn, M. Rohde, and E. Medina. 2011. Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. J Innate Immun 3(5):495-507.

Agerer, F., S. Lux, A. Michel, M. Rohde, K. Ohlsen, and C. R. Hauck. 2005. Cellular invasion by Staphylococcus aureus reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. J Cell Sci 118(Pt 10):2189-2200.

Almeida, R. A., K. R. Matthews, E. Cifrian, A. J. Guidry, and S. P. Oliver. 1996. Staphylococcus aureus invasion of bovine mammary epithelial cells. Journal of Dairy Science 79(6):1021-1026.

Almeida, R. A. and S. P. Oliver. 1995. Invasion of bovine mammary epithelial cells by Streptococcus dysgalactiae. J Dairy Sci 78(6):1310-1317.

Anderson, K. L. and R. L. Lyman. 2006. Long-term persistence of specific genetic types of mastitis-causing Staphylococcus aureus on three dairies. J Dairy Sci 89(12):4551-4556.

Aydin, A., M. Sudagidan, and K. Muratoglu. 2011. Prevalence of staphylococcal enterotoxins, toxin genes and genetic-relatedness of foodborne Staphylococcus aureus strains isolated in the Marmara Region of Turkey. Int J Food Microbiol 148(2):99-106.

Bannerman, D. D., M. J. Paape, J.-W. Lee, X. Zhao, J. C. Hope, and P. Rainard. 2004. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clinical and diagnostic laboratory immunology 11(3):463-472.

Barkema, H. W., Y. H. Schukken, and R. N. Zadoks. 2006. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. J Dairy Sci 89(6):1877-1895.

Bergdoll, M. S., F. S. Chu, C. R. Borja, I.-Y. Huang, and K. F. Weiss. 1967. The staphylococcal enterotoxins. Japanese journal of microbiology 11(4):358-368.

Berube, B. J. and J. Bubeck Wardenburg. 2013. Staphylococcus aureus alpha-toxin: nearly a century of intrigue. Toxins (Basel) 5(6):1140-1166.

Blowey, R. W. and P. Edmondson. 2010. Mastitis control in dairy herds. Cabi.

Bradley, A. J., J. E. Breen, B. Payne, V. White, and M. J. Green. 2015. An investigation of the efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in the United Kingdom. J Dairy Sci 98(3):1706-1720.

Brady, R. A., J. G. Leid, A. K. Camper, J. W. Costerton, and M. E. Shirtliff. 2006. Identification of Staphylococcus aureus proteins recognized by the antibody-mediated immune response to a biofilm infection. Infect Immun 74(6):3415-3426.



المنارة للاستشارات
www.manaraa.com

Bramley, A. J., A. H. Patel, M. O'Reilly, R. Foster, and T. J. Foster. 1989. Roles of alpha-toxin and beta-toxin in virulence of Staphylococcus aureus for the mouse mammary gland. Infect Immun 57(8):2489-2494.

Brussow, H., C. Canchaya, and W. D. Hardt. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev 68(3):560-602, table of contents.

Burke, F. M., N. McCormack, S. Rindi, P. Speziale, and T. J. Foster. 2010. Fibronectin-binding protein B variation in Staphylococcus aureus. BMC Microbiol 10:160.

Caiazza, N. C. and G. O'toole. 2003. Alpha-toxin is required for biofilm formation by Staphylococcus aureus. Journal of bacteriology 185(10):3214-3217.

Camussone, C. M. and L. F. Calvinho. 2013. [Virulence factors of Staphylococcus aureus associated with intramammary infections in cows: relevance and role as immunogens]. Rev Argent Microbiol 45(2):119-130.

Capurro, A., A. Aspan, K. Artursson, and K. P. Waller. 2010. Genotypic variation among Staphylococcus aureus isolates from cases of clinical mastitis in Swedish dairy cows. Vet J 185(2):188-192.

Cenci-Goga, B., M. Karama, P. Rossitto, R. Morgante, and J. Cullor. 2003. Enterotoxin production by Staphylococcus aureus isolated from mastitic cows. Journal of food protection 66(9):1693-1696.

Chao, G., G. Bao, Y. Cao, W. Yan, Y. Wang, X. Zhang, L. Zhou, and Y. Wu. 2015. Prevalence and diversity of enterotoxin genes with genetic background of Staphylococcus aureus isolates from different origins in China. Int J Food Microbiol 211:142-147.

Cifrian, E., A. Guidry, C. O'Brien, and W. Marquardt. 1995. Effect of alpha-toxin and capsular exopolysaccharide on the adherence of Staphylococcus aureus to cultured teat, ductal and secretory mammary epithelial cells. Research in veterinary science 58(1):20-25.

Clarke, S. R. and S. J. Foster. 2006. Surface adhesins of Staphylococcus aureus. Adv Microb Physiol 51:187-224.

Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa, and J. R. Penades. 2001. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol 183(9):2888-2896.

Cucarella, C., M. Á. Tormo, C. Ubeda, M. P. Trotonda, M. Monzón, C. Peris, B. Amorena, Í. Lasa, and J. R. Penadés. 2004. Role of biofilm-associated protein bap in the pathogenesis of bovine Staphylococcus aureus. Infection and immunity 72(4):2177-2185.

De Oliveira, A., J. Watts, S. Salmon, and F. M. Aarestrup. 2000. Antimicrobial susceptibility of Staphylococcus aureus isolated from bovine mastitis in Europe and the United States. Journal of Dairy Science 83(4):855-862.

DeGraves, F. J. and J. Fetrow. 1993. Economics of mastitis and mastitis control. The Veterinary Clinics of North America. Food Animal Practice 9(3):421-434.

den Reijer, P. M., M. Sandker, S. V. Snijders, M. Tavakol, A. P. Hendrickx, and W. J. van Wamel. 2017. Combining in vitro protein detection and in vivo antibody



المنارة للاستشارات
www.manaraa.com

detection identifies potential vaccine targets against Staphylococcus aureus during osteomyelitis. Med Microbiol Immunol 206(1):11-22.
Dhanawade, N. B., D. R. Kalorey, R. Srinivasan, S. B. Barbuddhe, and N. V. Kurkure. 2010. Detection of intercellular adhesion genes and biofilm production in Staphylococcus aureus isolated from bovine subclinical mastitis. Veterinary research communications 34(1):81-89.
Dinges, M. M., P. M. Orwin, and P. M. Schlievert. Staphylococcus aureus Exotoxins of.
Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of Staphylococcus aureus. Clin Microbiol Rev 13(1):16-34, table of contents.
Donlan, R. M. and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15(2):167-193.
Ellington, J. K., S. S. Reilly, W. K. Ramp, M. S. Smeltzer, J. F. Kellam, and M. C. Hudson. 1999. Mechanisms of Staphylococcus aureus invasion of cultured osteoblasts. Microb Pathog 26(6):317-323.
Febler, A., C. Scott, K. Kadlec, R. Ehricht, S. Monecke, and S. Schwarz. 2010. Characterization of methicillin-resistant Staphylococcus aureus ST398 from cases of bovine mastitis. Journal of Antimicrobial Chemotherapy 65(4):619-625.
Fitzgerald, J. R. 2012. Livestock-associated Staphylococcus aureus: origin, evolution and public health threat. Trends in microbiology 20(4):192-198.
Foster, T. J., J. A. Geoghegan, V. K. Ganesh, and M. Hook. 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol 12(1):49-62.
Fowler, T., S. Johansson, K. K. Wary, and M. Hook. 2003. Src kinase has a central role in in vitro cellular internalization of Staphylococcus aureus. Cell Microbiol 5(6):417-426.
Fowler, T., E. R. Wann, D. Joh, S. Johansson, T. J. Foster, and M. Hook. 2000. Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur J Cell Biol 79(10):672-679.
Fox, L. K. and D. D. Hancock. 1989. Effect of segregation on prevention of intramammary infections by Staphylococcus aureus. J Dairy Sci 72(2):540-544.
Gentilini, E., G. Denamiel, P. Llorente, S. Godaly, M. Rebuelto, and O. DeGregorio. 2000. Antimicrobial susceptibility of Staphylococcus aureus isolated from bovine mastitis in Argentina. Journal of dairy science 83(6):1224-1227.
Gillespie, B. E., S. I. Headrick, S. Boonyayatra, and S. P. Oliver. 2009. Prevalence and persistence of coagulase-negative Staphylococcus species in three dairy research herds. Vet Microbiol 134(1-2):65-72.
Goldmann, O., L. Tuchscherr, M. Rohde, and E. Medina. 2016. alpha-Hemolysin enhances Staphylococcus aureus internalization and survival within mast cells by modulating the expression of beta1 integrin. Cell Microbiol 18(6):807-819.
Gotz, F. 2002. Staphylococcus and biofilms. Mol Microbiol 43(6):1367-1378.
Graber, H. U., J. Naskova, E. Studer, T. Kaufmann, M. Kirchhofer, M. Brechbuhl, W. Schaeren, A. Steiner, and C. Fournier. 2009. Mastitis-related subtypes of bovine



المنارة للاستشارات
www.manaraa.com

Staphylococcus aureus are characterized by different clinical properties. J Dairy Sci 92(4):1442-1451.
Gudding, R., J. McDonald, and N. Cheville. 1984. Pathogenesis of Staphylococcus aureus mastitis: bacteriologic, histologic, and ultrastructural pathologic findings. American journal of veterinary research 45(12):2525-2531.
Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J Dairy Sci 77(7):2103-2112.
Hauck, C. R. and K. Ohlsen. 2006. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus. Curr Opin Microbiol 9(1):5-11.
Hennekinne, J.-A., M.-L. De Buyser, and S. Dragacci. 2012. Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol Rev 36.
Hensen, S., M. Pavičić, J. Lohuis, and B. Poutrel. 2000. Use of bovine primary mammary epithelial cells for the comparison of adherence and invasion ability of Staphylococcus aureus strains. Journal of dairy science 83(3):418-429.
Hillerton, J. and E. Berry. 2005. Treating mastitis in the cow–a tradition or an archaism. Journal of applied microbiology 98(6):1250-1255.
Holmes, M. A. and R. N. Zadoks. 2011. Methicillin resistant S. aureus in human and bovine mastitis. Journal of mammary gland biology and neoplasia 16(4):373-382.
Huynh, H. T., G. Robitaille, and J. D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res 197(2):191-199.
Jamali, H., B. Radmehr, and S. Ismail. 2014. Short communication: prevalence and antibiotic resistance of Staphylococcus aureus isolated from bovine clinical mastitis. J Dairy Sci 97(4):2226-2230.
Josse, J., F. Laurent, and A. Diot. 2017. Staphylococcal Adhesion and Host Cell Invasion: Fibronectin-Binding and Other Mechanisms. Front Microbiol 8:2433.
Kadariya, J., T. C. Smith, and D. Thapaliya. 2014. Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. BioMed research international 2014.
Kapur, V., W. M. Sischo, R. S. Greer, T. S. Whittam, and J. M. Musser. 1995. Molecular population genetic analysis of Staphylococcus aureus recovered from cows. J Clin Microbiol 33(2):376-380.
Keefe, G. 2012. Update on control of Staphylococcus aureus and Streptococcus agalactiae for management of mastitis. Vet Clin North Am Food Anim Pract 28(2):203-216.
Kenny, K., R. F. Reiser, F. D. Bastida-Corcuera, and N. L. Norcross. 1993. Production of enterotoxins and toxic shock syndrome toxin by bovine mammary isolates of Staphylococcus aureus. J Clin Microbiol 31(3):706-707.
Kerro, O. D. and H. Nederbragt. 2002. Factors involved in the early pathogenesis of bovine Staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. A review. The veterinary quarterly 24(4):181-198.



المنارة للاستشارات
www.manaraa.com

Knobloch, J. K.-M., M. A. Horstkotte, H. Rohde, and D. Mack. 2002. Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. Medical microbiology and immunology 191(2):101-106.

Kong, C., H.-m. Neoh, and S. Nathan. 2016. Targeting Staphylococcus aureus toxins: a potential form of anti-virulence therapy. Toxins 8(3):72.

Lammers, A., P. J. Nuijten, and H. E. Smith. 1999. The fibronectin binding proteins of Staphylococcus aureus are required for adhesion to and invasion of bovine mammary gland cells. FEMS microbiology letters 180(1):103-109.

Lasa, I. and J. R. Penadés. 2006. Bap: a family of surface proteins involved in biofilm formation. Research in microbiology 157(2):99-107.

Le Loir, Y., F. Baron, and M. Gautier. 2003. Staphylococcus aureus and food poisoning. Genetics and molecular research: GMR 1 (2), 63-76.(2003).

Loffler, B., L. Tuchscherr, S. Niemann, and G. Peters. 2014. Staphylococcus aureus persistence in non-professional phagocytes. Int J Med Microbiol 304(2):170-176.

Low, D. K. R. and J. H. Freer. 1977. Biological effects of highly purified B-lysin (sphingomyelinase) from S. aureus. FEMS letters 2:133-138.

Luini, M., P. Cremonesi, G. Magro, V. Bianchini, G. Minozzi, B. Castiglioni, and R. Piccinini. 2015. Methicillin-resistant Staphylococcus aureus (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: Genotyping of isolates. Vet Microbiol 178(3-4):270-274.

MATSUNAGA, T., S.-i. KAMATA, N. KAKIICHI, and K. UCHIDA. 1993. Characteristics of Staphylococcus aureus isolated from peracute, acute and chronic bovine mastitis. Journal of Veterinary Medical Science 55(2):297-300.

McCarthy, A. J. and J. A. Lindsay. 2010. Genetic variation in Staphylococcus aureus surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. BMC Microbiol 10:173.

McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover. 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. J Clin Microbiol 41.

McDougall, S., K. I. Parker, C. Heuer, and C. W. Compton. 2009. A review of prevention and control of heifer mastitis via non-antibiotic strategies. Vet Microbiol 134(1-2):177-185.

McMillan, K., S. C. Moore, C. M. McAuley, N. Fegan, and E. M. Fox. 2016. Characterization of Staphylococcus aureus isolates from raw milk sources in Victoria, Australia. BMC Microbiology 16(1):169.

Melchior, M., H. Vaarkamp, and J. Fink-Gremmels. 2006. Biofilms: a role in recurrent mastitis infections? The Veterinary Journal 171(3):398-407.

Mellenberger, R. and J. Kirk. 2001. Mastitis Control Program for Staph. aureus Infected Dairy Cows. Vetmed. Ucdavis. edu.

Middleton, J. R., C. D. Luby, and D. S. Adams. 2009. Efficacy of vaccination against staphylococcal mastitis: a review and new data. Veterinary microbiology 134(1-2):192-198.



المنارة للاستشارات
www.manaraa.com

Middleton, J. R., J. Ma, C. L. Rinehart, V. N. Taylor, C. D. Luby, and B. J. Steevens. 2006. Efficacy of different Lysigin formulations in the prevention of Staphylococcus aureus intramammary infection in dairy heifers. J Dairy Res 73(1):10-19.
Milanov, D., S. Lazić, B. Vidić, J. Petrović, D. Bugarski, and Z. Šeguljev. 2010. Slime production and biofilm forming ability by Staphylococcus aureus bovine mastitis isolates. Acta veterinaria 60(2-3):217-226.
Monecke, S., P. Kuhnert, H. Hotzel, P. Slickers, and R. Ehricht. 2007. Microarray based study on virulence-associated genes and resistance determinants of Staphylococcus aureus isolates from cattle. Veterinary microbiology 125(1-2):128-140.
Moon, J., A. Lee, H. Kang, E. Lee, Y. Joo, Y. H. Park, M. Kim, and H. Koo. 2007. Antibiogram and coagulase diversity in staphylococcal enterotoxin-producing Staphylococcus aureus from bovine mastitis. Journal of dairy science 90(4):1716-1724.
Moormeier, D. E. and K. W. Bayles. 2017. Staphylococcus aureus biofilm: a complex developmental organism. Mol Microbiol 104(3):365-376.
Neave, F., F. Dodd, R. Kingwill, and D. Westgarth. 1969. Control of Mastitis in the Dairy Herd by Hygiene and Management. Journal of Dairy Science 52(5):696-707.
O'Callaghan, R. J., M. C. Callegan, J. M. Moreau, L. C. Green, T. J. Foster, O. M. Hartford, L. S. Engel, and J. M. Hill. 1997. Specific roles of alpha-toxin and beta-toxin during Staphylococcus aureus corneal infection. Infection and immunity 65(5):1571-1578.
O'Gara, J. P. 2007. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS Microbiology Letters 270:179–188.
Otto, M. 2008. Staphylococcal biofilms. Curr Top Microbiol Immunol 322:207-228.
Otto, M. 2013. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu Rev Med 64:175-188.
Otto, M. 2014. Staphylococcus aureus toxins. Curr Opin Microbiol 17:32-37.
Oviedo-Boyso, J., R. Cortes-Vieyra, A. Huante-Mendoza, H. B. Yu, J. J. Valdez-Alarcon, A. Bravo-Patino, M. Cajero-Juarez, B. B. Finlay, and V. M. Baizabal-Aguirre. 2011. The phosphoinositide-3-kinase-Akt signaling pathway is important for Staphylococcus aureus internalization by endothelial cells. Infect Immun 79(11):4569-4577.
Oviedo-Boyso, J., J. J. Valdez-Alarcón, M. Cajero-Juárez, A. Ochoa-Zarzosa, J. E. López-Meza, A. Bravo-Patino, and V. M. Baizabal-Aguirre. 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. Journal of Infection 54(4):399-409.
Pantosti, A., A. Sanchini, and M. Monaco. 2007. Mechanisms of antibiotic resistance in Staphylococcus aureus.



المنارة للاستشارات
www.manaraa.com

Pereira, U. P., D. G. Oliveira, L. R. Mesquita, G. M. Costa, and L. J. Pereira. 2011. Efficacy of Staphylococcus aureus vaccines for bovine mastitis: a systematic review. Vet Microbiol 148(2-4):117-124.
Petrovski, K., M. Trajcev, and G. Buneski. 2006. A review of the factors affecting the costs of bovine mastitis. Journal of the South African Veterinary Association 77(2):52-60.
Prevost, G., P. Couppie, P. Prevost, S. Gayet, P. Petiau, B. Cribier, H. Monteil, and Y. Piemont. 1995. Epidemiological data on Staphylococcus aureus strains producing synergohymenotropic toxins. J Med Microbiol 42(4):237-245.
Rainard, P. 2003. The complement in milk and defense of the bovine mammary gland against infections. Veterinary research 34(5):647-670.
Rodgers, J. D., J. J. McCullagh, P. T. McNamee, J. A. Smyth, and H. J. Ball. 1999. Comparison of Staphylococcus aureus recovered from personnel in a poultry hatchery and in broiler parent farms with those isolated from skeletal disease in broilers. Veterinary microbiology 69(3):189-198.
Rogolsky, M. 1979. Nonenteric toxins of Staphylococcus aureus. Microbiol Rev 43(3):320-360.
Sabour, P. M., J. J. Gill, D. Lepp, J. C. Pacan, R. Ahmed, R. Dingwell, and K. Leslie. 2004. Molecular typing and distribution of Staphylococcus aureus isolates in Eastern Canadian dairy herds. J Clin Microbiol 42(8):3449-3455.
Sakwinska, O., M. Giddey, M. Moreillon, D. Morisset, A. Waldvogel, and P. Moreillon. 2011. Staphylococcus aureus Host Range and Human-Bovine Host Shift. Appl Environ Microbiol 77.
Savic, N. R., V. Katic, and B. VELEBIT. 2014. Characteristics of coagulase positive staphylococci isolated from milk in cases of subclinical mastitis. Acta Vet Beograd 64:115 - 123.
Sawant, A. A., B. E. Gillespie, and S. P. Oliver. 2009. Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. Vet Microbiol 134(1-2):73-81.
Schukken, Y. H., V. Bronzo, C. Locatelli, C. Pollera, N. Rota, A. Casula, F. Testa, L. Scaccabarozzi, R. March, D. Zalduendo, R. Guix, and P. Moroni. 2014. Efficacy of vaccination on Staphylococcus aureus and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds. J Dairy Sci 97(8):5250-5264.
Selbach, M. and S. Backert. 2005. Cortactin: an Achilles' heel of the actin cytoskeleton targeted by pathogens. Trends Microbiol 13(4):181-189.
Silva, N. C., F. F. Guimaraes, P. M. M. de, E. Gomez-Sanz, P. Gomez, J. P. Araujo-Junior, H. Langoni, V. L. Rall, and C. Torres. 2014. Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. Antonie Van Leeuwenhoek 106(2):227-233.
Simoons-Smit, A., P. Savelkoul, J. Stoof, T. Starink, and C. Vandenbroucke-Grauls. 2000. Transmission of Staphylococcus aureus between humans and domestic animals in a household. European Journal of Clinical Microbiology and Infectious Diseases 19(2):150-152.



المنارة للاستشارات
www.manaraa.com

Sinha, B., P. P. Francois, O. Nusse, M. Foti, O. M. Hartford, P. Vaudaux, T. J. Foster, D. P. Lew, M. Herrmann, and K. H. Krause. 1999. Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol 1(2):101-117.
Sischo, W. M., L. E. Heider, G. Y. Miller, and D. A. Moore. 1993. Prevalence of contagious pathogens of bovine mastitis and use of
mastitis control practices. Journal of the American Veterinary Medical Association 202:595-600.
Songer, J. G. and K. W. Post. 2004. Veterinary Microbiology-E-Book: Bacterial and Fungal Agents of Animal Disease. Elsevier Health Sciences.
Speziale, P., G. Pietrocola, S. Rindi, M. Provenzano, G. Provenza, A. Di Poto, L. Visai, and C. R. Arciola. 2009. Structural and functional role of Staphylococcus aureus surface components recognizing adhesive matrix molecules of the host. Future Microbiol 4(10):1337-1352.
Srinivasan, V., A. A. Sawant, B. E. Gillespie, S. J. Headrick, L. Ceasaris, and S. P. Oliver. 2006. Prevalence of enterotoxin and toxic shock syndrome toxin genes in Staphylococcus aureus isolated from milk of cows with mastitis. Foodbourne Pathogens & Disease 3(3):274-283.
Stephan, R., C. Annemüller, A. Hassan, and C. Lämmler. 2001. Characterization of enterotoxigenic Staphylococcus aureus strains isolated from bovine mastitis in north-east Switzerland. Veterinary microbiology 78(4):373-382.
Stewart, P. S. and J. W. Costerton. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358(9276):135-138.
Sutra, L. and B. Poutrel. 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus. J Med Microbiol 40.
Szweda, P., M. SCHIELMAnn, S. Milewski, A. FRAnKOWSKA, and A. JAKuBCZAK. 2012. Biofilm production and presence of ica and bap genes in Staphylococcus aureus strains isolated from cows with mastitis in the eastern Poland. Pol J Microbiol 61(1):65-69.
Takahashi, T., I. Satoh, and N. Kikuchi. 1999. Phylogenetic relationships of 38 taxa of the genus Staphylococcus based on 16S rRNA gene sequence analysis. Int J Syst Bacteriol 49 Pt 2:725-728.
Todd, J., M. Fishaut, F. Kapral, and T. Welch. 1978. Toxic-shock syndrome associated with phage-group-I Staphylococci. The Lancet 312(8100):1116-1118.
Türkyılmaz, S., S. Tekbıyık, E. Oryasin, and B. Bozdogan. 2010. Molecular Epidemiology and Antimicrobial Resistance Mechanisms of Methicillin-Resistant Staphylococcus aureus Isolated from Bovine Milk. Zoonoses and Public Health 57(3):197-203.
Valle, J., C. Latasa, C. Gil, A. Toledo-Arana, C. Solano, J. R. Penadés, and I. Lasa. 2012. Bap, a biofilm matrix protein of Staphylococcus aureus prevents cellular internalization through binding to GP96 host receptor. PLoS pathogens 8(8):e1002843.



المنارة للاستشارات
www.manaraa.com

van Leeuwen, W. B., D. C. Melles, A. Alaidan, M. Al-Ahdal, H. A. Boelens, S. V. Snijders, H. Wertheim, E. van Duijkeren, J. K. Peeters, and P. J. van der Spek. 2005. Host-and tissue-specific pathogenic traits of Staphylococcus aureus. Journal of bacteriology 187(13):4584-4591.

Vasudevan, P., M. K. M. Nair, T. Annamalai, and K. S. Venkitanarayanan. 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of Staphylococcus aureus for biofilm formation. Veterinary microbiology 92(1):179-185.

Wadstrom, T. 1974. Biological properties of extracellular proteins from staphylococcus. Ann N Y Acad Sci 236(0):343-361.

Wang, J. H., K. Zhang, N. Wang, X. M. Qiu, Y. B. Wang, and P. He. 2013. Involvement of phosphatidylinositol 3-Kinase/Akt signaling pathway in beta1 integrin-mediated internalization of Staphylococcus aureus by alveolar epithelial cells. J Microbiol 51(5):644-650.

Wellnitz, O., U. Berger, W. Schaeren, and R. Bruckmaier. 2012. Mastitis severity induced by two Streptococcus uberis strains is reflected by the mammary immune response in vitro. Schweiz Arch Tierheilkd 154(8):317-323.

Zadoks, R., W. Van Leeuwen, D. Kreft, L. Fox, H. Barkema, Y. Schukken, and A. Van Belkum. 2002. Comparison of Staphylococcus aureus isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. Journal of clinical microbiology 40(11):3894-3902.

Zecconi, A. 2010. Staphylococcus aureus mastitis: what we need to know to con. Israel Journal of Veterinary Medicine 65:93–99.

Zecconi, A., L. Cesaris, E. Liandris, V. Dapra, and R. Piccinini. 2006. Role of several Staphylococcus aureus virulence factors on the inflammatory response in bovine mammary gland. Microbial pathogenesis 40(4):177-183.

Zecconi, A. and F. Scali. 2013. Staphylococcus aureus virulence factors in evasion from innate immune defenses in human and animal diseases. Immunol Lett 150(1-2):12-22.



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# APPENDIX



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Table 1. Genes, primer sequences and PCR product size of enterotoxins evaluated by PCR.

| Gene | Toxin | direction | primers 5' to 3' | bp size |
|---|---|---|---|---|
| **seA** | seA | FWD | TTGCAGGGAACAGCTTTAGG | 251 |
| | seA | REV | GGTGTACCACCCGCACATTG | |
| **seB** | seB | FWD | GCAGAAAGCCAACCAGATCC | 617 |
| | seB | REV | CCTGGTGCAGGCATCATGTC | |
| **seC** | seC | FWD | CACCCAACGTATTAGCAGAG | 631 |
| | seC | REV | CCTGGTGCAGGCATCATATC | |
| **seE** | seE | FWD | GCCCTAACGTTGACAACAAGTCC | 572 |
| | seE | REV | ACCTTACCGCCAAAGCTGTCTGAG | |
| **seJ** | seJ | FWD | CTCCCTGACGTTAACACTAC | 462 |
| | seJ | REV | AGCGGAACAACAGTTCTGATGC | |
| **tsst1** | tsst1 | FWD | CGTAAGCCCTTTGTTGCTTG | 222 |
| | tsst1 | REV | ATAAGGCTGATGCTGCCATCTG | |

seA=Staphylococcal enterotoxin A; seB= Staphylococcal enterotoxin B; seC= Staphylococcal enterotoxin C; seE= Staphylococcal enterotoxin E; seJ = Staphylococcal enterotoxin J; and tsst1= Toxic shock syndrome toxin 1.



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Table 2. Distribution of PFGE types of *Staphylococcus aureus* in different farms.

| PFGE Types | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | Unknown Farm | Total isolate | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | - | - | - | - | - | - | - | 1 | - | - | - | - | 1 | 0.90 |
| B | - | - | 1 | - | - | - | - | - | - | - | - | 3 | 4 | 3.60 |
| C | - | - | 1 | - | - | - | - | - | - | - | 1 | - | 2 | 1.80 |
| D | - | - | - | 2 | - | - | - | - | - | - | - | - | 2 | 1.80 |
| E | - | - | - | - | 1 | - | - | 1 | - | - | - | 2 | 4 | 3.60 |
| F | - | - | 1 | - | - | - | - | - | - | - | - | - | 1 | 0.90 |
| G | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | 0.90 |
| H | - | - | 1 | - | - | - | - | - | - | - | - | - | 1 | 0.90 |
| I | - | - | - | - | 3 | 1 | 1 | - | 1 | - | 3 | - | 9 | 8.11 |
| J | 2 | - | - | - | 1 | - | - | - | - | - | - | - | 3 | 2.70 |
| K | - | - | - | - | - | - | - | - | - | - | - | 4 | 4 | 3.60 |
| L | - | - | 2 | - | - | - | - | - | - | - | - | - | 2 | 1.80 |
| M | 2 | 5 | 14 | - | 7 | 13 | - | 1 | 8 | 2 | 8 | 4 | 64 | 57.66 |
| N | - | - | - | - | - | - | - | - | 1 | - | - | - | 1 | 0.90 |
| O | - | - | 2 | - | 1 | 1 | - | - | 3 | 1 | 2 | 1 | 11 | 9.91 |
| P | 1 | - | - | - | - | - | - | - | - | - | - | - | 1 | 0.90 |
| **Total isolate** | **5** | **5** | **22** | **2** | **13** | **15** | **1** | **3** | **13** | **3** | **15** | **14** | **111** | **-** |
| **%** | 4.5 | 4.5 | 19.8 | 1.8 | 11.7 | 13.5 | 0.9 | 2.7 | 11.7 | 2.7 | 13.5 | 12.6 | **-** | **-** |

Farms are labeled 1-11 and unknown. PFGE types are labeled A-P.



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Table 3. Enterotoxin gene patterns in different PFGE type of *Staphylococcus aureus*.

| PFGE Types | b | bc | c | e,t | t | none | Total | % |
|---|---|---|---|---|---|---|---|---|
| A | - | - | - | - | - | 1 | 1 | 0.90 |
| B | - | - | - | - | - | 4 | 4 | 3.60 |
| C | 1 | - | - | - | - | 1 | 2 | 1.80 |
| D | - | - | - | - | - | 2 | 2 | 1.80 |
| E | - | - | - | - | 3 | 1 | 4 | 3.60 |
| F | - | - | - | - | - | 1 | 1 | 0.90 |
| G | - | - | - | - | - | 1 | 1 | 0.90 |
| H | - | - | - | - | - | 1 | 1 | 0.90 |
| I | 2 | 1 | - | - | - | 6 | 9 | 8.11 |
| J | - | - | - | - | - | 3 | 3 | 2.70 |
| K | - | - | - | 1 | 2 | 1 | 4 | 3.60 |
| L | - | - | 1 | - | - | 1 | 2 | 1.80 |
| M | 9 | - | 1 | - | 3 | 51 | 64 | 57.66 |
| N | - | 1 | - | - | - | - | 1 | 0.90 |
| O | 1 | - | 1 | - | - | 9 | 11 | 9.91 |
| P | - | - | - | - | - | 1 | 1 | 0.90 |
| **Total** | **13** | **2** | **3** | **1** | **8** | **84** | **111** | |
| **%** | 11.7 | 1.8 | 2.7 | 0.9 | 7.2 | 75.7 | | |

PFGE types are labeled A-P. b = *seb*, c=*sec*, e=*see* and t = *tsst-1*, bc =seb-sec, et=see-tsst-1.



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Table 4. Enterotoxin gene patterns in *Staphylococcus aureus* in different farms.

| Farms | b | bc | c | e,t | t | none | Total | % |
|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | - | 5 | 5 | 4.50 |
| 2 | 2 | - | - | - | - | 3 | 5 | 4.50 |
| 3 | 3 | - | 1 | - | - | 18 | 22 | 19.82 |
| 4 | - | - | - | - | - | 2 | 2 | 1.80 |
| 5 | 3 | - | - | - | - | 10 | 13 | 11.71 |
| 6 | 2 | - | - | - | - | 13 | 15 | 13.51 |
| 7 | 1 | - | - | - | - | - | 1 | 0.90 |
| 8 | - | - | - | - | 1 | 2 | 3 | 2.70 |
| 9 | 1 | 2 | 2 | - | - | 8 | 13 | 11.71 |
| 10 | 1 | - | - | - | - | 2 | 3 | 2.70 |
| 11 | - | - | - | - | - | 15 | 15 | 13.51 |
| Unknown | - | - | - | 1 | 7 | 6 | 14 | 12.61 |
| **Total isolates** | **13** | **2** | **3** | **1** | **8** | **84** | **111** | |
| **%** | 11.7 | 1.8 | 2.7 | 0.9 | 7.2 | 75.7 | **-** | |

Farms are labeled 1-11 and unknown. b = *seb*, c=*sec*, e=*see*, and t = *tsst-1*, bc =seb-sec, et=see-tsst-1.



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Table 5. The prevalence of slime production, hemolysin, enterotoxin gene and antimicrobial susceptibility/resistance by the three most widespread PFGE types.

| Virulence factors | Variables | PFGE I | % | PFGE M | % | PFGE O | % |
|---|---|---|---|---|---|---|---|
| Slime dose (n = 79) | low | 1 | 11.11 | 29 | 49.15 | 5 | 45.45 |
| | moderate | 5 | 55.56 | 15 | 25.42 | 5 | 45.45 |
| | severe | 3 | 33.33 | 15 | 25.42 | 1 | 9.09 |
| | ***Total*** | ***n=9*** | | ***n=59*** | | ***n=11*** | |
| Hemolysis (n =77) | alpha | 4 | 44.44 | 11 | 19.3 | 3 | 27.27 |
| | beta | 4 | 44.44 | 36 | 63.16 | 6 | 54.55 |
| | double | 1 | 11.11 | 10 | 17.54 | 2 | 18.18 |
| | ***Total*** | ***n=9*** | | ***n=57*** | | ***n=11*** | |
| AMR (n =80) | Susceptible | 4 | 44.44 | 41 | 68.33 | 10 | 90.91 |
| | Resistant | 5 | 55.56 | 19 | 31.67 | 1 | 9.09 |
| | ***Total*** | ***n=9*** | | ***n=60*** | | ***n=11*** | |
| Enterotoxin (n =84) | Present | 3 | 33.33 | 13 | 20.31 | 2 | 18.18 |
| | Absent | 6 | 66.66 | 51 | 79.69 | 9 | 81.81 |
| | ***Total*** | ***n=9*** | | ***n=64*** | | ***n=11*** | |

AMR= antimicrobial resistance.



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Table 6. *In vitro* adhesion and invasion of mammary epithelial cells by dominant PFGE types.

| PFGE Type | Number of *S. aureus* Inoculated | Adhesion (number of *S. aureus* attached) | Invasion (Number of *S. aureus* internalized) |
|---|---|---|---|
| I | $1.645x10^{+11}$ | $7.51x10^{+08}$ | $4.75x10^{+06}$ |
| M | $2.98333x10^{+11}$ | $7.33x10^{+08}$ | $5.51x10^{+06}$ |
| O | $1.44833x10^{+11}$ | $7.24x10^{+09}$ | $1.12x10^{+07}$ |
| p-value | | 0.0938 | 0.3365 |



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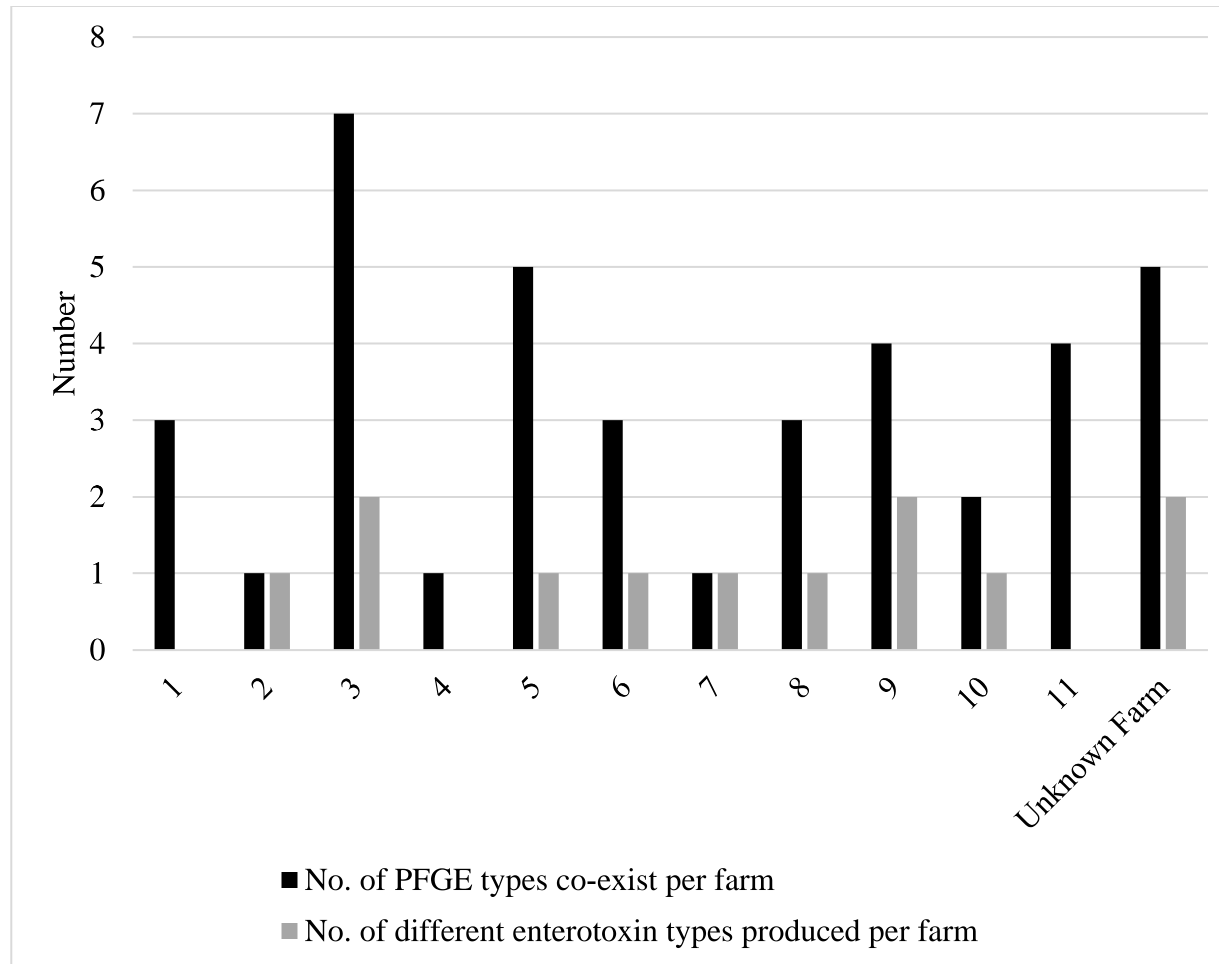


Figure 1. Number of PFGE types and enterotoxin types present per each farm.



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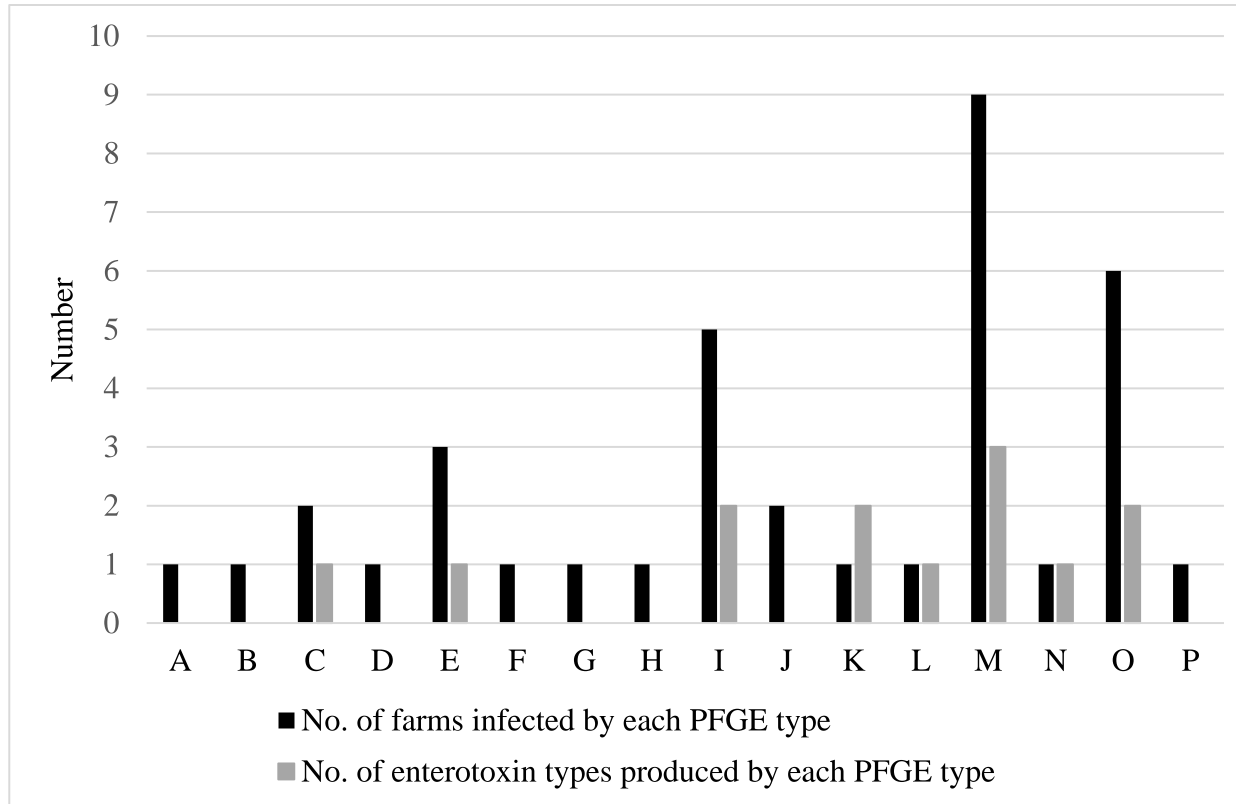


Figure 2. Number of positive farms, and enterotoxin types present in each PFGE types.

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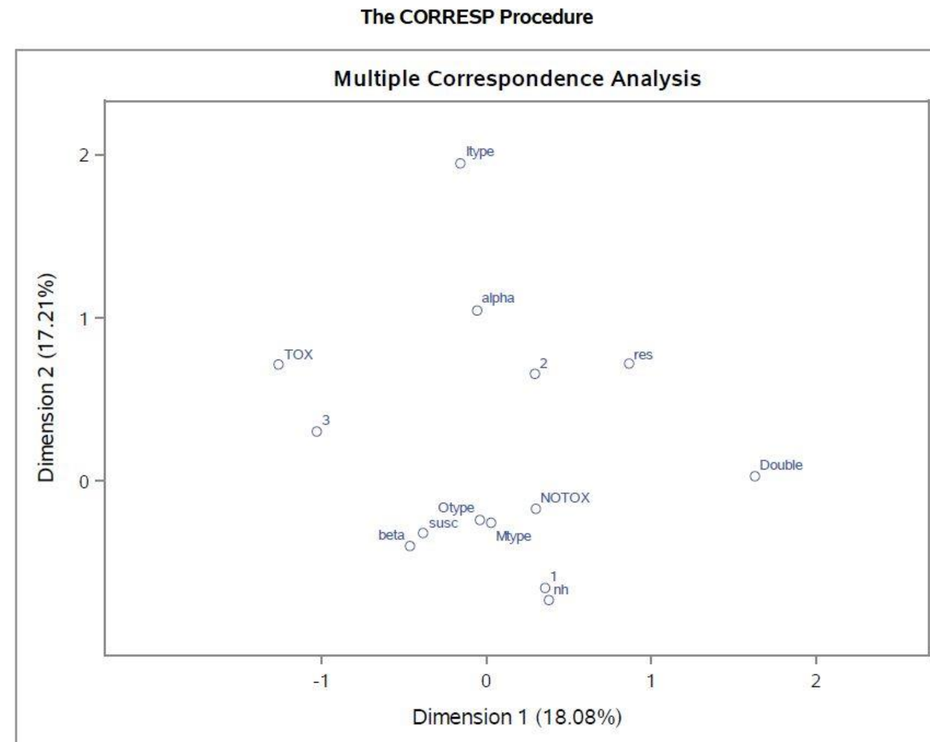


Figure 3. Multiple correspondence analysis.
The first two dimensions of this space are plotted to examine the associations among the categories. TOx= Positive for toxin gene, NOTOX= not positive for toxin gene, alpha= positive for alpha hemolysin, beta= positive for beta-hemolysin, double = produce both beta and alpha hemolysins, nh= negative for hemolysins, res= resistant to at least to one antimicrobial of 10 tested, susc= Susceptible to all 10 antimicrobials tested, 1= weak (low) slime producer, 2= medium (moderate) slime producer, 3 = strong (severe) slime producer .



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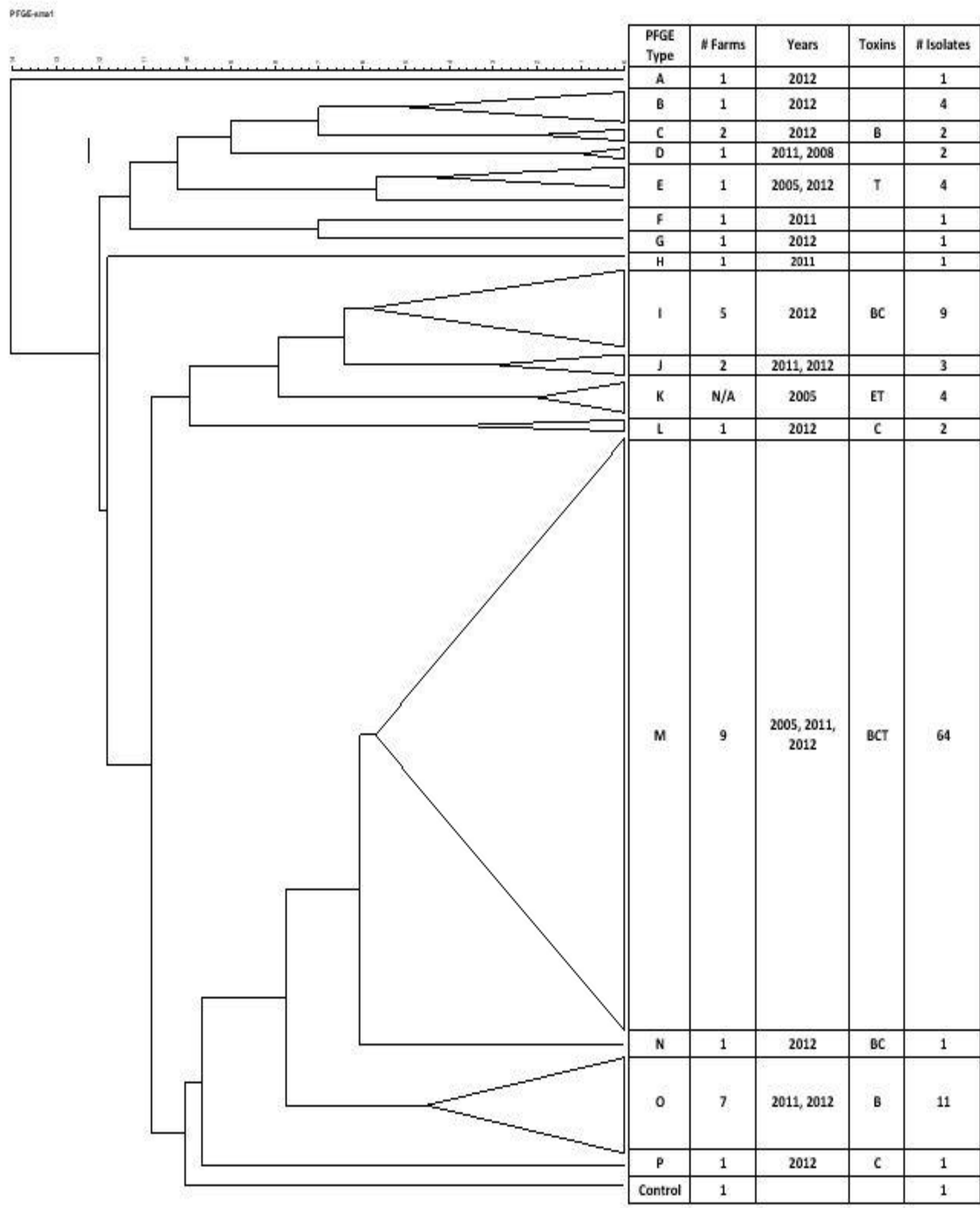

| PFGE Type | # Farms | Years | Toxins | # Isolates |
|---|---|---|---|---|
| A | 1 | 2012 | | 1 |
| B | 1 | 2012 | | 4 |
| C | 2 | 2012 | B | 2 |
| D | 1 | 2011, 2008 | | 2 |
| E | 1 | 2005, 2012 | T | 4 |
| F | 1 | 2011 | | 1 |
| G | 1 | 2012 | | 1 |
| H | 1 | 2011 | | 1 |
| I | 5 | 2012 | BC | 9 |
| J | 2 | 2011, 2012 | | 3 |
| K | N/A | 2005 | ET | 4 |
| L | 1 | 2012 | C | 2 |
| M | 9 | 2005, 2011, 2012 | BCT | 64 |
| N | 1 | 2012 | BC | 1 |
| O | 7 | 2011, 2012 | B | 11 |
| P | 1 | 2012 | C | 1 |
| Control | 1 | | | 1 |

Figure 4. Dendrogram of PFGE types with year, type of toxin, and number of isolates.



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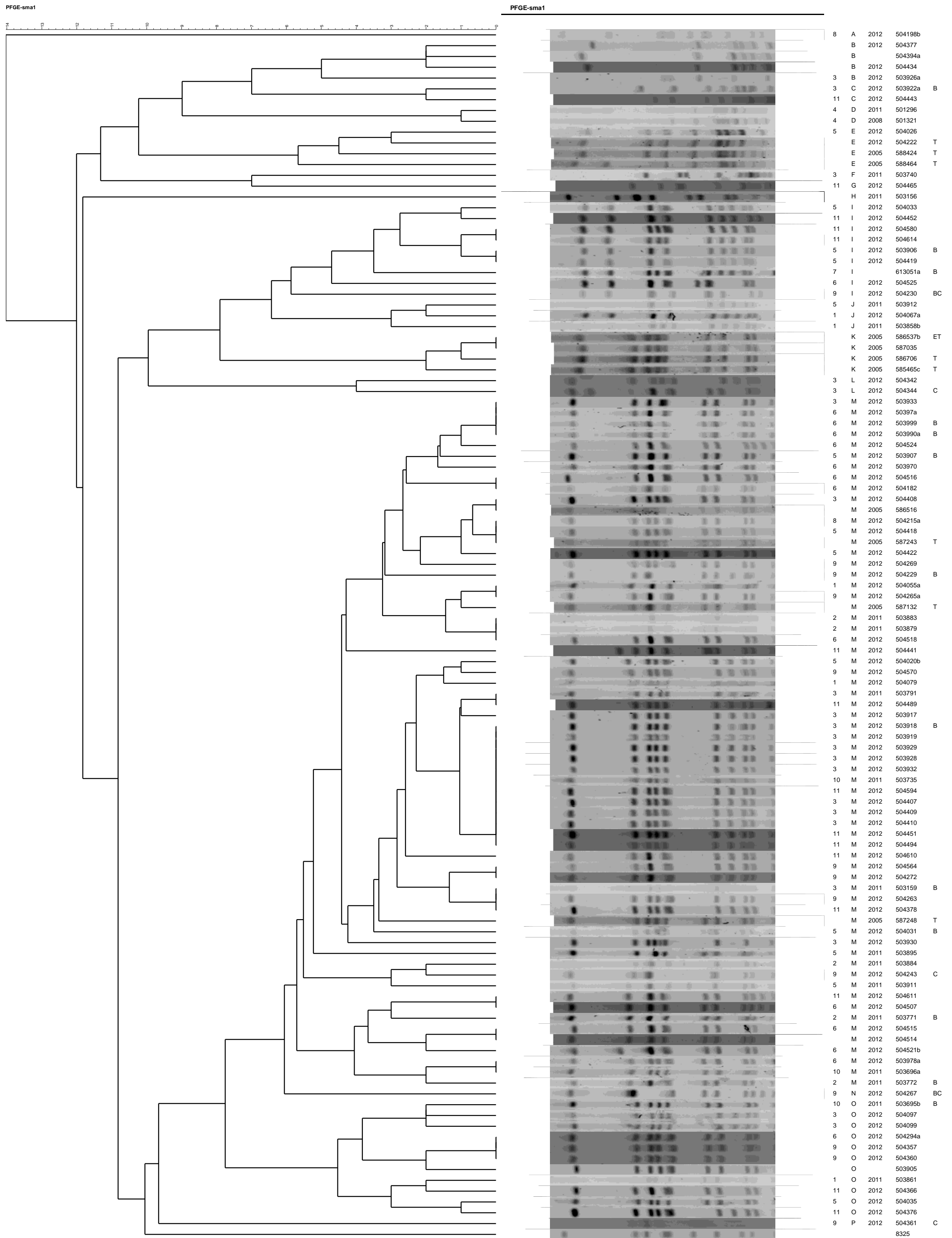


Figure 5. Dendrogram of PFGE types.



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**VITA**

Jacqueline Vaughn was born in Morristown, TN. Her parents are Daphne Rogers and Timothy Vaughn. All of her life, Jacque loved animals, but it wasn't until her mom married her step father Ronnie Rogers, a dairy farmer, that she fell in love with cows and other livestock. She became very involved in FFA throughout high school competing with many judging teams and then decided to pursue a bachelor's degree in agriculture focusing in animal science at the University of Tennessee at Martin in Martin, TN. While earning her Bachelors' degree Jacque continued gaining experience with livestock through collegiate livestock judging and working on the university's farm. Because she loved learning about animals so much, Jacque went on to pursue her Masters' degree in animal science at the University of Tennessee in Knoxville, TN studying dairy health and mastitis. Jacque plans to graduate in the summer of 2018 with a Master of Science degree in Animal Science.



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