Epidemiology, Genetic and Molecular Characterization of *Staphylococcus aureus* in Ohio Dairy Farms

_Dissertation_

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By

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ABSTRACT

Recognized as a significant cause of intramammary infections, *Staphylococcus aureus* (*Staph. aureus*) is the most common contagious pathogen affecting cows worldwide. Although practices to control this organism have been advocated for decades, identification of risk factors in herds is crucial in prevention and control of *Staph. aureus*. The objectives for **Chapter 2 and Chapter 3** were to estimate prevalence of *Staph. aureus* in Ohio dairies and to determine the association of herd characteristics and management practices with isolation of *Staph. aureus* in bulk tank milk (BTM). A questionnaire about herd characteristics, milking procedures, udder health and mastitis control, biosecurity and calf/heifer raising practices were mailed to 780 dairy producers, with a response rate of 49.2%. *Staph. aureus* prevalence was 48%, 64% and 69% when considering one two or three samples of BTM, respectively. Practices such as pre-stripping, pre- and post-dipping and use of single towels per cow as part of the milking routine were associated with significantly reduced detection of *Staph. aureus*. Dry-off practices such as abrupt cessation of milking, use of internal teat sealant or blanket dry treatment were associated with herd size. In addition to the presence of *Staph. aureus* in the infected udder and milk, the cow can also be colonized with the organism on different body sites. The objective of the **Chapter 4** was to assess
the role of teat skin colonization by *Staph. aureus* in *Staph. aureus* intramammary infections (IMI) by evaluating genetic relatedness of *Staph. aureus* isolates from milk and teat skin of dairy cows using pulsed-field gel electrophoresis (PFGE) and characterizing the isolates based on the carriage of virulence genes. Cows in four known *Staph. aureus* positive herds were sampled and *Staph. aureus* was detected from 20 cows and 43 quarters. Quarters with teat skin colonized with *Staph. aureus* were almost five times more likely to be diagnosed with *Staph. aureus* IMI than quarters not colonized on teat skin. Three main clusters (A, B, C) were identified with PFGE using a cutoff at 80% similarity. All clusters contained both milk and teat skin isolates with majority of isolates (72%) belonging to cluster B. Forty-two virulence factors were screened using PCR and presence of *clfA, clfB* genes may have contributed to the ability of certain isolates to become the predominant strain. The aim of the study in **Chapter 5** was to estimate antimicrobial resistance in *Staph. aureus* and to determine presence of methicillin-resistant *Staphy. aureus* (MRSA) in Ohio dairy BTM. *Staph. aureus* isolates for this study were those previously described in Chapters 2 and 3. Isolates were tested for antimicrobial resistance by Kirby-Bauer disc diffusion and MRSA confirmation was done by a duplex PCR using *femB* and *mecA* genes. *MecA* was detected in two *Staph. aureus* isolates from a single farm (herd prevalence = 0.95%), collected at different time points. Both isolates appeared highly clonal and belonged to SCC type IV, spa type t021, USA200. These results confirm that MRSA is present in BTM in US dairies
although in very low prevalence. Findings from this study show that *Staph. aureus* IMI is a multifactorial disease. Characteristics related to cow (e.g., colonization on teat skin), or practices performed at farm (pre-strip, pre-and post-dip) should be considered to control *Staph. aureus* IMI.
To my friends,

"Not to hurt our humble brethren [the animals] is our first duty to them, but to stop there is not enough. We have a higher mission: to be of service to them whenever they require it."

Saint Francis of Assisi

"The greatness of a nation and its moral progress can be judged by the way its animals are treated."

Mahatma Gandhi
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Fields of Study

Major Field: Comparative Veterinary Medicine
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Chapter 1

Introduction

1.1 Etiology and historical perspective of mastitis

Mastitis is defined as an inflammation of the mammary gland. In dairy cattle, it can be caused by a broad number of organisms comprising bacteria (Gram positive and negative), mycoplasma or mycotic organism that can opportunistically invade tissue and cause infection. However, the vast majority of mastitis in cows is of bacterial origin. Bacteria responsible for producing mastitis can be categorized based on the primary reservoir into two major groups: environmental and contagious (Blowey and Edmondson, 2010).

Environmental pathogens are best described as opportunistic invaders of the mammary gland. Relevant characteristics of this group are that bacteria typically have a tendency to be less adapted to udder, often produce mild to moderate immune response, but are also capable of causing severe mastitis. Control of environmental infection can be challenging because the causal bacteria can be found in bedding materials, moisture, soil, manure. Common pathogens of this group include Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* and Gram-positive bacteria as Coagulase negative *staphylococci* (CNS), *Streptococcus uberis* and *Streptococcus dysgalactiae*. 
Contrary to environmental organisms, contagious pathogens are considered organisms well adapted to survive within the host, in particular within the mammary gland. *Staphylococcus aureus*, an important pathogen, known in most dairy farms, is an example of a contagious pathogen.

*Staphylococcus aureus* is a Gram-positive coccus, facultative anaerobic, non-motile, non-sporulating, catalase-positive, and coagulase positive; and it is considered the most common contagious organism in most dairy farms. The primary method of spread of this pathogen is usually at milking time, from cow to cow; with the infected animal within a herd acting as a constant source of the organism. For the reason that the primary reservoir of contagious organism is the infected animal, the focus on control of the disease should be not just within but also between herds.

In the 1960s, numerous studies were published trying to understand the epidemiology of *Staphylococcus aureus* mastitis on dairy farms and to establish a control plan for contagious agents (Neave et al., 1966; Dodd et al., 1969; Neave et al., 1969). Founded on the results from these reports, a control plan entitled “Five-point mastitis control program”; was developed in the United Kingdom and later on, it was extended to a “ten point mastitis control plan”. This plan recommended: 1) establishment of goals for udder health, 2) maintenance of a clean, dry, comfortable environment; 3) proper milking procedures; 4) proper maintenance and use of milking equipment; 5) good record keeping; 6) appropriate management of clinical mastitis during lactation; 7) effective dry cow
management; 8) maintenance of biosecurity for contagious pathogens and marketing of chronically infected cows; 9) regular monitoring of udder health status and 10) periodic review of mastitis control program (www.nmconline.org/docs/NMCchecklistNA.pdf).

The impact of implementation of the practices included in this plan was a reduction in the prevalence of contagious agents such as *Staph. aureus* and practically the disappearance of *Streptococcus agalactiae*. Years have passed and even with the constant recommendation and implementation of these procedures, *Staph. aureus* remains a common cause of intramammary infection (IMI).

### 1.2. Managing mastitis in cows

One important step in managing mastitis is to monitor the somatic cell count values at both the herd and cow level on a regular basis. Evaluation of monthly SCC patterns for the herd can be helpful for troubleshooting subclinical mastitis problems. It is generally accepted that SCC from uninfected quarters is less than 200,000 cells/mL (Reneau, 1986; Torres et al., 2008). Large number of cows with chronically elevated SCC in a herd could be indicative of presence of pathogens that are transmitted in a contagious manner.

Due to the continued exposure of teat ends to milk containing mastitis causing organisms, the prevalence of subclinical mastitis usually increases as lactation progresses. In cases when contagious mastitis is suspected, consistent
use of good milking practices, regular observation of milking process with subsequent improvement as needed are essential for successful control of mastitis and prevention of transmission of pathogens via milking equipment. Studies have found correlations between cow cleanliness and milk quality (Schreiner and Ruegg, 2003; Reneau et al., 2005).

The premilking routines can vary greatly from farm to farm and even variations between individuals within a herd are common. Many factors (such as use of pre-dipping, examination of foremilk, preparation of teats, and use of post-dipping) are relevant and should be routinely assessed in a successful control plan for contagious pathogens. The use of pre-milking teat-dipping with a disinfectant is the most effective method to clean teats, provided that disinfectant is applied onto teats clean of dirt, it is in contact with teat skin for a sufficient time to adequately kill bacteria, is properly formulated and is stored in clean containers (Galton et al., 1984; Galton et al., 1986a; Elmoslemany et al., 2010).

The benefit of examining foremilk before milking units are attached is identification of intramammary infections at early stage where the only sign may be the presence of slightly abnormal milk. Disinfecting the teats by dipping is a well-established and recognized practice in mastitis prevention plan in US dairies and studies have reported reduction in the incidence of clinical mastitis when appropriate teat disinfection is performed (Pankey and Drechsler, 1993; Dufour et al., 2012). Also drying of teats is an important step in pre-milking preparation. It has been demonstrated that drying teats using a towel was able to reduce
bacterial counts on teat ends almost 3-fold (from 35,000–40,000 colony-forming unit (cfu/mL) for teats cleaned but not dried to 11,000-14,000 cfu/mL for teats cleaned and dried (Galton et al., 1986b). The use of a single towel to dry udders on more than one cow is not recommended; Rodrigues et al., (2005) reported that clinical mastitis rate in herds using one towel per cow was 7.8% compared to 12.3% in herds that used a same towel on more than one cow.

Manual application of disinfectant as a dip provides more consistent teat coverage than the use of spray and is therefore recommended (Keefe, 2012). Teats should be fully covered to provide adequate disinfection and teat skin conditioning to all parts of the teat that are in contact with the milking unit. This is particularly important with pathogens that colonize teat skin like *Streptococcus agalactiae* and *Staphylococcus aureus* (Keefe, 2012).

### 1.3. Impact of stage of lactation on mastitis occurrence

Regarding the entire production cycle, the dry period has been recognized as a high risk period for acquisition of new intramammary infections, with over 60% of new environmental IMIs occurring at this time (Cousins et al., 1980; Smith et al., 1985 Bradley and Green, 2004). The dry period consists of three phases during which susceptibility to infection varies: the early dry period (involution or first 2 weeks after dry-off), steady stage and the late (colostrogenesis or 2 weeks before calving). During the first and third phase cows are highly susceptible to bacterial infections (Oliver and Sordillo, 1988;
Smith, 1995; Bradley and Green, 2004). Furthermore, cases of clinical mastitis in the early lactation are often associated with IMI acquired during previous dry-off (McDonald and Anderson, 1981; Bradley and Green, 2000).

The most commonly employed method of drying off cows in the US is abrupt cessation of milking at the end of lactation. Especially in high-producing dairy cows this may cause significant pressure in the mammary gland and on the teat canals, causing milk to leak and teat canals to stay open longer (Williamson et al., 1995; Dingwell et al., 2004; Odensten et al., 2007).

Schukken et al. (1993) reported that cows with milk leakage after dry-off (as a result of incomplete teat canal closure) were 4-fold more likely to develop clinical mastitis than cows that did not leak. Teat end condition (Neijenhuis et al., 2000), delay in teat canal closure (Dingwell et al., 2002) or increased milk yield at dry-off (Rajala-Schultz et al., 2005) have been found to have a significant relationship with IMI at calving. The closure of the streak canal by a keratin plug is one of most important factors during the early dry period that influence the susceptibility of the gland to IMI (Bradley and Green, 2004). The plug works not only as a physical barrier against outside pathogens but it also inhibits bacterial growth due to its composition (Comalli et al., 1984; Hogan et al., 1987).

1.4. Prevalence and impact of mastitis

Mastitis is one of the most important and costly disease affecting dairy farms. A USDA NAHMS Dairy survey (2008) estimated *Staph.aureus* herd
prevalence to be 43% using a single bulk tank milk sample per herd and Olde Riekerink et al. (2010) reported *Staph. aureus* herd prevalence of 74% from Canada. The cow prevalence has been reported to be 6.4% in Switzerland (Moret-Stalder et al., 2009), 22% in Norway (Østerås et al., 2006) and ranging from over 3 to 15% in Dutch dairy farms (Sampimon et al., 2009).

Controlling mastitis is important for the US dairy industry because the condition has significant economic implications. It is difficult to estimate the losses associated with clinical mastitis, which arise from the costs of treatment, decreased milk production and milk quality, and it has also been cited as the most common cause of death in adult dairy cows (Esslemont and Kossaibati, 1997).

Mastitis presents itself in variety of forms, ranging from mild with no visible changes in milk or mammary gland tissue to a clinical onset where signs of inflammation such as swelling, redness, and pain are present. Usually *Staph. aureus* infection is sub-clinical which make the quantification of losses more difficult to estimate. Infection caused by *Staph. aureus* is a complex disease representing a challenge to producers, veterinarians and researchers.

Recently, concern for animal welfare and comfort has been expressed in the press uncountable times. Mastitis can be a painful condition (Medrano-Galarza et al., 2012), and while severe cases of mastitis (presenting septicemia or necrosis of mammary tissue) are less frequent in well managed herds, negative effects of mastitis may have welfare consequences (Leslie and
Petersson-Wolfe, 2012). Animals experiencing pain associated with intramammary infection often reduce feed intake and consequently have decreased productivity. Pain may also affect immune system, increasing animal susceptibility to other infections (e.g. reproductive) and pose a risk for the animal to be prematurely culled from the herd.

1.5. Antibiotic use and risks to human health

It is well known that antibiotic residues in milk intended for human consumption are not allowed. Initially, the focus of public health representatives was to protect hypersensitive individuals from developing an allergic response after been exposed to milk containing drug residues; however, in the last years concern has shifted to the potential development and/or transmission of resistant bacteria in milk (Mitchell et al., 1998). The potential significance of bovine mastitis indirectly impacting public health should not be disregarded. In modern dairy cattle operations, most antimicrobials are administered for therapeutic purposes. Mastitis is one of the most common disease of adult dairy cows and accounts for most antibiotic usage in dairy herds (Pol and Ruegg, 2007; Saini et al., 2012). The use of antibiotics and possible implications for human health, through an increased risk of bacteria resistant to antibiotics reaching the food chain, or presence of antibiotic residues in milk has been a topic of discussion (National Institute for Animal Agriculture, 2011). Also potential dissemination of
zoonotic organisms via milk through consumption of raw milk or unpasteurized dairy products is a concern.

National Animal Health Monitoring System (USDA, 2008a) Dairy study about use of antimicrobials on U.S. dairy farms revealed that β-lactam antibiotics (primarily penicillin and cephalosporins) are the most commonly used antimicrobials on dairy farms and that drug classes important for human treatment (such as fluorquinolones) have no approved usages in adult dairy cows and are rarely used on dairy farms. The FDA is responsible for attesting that the industry is following the regulations and takes regulatory action if needed, while the dairy industry endures the responsibility for the safety of milk and milk products. Pasteurized Milk Ordinance (legal rules that define standards pertinent to production, transportation and processing of Grade “A” milk) requires that every tanker of milk must be screened for β-lactam residues prior to unloading. Individual bulk milk samples from every farm shall include at least four samples collected in at least four separate months, during any consecutive six months. During fiscal year 2012 (October 1, 2012 to September 30, 2013), 3,761,500 samples (defined as a load or lot of milk sampled), were analyzed for animal drug residues. Of these 731 samples (0.02%) were positive for a drug residue (National Institute for Animal Agriculture NIAA, 2011).

The most cited reasons for antibiotic residues in milk in the farm are the use of intramammary antibiotics and mistakes regarding withholding periods of milk (McEwen et al., 1991; Wilson et al., 1998). Herds with higher proportion of
infected cows (higher bulk tank SCC) show greater risk for the occurrence of residues (Ruegg and Tabone, 2000).

On dairy farms the majority of antimicrobial usage is related with treatment and prevention of infectious diseases. Data from USDA (2008) shows that approximately 3% of adult dairy cows were treated for respiratory problems, 7% for foot infections, 7% for reproductive disorders and 16% for mastitis. Practically all conventional dairy farms report some consistent usage of antibiotics (Oliveira et al., 2012). However, mastitis pathogens in general do not appear to be becoming more resistant to commonly used antimicrobials (Erskine et al., 2002; Makovec and Ruegg, 2003).

1.6. Methicillin resistant *Staphylococcus aureus* (MRSA)

Despite the fact that much research and effort has been dedicated to mastitis control, *Staph. aureus* has many characteristics that have helped its remarkable success to persist over the years. Among these are its virulence, genetic diversity and ability of acquiring new exogenous genes allowing it to adapt to a variety of environmental conditions (Moellering, 2012). More than fifty years had passed since Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described, only 2 years after the initial clinical use of methicillin (Jevons 1961; Barber 1961). These penicillin-resistant strains produce β-lactamase, an enzyme that hydrolyses the β-lactam ring in the β-lactam class of antibiotics, thus inactivating the antibiotic. In the late 1940’s and throughout the 1950’s, *Staph.*
*Staphylococcus aureus* developed resistance to penicillin. Methicillin, a form of penicillin, was introduced to counter the increasing problem of penicillin-resistant *Staph. aureus*. However, the desired relief was short when British scientists identified the first strains of *Staph. aureus* bacteria that were resistant to methicillin (MRSA) (Jevons, 1961; Barber, 1961).

Methicillin resistance is conferred by the acquisition of *mecA* gene which encodes for a production of a unique penicillin-binding protein (PBP2a or PBP2’) that has reduced affinity for the whole group of β-lactams, providing *Staph. aureus* to be resistant to this significant class of antimicrobial. Methicillin resistance in *Staph. aureus* has emerged as a major public health problem, the resistant organisms first described in health care-associated infections (HA-MRSA) later it expanded to community-acquired infections (CA-MRSA) posing a challenge for infectious disease medicine (Holmes and Zadoks, 2011).

During the 1970s, the first report of MRSA infection in animals was described in Belgium in 1972 from a cow with mastitis (Devriese et al., 1972). For the next 3 decades, no reports of *mecA* positive *Staph. aureus* in dairy cattle were published and limited information was available on the prevalence of MRSA in dairy herds.

Recently, an increased number of reports have been published on outbreaks in companion and livestock animals, as well as in humans who are in close contact with these (Catry et al., 2010; Spoor et al., 2013). Farmers, veterinarians and people who work with animals may be at risk of being exposed
to MRSA as has been shown in multiple reports (van Loo et al., 2007; Wulf et al., 2008; Denis et al., 2009; Garcia-Graells et al., 2012). There is an increased risk for colonization and infection with MRSA strain in people with close contact with dairy farm animals (Spohr et al., 2011).

1.7. Understanding the epidemiology of Staphylococcus aureus

As stated above Staph. aureus is a major pathogen that causes a wide variety of diseases not only in animals but also in humans. This variety is related to a number of virulence factors that allow the organism to adhere to surface, invade or avoid the immune system, causing harmful effects to the host (Bien et al., 2011). In cattle, Staph. aureus is able to produce large array of virulence factors that are supposed to contribute to the manifestation and severity of staphylococcal infections or pathogenesis of mastitis (Sutra and Poutrel, 1994).

As a contagious pathogen, Staph. aureus dissemination is typically associated with milking practices or defective equipment in the milking parlor. However, heifers that have never been milked can be positive for Staph. aureus (De Vliegher et al., 2012) demonstrating that possible others sources are involved in transmission of this pathogen. Studying differences in Staph. aureus strains Sommerhäuser et al. (2003) noted that some strains behave more as environmental strains with little or no tendency of spread from quarter to quarter while other strains differed in their ability of infect udder quarters.
An association between genotype and severity of intramammary infection was noted when *Staph. aureus* isolates from cows with IMI were analyzed using pulsed-field gel electrophoresis (PFGE) to study genetic relationship between severity, persistence of infection and bacterial genotype (Haveri et al., 2005). Zadoks et al. (2002) concluded after using different molecular techniques that some strains of *Staph. aureus* are found on the skin but most *Staph. aureus* mastitis cases are caused by strains highly adapted to the mammary gland and different from skin isolates. However, another study indicated that most *Staph. aureus* isolates from teat skin and teat canal were genetically indistinguishable from those isolated from infected mammary glands (Haveri et al., 2008). Thus, results from published literature regarding the role of teat skin as a source of *Staph. aureus* intramammary infections are conflicting as the involvement of teat skin in *Staph. aureus* epidemiology is poorly understood.

Despite all advances in management, and therapeutics, mastitis caused by *Staph. aureus* is still an important disease with significant economic losses to dairy industry in general and it remains a major problem and challenge in US dairy farms thus providing the theme for this dissertation.

The objectives of this study were:

Chapter 2 - to estimate the prevalence of *Staph. aureus* in Ohio dairy bulk tank milk samples, to determine herd characteristics and management practices
associated with isolation of Staph. aureus, and to describe educational needs of dairy producers in Ohio.

Chapter 3 - to survey dairy herds regarding their dry-off and raising practices and assess their association with the likelihood of a herd being positive for Staph. aureus in dairy bulk tank milk.

Chapter 4 - to compare genetic relatedness of Staph. aureus isolates from milk and teat skin using pulsed-field gel electrophoresis (PFGE) and to characterize isolates based on carriage of virulence factors;

Chapter 5 - to estimate antimicrobial (specifically methicillin) resistance levels among Staph. aureus isolated from bulk tank milk samples in Ohio.

1.8. References


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www.nmconline.org/docs/NMChecklistNA.pdf

Chapter 2

Management practices associated with presence of *Staphylococcus aureus* in bulk tank milk from Ohio dairy herds

2.1. Introduction

*Staphylococcus aureus* (*Staph. aureus*) is the most common contagious pathogen affecting dairy cows in North America with herd prevalence estimated between 43% and 74% (USDA, 2008; Olde Riekerink et al., 2010). Importance of management practices to control mastitis is unquestionable and for a successful control plan, a fundamental first step is knowledge about the organism's presence and prevalence in a herd as well as understanding of the prevailing risk factors.

*Staphylococcus aureus* resides primarily in the udder (Roberson, 1999); hence its presence in bulk tank milk (BTM) when sampled correctly provides evidence about infection status of all cows that were milked prior to sample collection (Jayarao et al., 2004). Even though culture of quarter-milk samples from all milking cows provides a more accurate estimation of within-herd prevalence, this method has the disadvantage of being costly and laborious. Cited by various studies as a useful tool for evaluating milk quality, and monitoring udder-health status, herd-level prevalence of *Staph. aureus* has been
estimated by culturing BTM samples (Farnsworth, 1993; Jayarao and Wolfgang, 2003; Jayarao et al., 2004). In a Canadian study in the 1990’s, 58 out of 59 farms had at least one BTM sample positive for Staph. aureus (Kelton et al., 1999) and more recently prevalence of Staph. aureus was estimated at 74% in herds where at least one of three BTM samples cultured was Staph. aureus positive (Olde Riekerink et al., 2010).

Although Staph. aureus can be isolated from environmental sources, e.g. bedding contaminated by milk leakage from infected cows (Capurro et al., 2010), the major mode of transmission for Staph. aureus is from cow-to-cow via fomites, such as milking unit liners, at the time of milking (Zadoks et al., 2002). Proper milking procedures, improved milking hygiene, milking system maintenance, employee training and also fly control have been cited as components for prevention of new mastitis cases (Rodrigues et al, 2005; Anderson et al., 2012, Keefe, 2012).

Considerable changes in housing and management of cows have occurred in the past decades in the US dairy industry, impacting prevalence and incidence of mastitis in the herds. Milk production per cow has nearly doubled, and the average herd size has increased six-fold from approximately 19 cows to 120 cows in the US from 1970 to early 2000’s (ERS, 2000). Dairy industry in Ohio has undergone similar changes and data available from Ohio herds regarding management practices or prevalence of contagious mastitis organisms may no longer be current (Bartlett et al., 1992; Khaitsa et al., 2000).
The objectives of the present study were: (1) to estimate the prevalence of Staph. aureus in Ohio dairy bulk tank milk samples, (2) to determine herd characteristics and management practices associated with presence of Staph. aureus in BTM, and (3) to describe educational needs of dairy producers in Ohio. We hypothesized that likelihood of being positive for Staph. aureus is associated with determined management practices.

2.2. Materials and Methods

2.2.1. Target population and questionnaire instrument

A questionnaire was developed to survey dairy producers regarding herd characteristics and management practices as well as to inquire educational needs and interests of dairy producers in Ohio.

According to Ohio Department of Agriculture there are 2,810 dairy farms in Ohio. In the current study, questionnaires were mailed with a paid return envelope to 780 randomly selected dairy producers located across the state of Ohio. This was done in collaboration with their milk testing laboratory which is responsible for raw milk testing for 95% of Grade A and Grade B milk producers in the state. Information about general management practices in dairy farms was collected through an 8-page survey containing 42 questions about herd characteristics, milking procedures, mastitis control and biosecurity practices (Table 2.1). Additionally, producers’ interest level on 21 topics, ranging from manure nutrient management to dairy facilities, cow comfort and record keeping...
systems, was assayed with responses categorized as high, moderate, little and no interest to producers. In the questionnaire permission to test their BTM was also requested.

Questionnaire followed Dillman’s survey method with some adjustments (Dillman, 2000). Briefly, an introductory letter was sent to producers a week prior to the actual questionnaire, describing the goals of the study. Before mailing to dairy producers, the questionnaire was pilot-tested with a group of dairy veterinarians, dairy producers, and faculty and graduate students with dairy interest. It was designed with closed or semi-closed questions. Two reminders, a month apart, were sent via mail to those producers who had not yet responded. In addition to the answers provided by the producers through the questionnaire, their milk testing laboratory provided to the researchers data on somatic cell count (SCC) on the herds from previous 12 months. The identity of producers was unknown to the investigators throughout the entire study.

2.2.2. Sample collection and laboratory processing

Bulk tank milk samples were collected by the milk testing laboratory personnel at three different time points in a monthly interval. In the first sampling 226 BTM were collected, the second time 260 BTM samples and the third time 281 samples were received. Despite of the initial intention of sampling and culturing three BTM samples from each herd, due to logistic challenges, a full set of three samples was not received from all herds.
Milk samples were frozen at arrival and kept at -20°C until further processing. One hundred microliters of milk was plated on Baird Parker media (Remel™ Inc., Lenexa, KS). The plates were incubated at 37°C, examined for growth at 24 and 48 hours and suspect colonies (black colonies with or without halo) were further subcultured on blood agar plates. Identification of Staph. aureus was based on colony morphology, Gram stain, positive catalase test, and a positive tube coagulase rabbit plasma test read at 4 and 24 hours. All isolates identified as Staph. aureus were stored in milk/glycerol solution at -80°C.

2.2.3. Statistical analysis

Descriptive statistics on herd characteristics and different management practices implemented in the herds were summarized and compiled using PROC FREQ procedure of SAS (SAS Institute Inc. Cary, NC). A herd was considered positive for Staph. aureus when at least one BTM sample was positive on culture. Prevalence was calculated two ways: First, using data from all herds, regardless of how many samples were cultured from each herd. Second, prevalence was also calculated by only using data from herds that provided three BTM samples for culture. Initial screening to identify management practices associated with presence of Staph. aureus in BTM samples was performed using chi-square test (or Fisher’s exact test, as needed). This was done using data from all herds. All variables associated with Staph. aureus in the initial screening with P-value <0.25 were included in a full model. Multivariate analysis was run using PROC GLIMMIX of SAS, with Staph. aureus status of the herd (positive –
yes/no) as the outcome. Non-significant variables were eliminated from the full model one at a time starting with the least significant until all variables remaining in the model had $P$-value $<0.1$. The fit of the model was assessed using Hosmer-Lemeshow goodness-of-fit test.

2.3. Results

2.3.1. Prevalence of Staphylococcus aureus

A total of 384 surveys were returned, resulting in a response rate of 49.2%. However, four questionnaires could not be included in the analysis (one producer had exited the dairy business and three questionnaires were returned empty) giving a total of 380 responses. Some questions in the survey were not answered by all responders, and for those questions the denominator will be provided when responses (as a percentage) to these questions are shown. Thus, unless the denominator is mentioned, 380 was the number used to calculate the proportions of those responding producers.

Of the 380 herds included in the analysis, 307 provided milk samples for culture; however, herds contributed a different number of BTM samples: 39 (13%) herds provided only one sample, 78 (25%) herds provided two samples and 190 (62%) herds provided three BTM samples, resulting in 765 BTM cultured. In total, 73 of the responding herds did not agree to have their BTM sampled.
If all herds were included in the analysis, regardless of the number of samples collected and if considering only the first BTM sample per herd, *Staph. aureus* prevalence was 48% (146/307). If the first two samples were interpreted in parallel, 198 of the 307 herds were found to be positive (64% prevalence). If all three samples were considered and if any one of them was positive, a total of 213 herds (69%) was found positive for *Staph. aureus*.

If only including herds with all three samples (n=190) in the analysis and considering only the first sample from each herd, *Staph. aureus* prevalence was 47% (90/190). If the first two samples were considered and interpreted in parallel, 122 herds were positive (122/190=64%). When all three samples were considered and interpreted in parallel, 137 out of 190 (72%) were positive. In other words, 28% (53 of these 190 herds) were negative in all three samplings.

### 2.3.2. Herd Characteristics

Of the responding producers, 28% had less than 50 cows, 61% had between 50-199 animals, and the remaining 11% had 200 or more cows (with 4% of herds with more than 500 cows). Majority of producers (80%) reported milking Holstein cows, 9% Jerseys and remaining producers had other or cross breeds in their herds (Table 2.2). A small percentage (4%) of the responders indicated to operate an organic dairy farm while 96% had a conventional farm. Thirty-eight percent reported not being part of Dairy Herd Improvement Association (DHIA). The smallest herds were least likely to belong to DHIA.
(P=0.01): 48% of herds with less than 50 cows, 36% of herds with 50-199 cows, 28% of herds with 200-499 cows and 27% with 500 or more cows reported not being part of DHIA and testing on a regular basis.

Most farms (91%) reported milking cows twice per day, the remaining 9% milked more frequently than twice a day. Herringbone was the predominant parlor type, used in 52% of the herds. Milking cows were housed in tie stall barns in 10% of the responding herds (all herds with tie stalls had less than 200 cows: 75% of them had less than 50 cows and 25% 50-199 cows). In 75% of the responding herds, milking cows were housed in free stall barns. In the remaining 15% of the herds, other housing arrangements (e.g., loose housing/manure pack, combination of different facility solutions) were available for milking cows. Dry cows were kept in loose housing/manure pack in 55% of the herds, in 5% of the herds cows stayed in the tie stalls through the dry period and in the rest of the herds dry cows were in free stalls (Table 2.2).

Straw was used as the only type of bedding in 40% of herds, while sand was used in 16% of the herds. Sawdust only was used in 9% of the responding herds. In the remaining 35% of the herds, combinations of these bedding materials or other types of bedding were used. In 67% of the herds producers let cows go to pasture when it was available (46% reported to let all cows to pasture, 15% only dry cows and 6% only milking cows). In 18% of the herds cows had access to an outside exercise lot. In 15% of the herds, cows had no access neither to pasture nor exercise lot at any time (Table 2.2). Detection of
*Staph. aureus* was more common among herds that provided cows access to pasture or exercise lot (71% positive for *Staph. aureus*) compared to herds that did not allow access to outside (60% positive for *Staph. aureus*), however, the difference was not statistically significant ($P=0.11$).

In 44% of the responding herds, average milk production was reported being between 15,001 to 20,000 lbs per year followed by 43% which produce between 20,001 to 30,000 lbs per year. In 11% of the responding herds, herd average production was less than 15,000 lbs and in 2% production was over 30,001 lbs.

None of the above mentioned herd characteristics were significantly associated ($P>0.05$) with the presence of *Staph. aureus* in BTM.

### 2.3.3. Udder health, milking procedures and biosecurity practices

Adoption of the recommended milking/mastitis-management procedures can be summarized as follows: 57% of the herds reported checking foremilk (pre-strip), 82% used pre-milking teat disinfection, 97% used post-milking teat disinfection, 92% used single towels per cow (either paper or washable cloth towels) and 38% segregated known infected cows or milked those cows last. A reduced percentage of the 380 herds, though, performed all these procedures at the same time: for instance, 49% of the herds practiced pre-strip, pre-dip, post-dip and used individual towels for each cow. Only 16% reported milking cows
known to be *Staph. aureus* positive last in addition of doing pre-strip, pre-dip, post-dip and using single/washable towels.

Owners were responsible for milking cows in 64% of the farms (69% of these herds were positive for *Staph. aureus*), while the remaining 36% of herds had hired employees to assist with milking (68% positive for *Staph. aureus*). In only ten percent of the farms, hired workers were reported to be solely responsible for milking of cows (80% positive for *Staph. aureus*). Detection of *Staph. aureus* in BTM comparing herds where only hired employees were responsible for milking with herds where milking was done with participation of owners was not significant ($P=0.14$).

Based on producer reporting, 62% of the responding herds had average SCC during the three months prior to answering the survey between 150,001 and 300,000 cells/mL. This agreed well with the actual average SCC from the previous year provided by the milk testing laboratory: Ten percent of the herds had average SCC less than 150,000 cells/mL and 51% of herds had average SCC between 150,001 and 300,000 cells/mL (Table 2.2).

Of the 372 responding herds, 37% reported to have never cultured milk from clinical mastitis cases and in 45% of 367 responding herds BTM was not cultured to monitor udder health in the herd. Forty-nine percent (159/327) of the responders did not recall any contagious pathogens to ever been detected in their herds, but of those herds, 64% were found to be positive for *Staph. aureus*. Fifty-one (168/327) percent of responding producers recalled *Staph. aureus*
having been cultured from their cows earlier and 71% of these herds were found to be positive for *Staph. aureus* in the current study. No difference was found between these two groups (*Staph. aureus* having been cultured previously or not \( P=0.21 \)).

Thirty-eight percent (141/372) of the producers considered themselves to have an open herd, and reported to purchase animals from outside, raise heifers outside the home farm and/or take animals to fairs and shows (66% were positive for *Staph. aureus*). The remaining 62% (229/372) considered themselves as closed herds (they raise all replacements at farm and do not buy animals) and 70% of them were positive for *Staph. aureus* (\( P=0.61 \), Table 2.2).

### 2.3.4. Variables associated with *Staph. aureus* in BTM

Probability of finding *Staph. aureus* in BTM was significantly lower (\( P<0.01 \)) in herds where BTM SCC was less than 150,000 cells/mL during the past 12 months than in those herds with BTM SCC higher than 150,000 cells/mL (Table 2.3).

Detection of *Staph. aureus* in herds that reported to practice prestriping, pre- and post- dipping and use of single towels (either paper or washable cloths) was significantly lower (\( P=0.04 \)) when compared to responding herds where all of those practices were not part of the milking routine. The probability of detection of *Staph. aureus* in these herds was 64% versus 74%, respectively (Table 2.3).
Of the open herds bringing in new animals from outside, 80% did not quarantine incoming animals before introducing them to the existing herd, and 72% of those were positive for *Staph. aureus*. Of the herds that did quarantine (20%) newly purchased animals before introducing them to the existing herd, 54% were positive for *Staph aureus* ($P=0.06$; Table 2.3).

Final model with variables associated with detection of *Staph. aureus* is presented in Table 2.4.

2.3.5. Educational needs of dairy producers

Interests and current needs for educational opportunities among Ohio dairy producers are listed in Table 2.5. Those topics were selected by The Ohio State University Veterinary Extension service to assist dairy producers with educational training. Mastitis control and troubleshooting high SCC were rated first in importance with 74% of the responding producers mentioning it as highly important theme followed by nutrition with 54%, foot health/lameness with 53% and poor reproductive performance with 53% of the producers indicating interest in those topics.

On-farm biosecurity protocols were of no or little interest for more than half (55%) of the responders, 28% considered them of moderate importance and remaining 17% as highly important topic.

2.4. Discussion
2.4.1 Prevalence of *Staphylococcus aureus*

The herd prevalence of *Staph. aureus* based on BTM found in the current study (69%) is in agreement with other US and Canadian studies, which have reported *Staph. aureus* prevalence in BTM to range from 43% to 74%, respectively (USDA, 2008; Olde Riekerink et al., 2010). The USDA NAHMS study used a single BTM while the Canadian study used four samples per herd collected at different time points. If only one BTM was used to estimate herd prevalence in the current study, as in the USDA NAHMS study, the prevalence of *Staph. aureus* was 48%, but when considering all three BTM, when available, cumulative prevalence increased to 69%. A limitation of the current study was that three samples were not obtained from all herds, thus 69% is likely an underestimation of prevalence of *Staph. aureus* in Ohio dairy herds. This is corroborated by the observation in the current study that herd prevalence increased to 72% when a subset of data only from herds with three bulk tank samples was used to calculate the portion of herds positive for *Staph. aureus* in BTM. This agrees with earlier studies reporting that the sensitivity of a single BTM for detection of *Staph. aureus* is low (between 21 and 42%) and probability of detecting *Staph. aureus* from the BTM is likely dependent on within-herd prevalence of *Staph. aureus* IMI (Godkin and Leslie, 1993; Olde Riekerink et al., 2010) with increased sensitivity in high prevalence herds. However, determining the within-herd prevalence involves culturing quarter/composite milk samples from all cows which is associated with higher cost and more labor, contrary to
BTM samples that are readily available and include cows milked in the herd at that sampling time.

2.4.2. Detection of *Staphylococcus aureus* associated with herd characteristics, udder health and milking management practices

*Staph. aureus* is considered a major pathogen mainly because of its impact on milk quality and SCC (Keefe, 2012). Findings for the current study demonstrated that with increasing BTM SCC the odds of detecting *Staph. aureus* in the BTM also increased.

A decreased probability of detecting *Staph. aureus* in BTM was found in herds that reported implementing four of the recommended practices on milking routine (pre-strip, pre- and post-dip and use of a single towel per cow to dry teats). Herds that did not use these four procedures had almost two times higher odds of detecting *Staph. aureus* in their BTM than herds that did. Some of the benefits of these practices are associated with improving milk letdown, assistance in identification of abnormal milk (forestripping), and reduction of the microbial count on teats prior to milking (pre-dipping; Zucali et al. 2011). The use of teat dipping and a single towel per cow can be important as *Staph. aureus* teat colonization has been demonstrated to be an important factor for *Staph. aureus* IMI (da Costa et al., 2014).

Some practices such as milking known infected cows last has proven to reduce the spread of this contagious pathogen and to significantly decrease the
prevalence of *Staph. aureus* mastitis and bulk tank SCC (Wilson et al., 1995; Zecconi et al., 2003). Producers who reported to have cultured *Staph. aureus* in their farm at some point, had higher probability of having *Staph. aureus* detected in BTM (71%) in their herds compared to herds where this organism was not reported (64%). Segregation of infected cows can be logistically demanding in some farms, since creation of such an additional group would require extra space, time, and effort, in addition to the initial cost of diagnosing those infected cows. This could be one reason why only 38% of the producers in this study reported to milk infected animals last.

When asked about topics that producers would like to have more information, mastitis control and troubleshooting high SCC were identified as high priority by almost 60% of responders. Producers appear to be aware of the importance of proper milking practices, as the percentage of herds performing these practices individually was high. However, implementation of these milking procedures together seemed difficult to accomplish. Based on the results presented here, many individual milking procedures were reported to be followed, but maybe not executed appropriately. Despite the apparent simplicity of procedures involved in mastitis prevention, consistency of protocol implementation over time is responsible for herd performance, increased parlor efficiency and reduction on mastitis incidence (Taylor, 2012).

Implementation of recommended management practices depends on the ability of the producer to motivate farm employees (Rodrigues et al., 2005).
Practices used on farms are likely to remain constant over time, contrary to attitudes, motivation and knowledge that have the tendency to progress (Dufour et al., 2012). Detection of *Staph. aureus* in herds where hired employees were the only ones responsible for milking cows was 12% higher (80% versus 68%) when compared to herds where owner was also involved in milking. Limited studies have focused on investigating the relationship between personnel motivation and milk quality, but frequent training of employees has been reported to result in better milking performance and lower incidence of clinical mastitis cases (Rodrigues and Ruegg, 2005; Rodrigues et al., 2005).

Increased detection of *Staph. aureus* was found in herds that provided access to pasture to all cows in the current study, even though this finding was not significant. A study from the southern United States has highlighted the role of flies as a source of *Staph. aureus* IMI when cattle have access to pasture as flies could be involved in the transmission of *Staph. aureus* between infected and uninfected heifers (Anderson et al., 2012). Another report showed that presence of biting flies (*Haematobia irritans*) that cause teat lesions is associated with a high level of *Staph. aureus* IMI, and suggested that fly control should be included in a mastitis management plan (Ryman et al., 2013). Heifers from herds using fly control had lower prevalence of IMI when compared to herds where no fly control was used (Nickerson et al., 1995). While no questions were asked about flies or fly control in the current study, it is possible that the increased detection of *Staph. aureus* in pasturing herds was related to flies.
2.4.3. *Staphylococcus aureus* associated with biosecurity

Although biosecurity practices have been a recommendation for mastitis control (Dinsmore, 2002) their implementation on farms may be challenging. In the current study, open herds that did not quarantine purchased animals were more frequently found to be positive for *Staph. aureus* in BTM than herds where quarantine was applied. As US dairies grow in size with consequent acquisition of replacement animals from outside herds, biosecurity is as important practice to prevent costly outbreaks of contagious mastitis caused by *Staph. aureus* (Dinsmore, 2002). *Staph. aureus* can be introduced by the entry of new animals, but in most cases it is already present in the herd, as shown in this study. An IMI caused by *Staph. aureus* often occurs in a chronic asymptomatic form, and entry and spread of this organism could not be effectively controlled by quarantine alone. Concomitant testing prior to purchase is necessary.

Although biosecurity practices are beneficial to herds in reducing the entry and spread of contagious pathogens (Dinsmore, 2002), responding producers in the current study showed only moderate interest in this theme. Management practices used to prevent the entry of disease such as quarantine was only practiced in 20% of responding open herds, but the proportion of herds positive for *Staph. aureus* was 18% lower in herds that implemented the practice (54%)
than in those that did not (72%). Unfortunately, we did not include in our questionnaire an assessment of whether or not milk culture was part of a quarantine practice for these herds. Given the borderline significance of quarantine in this study, it is possible that the practice of quarantine is a covariate of other more impactful combinations of good herd management.

As cited before, intramammary infections caused by *Staph. aureus* are usually subclinical, and thus, *Staph. aureus* can remain unnoticed by producers. It is known that the movement of animals carrying contagious mastitis organisms such as *Staph. aureus* is a risk factor for spreading this pathogen between herds (Barkema et al., 2006) and the probability of infection is dependent, among other factors, on the number of infected cows within the herd (Dohoo et al., 2003). Thus, caution should be used when introducing purchased animals into the farm or when participating in fairs (possible that cows will be milked in common parlor in fair grounds). Continuous efforts should be made to reduce the spread of *Staph. aureus* and to keep the occurrence of new infections to a low level.

Bulk tank milk samples for the current study were collected from herds located across the state, and considering the characteristics such as herd size, type of housing system, and milk production, these herds were representative of Ohio herds.
2.5. Conclusion

   The prevalence of *Staph. aureus* was 69% in Ohio BTM. Herds with SCC <150,000 cells/mL had significantly decreased probability of having *Staph. aureus* in BTM when compared to herds with greater SCC. Adoption of the commonly recommended milking procedures individually (pre- and post-dipping and use of single towels) was relatively high in the herds responding to this survey and a protective effect was observed on farms where milking routine included pre-stripping, pre-and post-dipping and a use of a single towel per cow. Herds that practice quarantine were associated with lower detection of *Staph. aureus*.

2.6. References


Table 2.1. Summary of selected management practices included in the questionnaire about herd characteristics, udder health and biosecurity submitted to Ohio dairy farms.

<table>
<thead>
<tr>
<th>Herd characteristics</th>
<th>Udder health, mastitis control, milking procedures</th>
<th>Biosecurity practices</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Size</td>
<td>• Person responsible for milking the cows (owner, hired)</td>
<td>• Closed herd (raise heifers on farm and do not buy outside animals) or open herd (purchase animals, take to shows bringing back to original farm)</td>
</tr>
<tr>
<td>• Breed</td>
<td>• Test milk for clinical mastitis or culture BTM</td>
<td>• Practice quarantine test purchased animals before bringing to the farm</td>
</tr>
<tr>
<td>• Average milk production</td>
<td>• Milking routine (Pre-strip, pre/post-dip, single towels, milking infected cows last or separately, knowledge about the presence of contagious pathogens in the herd)</td>
<td>• Test purchased animal for Johne’s disease, contagious pathogens</td>
</tr>
<tr>
<td>• Nature of the herd (conventional, grazing or organic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Average SCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Test milk samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Type of housing for milking and dry cows</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Frequency of milking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Type of parlor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Type bedding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Access to pasture</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Characteristics of Ohio dairy farms provided by responding producers.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Proportion of Farms %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size (n=380)</strong></td>
<td></td>
</tr>
<tr>
<td>Less than 49 cows</td>
<td>28</td>
</tr>
<tr>
<td>Between 50-199 cows</td>
<td>61</td>
</tr>
<tr>
<td>Between 200-499 cows</td>
<td>7</td>
</tr>
<tr>
<td>More than 500 cows</td>
<td>4</td>
</tr>
<tr>
<td><strong>Housing for Milking Cows (n=380)</strong></td>
<td></td>
</tr>
<tr>
<td>Free stall</td>
<td>75</td>
</tr>
<tr>
<td>Tie stall</td>
<td>10</td>
</tr>
<tr>
<td>Loose housing/manure pack</td>
<td>15</td>
</tr>
<tr>
<td><strong>Housing for Dry Cows (n=380)</strong></td>
<td></td>
</tr>
<tr>
<td>Tie stall</td>
<td>5</td>
</tr>
<tr>
<td>Free stall</td>
<td>40</td>
</tr>
<tr>
<td>Loose housing/manure pack</td>
<td>55</td>
</tr>
<tr>
<td><strong>Past 12-month Somatic Cell Count (cells/mL n=380)</strong></td>
<td></td>
</tr>
<tr>
<td>Less than 150,000</td>
<td>10</td>
</tr>
<tr>
<td>150,001- 300,000</td>
<td>51</td>
</tr>
<tr>
<td>300,001- 500,000</td>
<td>37</td>
</tr>
<tr>
<td>Over than 500,000</td>
<td>2</td>
</tr>
<tr>
<td><strong>Outside access (n=380)</strong></td>
<td></td>
</tr>
<tr>
<td>All cows on pasture</td>
<td>46</td>
</tr>
<tr>
<td>Only dry cows on pasture</td>
<td>15</td>
</tr>
<tr>
<td>Only milking cows on pasture</td>
<td>6</td>
</tr>
<tr>
<td>Access to outside exercise lot</td>
<td>18</td>
</tr>
<tr>
<td>No access to pasture/exercise lot</td>
<td>15</td>
</tr>
<tr>
<td><strong>Culture clinical cases (n=372)</strong></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>37</td>
</tr>
<tr>
<td>Occasionally</td>
<td>51</td>
</tr>
<tr>
<td>From all clinical cases</td>
<td>6</td>
</tr>
<tr>
<td>Only from chronic cases</td>
<td>6</td>
</tr>
<tr>
<td><strong>Culture bulk tank milk (n=367)</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>45</td>
</tr>
<tr>
<td>Occasionally</td>
<td>17</td>
</tr>
<tr>
<td>Regularly (Monthly)</td>
<td>38</td>
</tr>
<tr>
<td><strong>Open or closed herd (n=378)</strong></td>
<td></td>
</tr>
<tr>
<td>Closed</td>
<td>62</td>
</tr>
<tr>
<td>Open (purchase outside animals)</td>
<td>21</td>
</tr>
<tr>
<td>Open (participate in shows/fairs)</td>
<td>17</td>
</tr>
</tbody>
</table>
Table 2.3. Proportions of Ohio dairy farms implementing certain management practices and their association with being positive for *Staph. aureus*. Practices with *P*-value less than 0.25 based on Chi-square or Fisher’s exact test are listed in the table.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Proportion of Farms (%)</th>
<th><em>Staph.aureus %</em></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Somatic cell count &lt;150,000 cells/mL (n=380)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>No</td>
<td>90</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td><strong>Milking routine procedures (n=380)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Practice pre-strip</td>
<td>57</td>
<td>66</td>
<td>44</td>
</tr>
<tr>
<td>No pre-strip</td>
<td>43</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>Use pre-dip</td>
<td>82</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>No pre-dip</td>
<td>18</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Use pre-strip, pre- and post-dip do not use</td>
<td>53</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>Use pre-strip, pre-, post- dip and single towel do not use</td>
<td>47</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td><strong>Responsible for milking (n=380)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only hired employees</td>
<td>10</td>
<td>80</td>
<td>19</td>
</tr>
<tr>
<td>Owner and hired</td>
<td>90</td>
<td>68</td>
<td>32</td>
</tr>
<tr>
<td><strong>Practice Quarantine (n=141)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>No</td>
<td>80</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td><strong>Have had Staph. aureus cultured before (n=254)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>51</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>No</td>
<td>49</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td><strong>Access to pasture and/or exercise lot (n=380)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outside access</td>
<td>85</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>No outside access</td>
<td>15</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td><strong>Bedding (n=380)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>32</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>No sand</td>
<td>68</td>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>

*p*-values refer to association of practice and being positive for *Staph.aureus*.
Table 2.4. Final logistic regression model with presence of *Staph. aureus* in BTM sample as the outcome. A total of 307 Ohio dairy herds provided BTM samples for detection of *Staph. aureus* and answered survey questions related to their management practices.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>Pr value</th>
<th>OR</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;300,000 cells/mL</td>
<td>1.52</td>
<td>0.43</td>
<td>&lt;0.001</td>
<td>4.61</td>
<td>1.9-10.8</td>
</tr>
<tr>
<td>150-300,000 cells/mL</td>
<td>1.38</td>
<td>0.42</td>
<td>0.001</td>
<td>3.98</td>
<td>1.7-9.1</td>
</tr>
<tr>
<td>&lt;150,000 cells/mL</td>
<td>ref</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Milking procedures</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do not practice</td>
<td>0.66</td>
<td>0.26</td>
<td>0.01</td>
<td>1.98</td>
<td>1.1-3.2</td>
</tr>
<tr>
<td>Practice</td>
<td>ref</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Responsible for milking</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hired employee only</td>
<td>0.84</td>
<td>0.49</td>
<td>0.08</td>
<td>2.31</td>
<td>0.8-6.1</td>
</tr>
<tr>
<td>Owner and hired</td>
<td>ref</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup>Milking procedures consist of pre-strip, pre- and post-dip and use of single towel implemented concomitantly.
Table 2.5. Summary of responses (n) according to the level of importance (%) of educational needs of dairy producers in Ohio.

<table>
<thead>
<tr>
<th>Topics</th>
<th>Importance (%)</th>
<th>Number of responders (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Mastitis control and SCC</td>
<td>74</td>
<td>22</td>
</tr>
<tr>
<td>Troubleshooting nutrition problems</td>
<td>54</td>
<td>37</td>
</tr>
<tr>
<td>Troubleshooting poor reproduction</td>
<td>53</td>
<td>36</td>
</tr>
<tr>
<td>Foot health care and lameness</td>
<td>53</td>
<td>34</td>
</tr>
<tr>
<td>Effective reproductive strategies</td>
<td>51</td>
<td>37</td>
</tr>
<tr>
<td>Herd health and vaccination</td>
<td>48</td>
<td>39</td>
</tr>
<tr>
<td>Facilities and cow comfort</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>ID animals of medical attention</td>
<td>42</td>
<td>33</td>
</tr>
<tr>
<td>Transition cow management</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>Best management and welfare</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Management of calves and heifers</td>
<td>37</td>
<td>49</td>
</tr>
<tr>
<td>Economics of dairy business</td>
<td>36</td>
<td>43</td>
</tr>
<tr>
<td>Dry-off therapy</td>
<td>33</td>
<td>49</td>
</tr>
<tr>
<td>Calving management</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>Record-keeping</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>Economics of alternative feeds</td>
<td>26</td>
<td>53</td>
</tr>
<tr>
<td>Manure nutrient management</td>
<td>29</td>
<td>47</td>
</tr>
<tr>
<td>On-farm biosecurity protocols</td>
<td>18</td>
<td>46</td>
</tr>
<tr>
<td>Buying sound animals</td>
<td>17</td>
<td>28</td>
</tr>
<tr>
<td>Grazing and economics</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>Management of organic production</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
Chapter 3
Dry-off Practices and Prevalence of *Staphylococcus aureus* in Ohio Dairy Farms

3.1. Introduction

The importance of the dry period (DP) has long been recognized in the production cycle of a dairy cow for the acquisition and elimination of intramammary infections (IMI; Bradley and Green, 2000). Cows are highly susceptible to bacterial infections immediately after cessation of milking (involution or first 2 weeks after dry-off) as well as just before calving (colostrogenesis or 2 weeks before calving) (Smith et al., 1985; Oliver and Sordillo, 1988; Smith, 1995; Bradley and Green, 2004).

The rate of new IMI is higher during DP when compared to rates during the lactation (Cousins et al., 1980; Smith et al., 1985). Several factors have been cited to contribute to this increased susceptibility: teat end condition (Neijenhuis et al., 2000), delay in teat canal closure (Dingwell et al., 2002) and increased milk yield at dry-off (Rajala-Schultz et al., 2005) were found to have a significant relationship with IMI at calving. Furthermore, cases of clinical mastitis in the early lactation are often associated with IMI acquired during the dry period (McDonald and Anderson, 1981; Bradley and Green, 2000).
Use of abrupt cessation of milking at the end of lactation has been reported since 1933 (Wayne and Macy, 1933) and currently it is still the most commonly employed method of drying off cows in the US (USDA, 2008a). In high-producing dairy cows this may cause significant pressure in the mammary gland and on the teat canals, causing milk to leak and teat canals to stay open longer, allowing bacteria to enter into the gland and consequently to cause an IMI (Williamson et al., 1995; Dingwell et al., 2004; Odensten et al., 2007).

With the objectives of eliminating existing infections and prevention of new infections, blanket dry cow therapy (DCT), treatment of all quarters of all cows with antimicrobial drugs at dry-off, has been recommended as part of mastitis control programs in many countries. In fact, DCT has played an important role in reduction of contagious pathogens lowering of somatic cell count (SCC) in bulk tank (Bradley, 2002; Ruegg, 2012). However, it has been questioned whether the use of blanket DCT is still necessary in all farms or should its use be dependent of characteristics of each farm, such as presence of major mastitis pathogens or high bulk tank milk SCC (Rajala-Schultz et al., 2011; Cameron et al., 2014).

Additionally to the negative impact of *Staph. aureus* infection on adult cows, heifers can also be detected with *Staph aureus* IMI already before and at their first calving (Fox, 2009; De Vliegher et al., 2012). Although it is poorly understood how heifers that have never been exposed to milking process become infected with *Staph. aureus*, some recommendations to control heifer
mastitis include separating preweaned calves to prevent suckling, fly control, and segregation of pregnant heifers from dry cows. Recently researchers concluded that strains isolated from colostrum of heifers and mastitic milk of lactating cows (from the same herd) present similar phenotypic and genotypic characteristics, suggesting transmission between heifers and mature cows (Stalder et al., 2014).

Analysis of bulk tank milk (BTM) has been demonstrated to be a valuable tool to identify mastitis pathogens, evaluate milk quality and monitor udder-health status in a herd (Farnsworth, 1993; Jayarao and Wolfgang, 2003; Jayarao et al., 2004). Identification of management practices that enhance udder health can have an impact on profitability of dairy farms. The objective of this study was to survey dairy herds regarding their dry-off and heifer raising practices and assess their association with the likelihood of a herd being positive for Staph. aureus in dairy BTM. The hypothesis was that presence of Staph. aureus in dairy farms was associated with some of these practices.

3.2. Materials and Methods

3.2.1. Target population and questionnaire instrument

A questionnaire following Dillman’s survey method (Dillman, 2000) was sent to dairy producers to obtain information regarding dry-off management and calf/heifer raising practices in Ohio dairies. These questions were part of an 8-page survey about general management practices about udder health, milking procedures, herd characteristics and biosecurity (da Costa et al., 2014 unpublished).
An introductory letter was sent to producers a week prior to the actual questionnaire, describing the goals of the study. Designed with closed or semi-closed questions, the questionnaire was pilot-tested by a group of individuals with dairy interest and expertise before mailing to dairy producers. Upon testing, the questionnaire was revised as necessary and final version was mailed with a paid return envelope to 780 randomly selected dairy producers located across the state of Ohio. This was done in collaboration with their milk testing laboratory which is responsible for raw milk testing for 95% of Grade A and Grade B milk producers in the state. Two postcard reminders, a month apart, were sent to producers that had not responded previously. A permission to test their BTM was requested in the questionnaire. During the entire study, identity of the producers was unknown to the investigators.

3.2.2. Sample collection and laboratory processing

Bulk tank milk samples were collected at three different time points in a monthly interval from 2011. During the first sampling 226 BTM were collected, during the second 260 BTM and during the third sampling 281 BTM samples were received.

Samples were collected by milk testing laboratory personnel and were kept frozen until transported to the Ohio State University Mastitis laboratory, where they were kept frozen at -20°C until further processing.
For culturing, milk was thawed at room temperature and one hundred microliters was plated on Baird Parker media (Remel™ Inc., Lenexa, KS). The plates were examined for growth at 24 and 48 hours after being incubated at 37\(^\circ\)C, and suspect colonies (black colonies in Baird Parker) were further subcultured on blood agar plates. Phenotypic identification of \textit{Staph. aureus} was based on colony morphology, Gram stain, positive catalase test, and a positive tube coagulase rabbit plasma test read at 4 and 24 hours.

\textbf{3.2.3. Statistical analysis}

Survey data was entered into a database and herds were considered positive if \textit{Staph. aureus} was detected from at least one BTM sample obtained from the herd.

Descriptive statistics were compiled using PROC FREQ procedure in SAS 9.2 (SAS Inst. Inc. Cary, NC). Association between variables was tested using chi-square test of independence or Fisher’s exact test when necessary to detect management practices associated with presence of \textit{Staph. aureus} in BTM.

\textbf{3.3. Results}

\textbf{3.3.1. Descriptive dry-off practices and Prevalence of \textit{Staph. aureus}}

With a response rate of 49.2\%, a total of 384 surveys were returned. Four questionnaires were not included in the analysis (one producer had exited the dairy business and three questionnaires were returned empty) given a total of 380 responses. For questions that were not answered by all responders, a denominator will be provided when percentage is shown. Prevalence of \textit{Staph.}
*aureus* in herds participating in the current study was estimated at 48% (146/307) if only the first BTM sample per herd was considered, 64% (198/307) if two samples were interpreted in parallel, and 69% if all three samples were considered and if any one of them was positive (Da Costa et al., 2014, unpublished).

The majority of the responding farms (81%, 303/374) reported to treat all quarters of all cows with intramammary antibiotics at the time of dry-off, 9% (34/374) responded they do not treat any cows at dry-off, and 10% (37/374) of the farms reported to use selective treatment. Internal teat sealant was applied to all cows in 30% (110/366) of the herds, to selected cows in 4% (14/366) and not used at all in 66% (242/366) of the responding herds. In 55% of responders’ herds, dry cows were kept in loose housing/manure pack, 40% in free stall and 5% in tie-stall barns (Table 3.1).

Close to 20% (66/373) of the responding producers consider cows’ milk yield level in their dry-off decision, which is mostly based on expected calving date. Regarding milk production at dry-off and length of the dry period, cows in close to two-thirds of the responding herds produce between 21 to 40 lbs (63%, 234/372) and have dry period between 45 to 60 days (69%, 259/376) Table 3.1.

Abrupt cessation of milking was the most common method to dry cows off (used by 73% (272/372) of the responding producers). In 26% of the herds, producers reduced frequency of milking prior to dry-off and 20% of the responding producers changed ration fed to cows to reduce energy intake. Many
herds used these practices in combination (thus the percentages add up to more than 100%). Small percentage (less than 2%, 6/372) mentioned reducing amount of water being offered to cows. Comparing the practices of abrupt cessation, reduction on frequency of milking and change of ration, detection of Staph. aureus were respectively 62%, 69% and 68%. None of these above practices showed a significant association with detection of Staph. aureus in BTM ($P=0.56$).

In responding herds that reported to use of internal teat sealant (ITS) in all cows, detection of Staph. aureus was 67% compared to 71% when no sealant was used ($P=0.54$). When analyzing herds that use blanket dry cow therapy versus those herds which use selective or no DCT, 70% and 68%, respectively, were positive for Staph. aureus ($P=0.61$).

Larger herds were more likely to treat all cows with antibiotics at dry-off, to use internal teat sealant and to use method of abrupt cessation of milking. The surveyed dry-off practices that were significantly associated with herd size are presented in Table 3.2.

3.3.2. Calf and heifer raising practices

Over 60% (223/369) of the producers reported to move calves immediately after birth from their mothers and not allow them to nurse. In 15% (54/369) of the herds calves could stay with their dams to nurse before being separated and in 25% (92/369) of the herds calves could stay for a day before
being removed. Higher proportion of herds where calves were allowed to nurse was positive for *Staph. aureus* (75%) compared to herds where nursing was not allowed, with 66% of this group being positive for *Staph. aureus* ($P= 0.10$).

In 86% of the responding herds, dam’s fresh colostrum was used as an only source of newborn feed and 13% of the herds reported pooling colostrum from several cows. Only 1% of the herds pasteurized colostrum before feeding to calves.

Milk replacer was the most popular option in calf feeding used by 66% (245/371) of the producers (54% used medicated replacer, 6% non-medicated replacer milk and remaining 6% either medicated or not medicated replacer). Milk from cows with mastitis was fed to calves in 17% (63/371), salable milk was used by 17% of herds and 1% pasteurized milk to fed calves.

Pre-weaned calves were housed individually in 79% of the herds either in pens (32%), in hutches (42%) or in stalls (5%); the remainder of the herds (21%) raised pre-weaned calves in groups. In 95% of the responding herds calves were raised from birth to weaning at the farm. In almost half (49%) of the farms, calves were weaned at eight weeks or older age and then housed in group pens in 90% (333/370) of the herds. More information about calf and heifer raising in the responding herds can be found in Table 3.3.

Seventy percent of producers (257/369) reported that cross-suckling among heifer calves occur on their farms whereas 30% (112/369) reported not to have observed cross-suckling to happen. Herds where producers reported
heifers suckling each other were more likely to be positive for *Staph. aureus* (71%) than herds where no suckling had been noticed (65%), however, the difference was not significant (*P* =0.28). The use of anti-suckling devices was not a common practice; 64% (235/370) never used, 23% (87/370) used occasionally and 13% (48/370) used them routinely. In herds where anti-suckling devices were used, the presence of *Staph. aureus* was higher (82%) compared to herds that do not use it (65%, *P*=0.03, Table 3.4). Calf and heifer raising practices were not found to be associated with herd size.

3.4. Discussion

3.4.1. Dry-off practices in Ohio dairy herds

The objective of this study was to survey dairy herds regarding their dry-off practices and heifer raising practices and assess if these were associated with the likelihood of a herd being positive for *Staph. aureus* in dairy bulk tank milk.

All examined dry-off practices were significantly associated with herd size, with larger herds being more likely to use blanket DCT, ITS and dry cows off abruptly. Higher proportion of small herds (with less than 50 cows) reduced milking frequency as part of their dry-off practices. Possible explanations to this fact could be that it is logistically easier for large herds to implement routine, standard procedures, without e.g., needing to make decisions about selective
treatment. None of the dry-off practices, however, were significantly associated with presence of \textit{Staph. aureus} in the current study.

A study supports the use of intermittent milking when reporting the influence of method of cessation on high producing Jersey cows (Oliver, et al., 1990). In the Oliver et al study (1990), cows were assigned to be dried off by abrupt cessation or intermittent milking once a day for seven days. The authors concluded that intermittent milking followed by dry cow antibiotic therapy had an effect on incidence of mastitis cases and might be the best approach to dry off cows. In agreement with this, Newman et al. (2010) reported that intermittent milking decreased milk yield and cows with lower milk yield at the end of lactation had significantly lower risk of infections at calving. In the current study no difference was found comparing methods of dry-off and being positive for \textit{Staph. aureus} in BTM. This finding, however, does not necessarily reduce the importance of appropriate dry-off practices in lowering the risk of infections or maintaining udder-health status in a herd. Differently from the other studies mentioned above, the current assessment was done at herd level with use of culture of BTM instead of cow level (individual cows or quarters) and is likely a reason why no relationship between diverse dry-off practices and presence of \textit{Staph. aureus} was found.

Gradual reduction of milking frequency in high-producing cows has been shown to result in reduced milk leakage after dry-off (Zobel et al., 2013). Also, research on the length of dry period has revealed that cows with extended dry
period (143 to 250 d) had higher odds to have a subclinical infection during early lactation compared with cows with a dry period of 53 to 76 days (Pinedo et al., 2011). Findings from the current study revealed no statistical difference for presence of *Staph. aureus* in BTM when comparing the milk yield at dry-off and length of the dry period.

Blanket dry-cow therapy or the use of antimicrobials in all cows at dry-off has been recommended for decades and was associated with lower prevalence of *Staph. aureus* in a recent Canadian study (Olde Riekerink et al., 2010). Prophylactic treatment has been advocated to be used in the cow rather than at the quarter level since this not only reduces the number of new infections but also the number of cows with new infections in multiple quarters (Berry et al., 2003; Robert et al., 2006).

However, due to concerns about possible emergence of antibiotic resistant organisms and antimicrobial residues in the food chain, selective use of DCT has received more attention in recent years (Dingwell et al., 2003; Osteras et al., 2006; Torres et al., 2009). Cameron et al. (2014) found that risk of IMI at calving and risk of clinical mastitis in the first 120 days in milk did not differ between groups of low SCC cows either receiving blanket DCT or being treated selectively at dry-off. These authors concluded that using selective DCT in treatment and prevention of IMI over the dry period was as effective as using blanket DCT. Similar results were reported by Rajala-Schultz et al. (2011) with no difference found in milk yield or SCC between blanket and selective DCT. In the
current study, the fact that prevalence of *Staph. aureus* was not statistically different in BTM from herds where producers reported to treat all cows at dry-off compared to those that treat only selected cows support those findings and can lead to more studies to develop a system to guide strategic treatment decisions of cows at dry-off.

3.4.2. Calf and heifer raising practices

Findings from the current study showed that proportion of herds positive for *Staph. aureus* was higher if calves were allowed to nurse than if calves were not allowed to nurse, leading to speculation that involvement of teat skin colonized by *Staph. aureus* can play a role as studies had demonstrated the involvement of teat skin colonized by *Staph. aureus* as a source of *Staph. aureus* IMI (Haveri et al., 2008; Capurro et al., 2010).

Another factor cited in the literature that requires attention is pre-weaned calves suckling/licking each other or themselves. As a result of this practice an increase of *Staph. aureus* colonization on teat skin or transfer of mastitis causing bacteria among the group can increase the probability of IMI (Ruegg, 2011; Petzer et al., 2013). Heifers colonized on oral mucosa were 4.1 times more likely to have a mammary quarter infected with *Staph. aureus* after calving than non-colonized heifers (Rajala-Schultz et al. 2010). In the current study, the proportion of herds that were positive for *Staph. aureus* was higher in herds that had noticed cross-suckling than among herds that had not noticed it, however, it was not
statistically significant. This could be a matter of sample size and the fact that finding *Staph. aureus* in BTM was not fine enough of a measure to detect such difference. However, the proportion of herds positive for *Staph. aureus* was statistically significantly higher among those that reported using anti-suckling devices than among herds where such tools were not applied. Although the frequency of suckling was not asked in the questionnaire, it could be that producers have had *Staph. aureus* IMI before and they simply wanted to decrease the occurrence of this habit and to prevent spread by this organism via this route.

No significant difference was found in detection of *Staph. aureus* in BTM when comparing herds that offered milk replacer and herds that offered milk from cows with IMI to calves. However, this finding may be reasoned by the overall high prevalence of *Staph. aureus* in the current study, with both groups presenting similar prevalence amid them.

Limitation of the current study is that routine bacteriological culture of BTM is known to have low sensitivity for *Staph. aureus* (Godkin and Leslie, 1993). In view of the overall prevalence of *Staph. aureus* at 69%, any given herd was more likely to be positive than not, and assessment of BTM culture was likely not discriminatory enough to find differences. However, sampling large number of herds at cow level may not be feasible. Considering this, multiple bulk tank milk samples (up to 3) were screened in this study and also a larger volume of milk was plated (0,1mL), thus increasing the likelihood of detecting *Staph. aureus* in
the BTM as demonstrated when analyzing prevalence of *Staph. aureus* in a subset of herds where three samples were provided and prevalence had increase to 72%. Despite this, *Staph. aureus* prevalence may be an underestimation, especially since three consecutive samples were not available from all herds.

Considering the characteristics such as herd size, type of housing system, and milk production, the herds that participated in this project were representative of Ohio herds. However, some biases may exist and might have influenced the current results. For instance, those producers who did respond to the questionnaire (volunteer bias) may be more proactive, and the management practices applied on those farms could be different from other herds, as discussed elsewhere (Dufour et al. 2010).

### 3.5. Conclusion

The surveyed dry-off practices were significantly associated with herd size, with larger herds being more likely to use internal teat sealant, blanket dry cow treatment, and to dry cows off abruptly, but none of the dry-off practices were significantly associated with *Staph. aureus* presence in BTM. Identification of effective alternatives to reduce the use of antimicrobials and further research in this area is needed. *Staph. aureus* was found in higher proportion of herds where calves were allowed to nurse or the habit of cross-suckling was noticed.
compared to herds where calves did not nurse or where cross-suckling had not
been observed.

3.6. References

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Table 3.1. Summary of dry-off practices implemented by Ohio dairy who had responded to the questionnaire.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Proportion of Farms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Use of intramammary dry cow therapy (n=374)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes, routinely all cows are treated</td>
<td>81</td>
</tr>
<tr>
<td>Yes, but only in selected cows</td>
<td>10</td>
</tr>
<tr>
<td>Do not treat any cows</td>
<td>9</td>
</tr>
<tr>
<td><strong>Use internal teat sealants (ITS) at dry-off (n=366)</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>66</td>
</tr>
<tr>
<td>Yes, all cows are treated with ITS</td>
<td>30</td>
</tr>
<tr>
<td>Yes, selected cows are treated with ITS</td>
<td>4</td>
</tr>
<tr>
<td><strong>Decision when to dry cows off (n=373)</strong></td>
<td></td>
</tr>
<tr>
<td>Based on expected calving date</td>
<td>96</td>
</tr>
<tr>
<td>Based on milk yield</td>
<td>19</td>
</tr>
<tr>
<td><strong>How much cows typically milk at the time of dry off (n=372)</strong></td>
<td></td>
</tr>
<tr>
<td>Less than 20 lbs</td>
<td>20</td>
</tr>
<tr>
<td>Between 21 and 40 lbs</td>
<td>63</td>
</tr>
<tr>
<td>Between 41 and 60 lbs</td>
<td>22</td>
</tr>
<tr>
<td>More than 60 lbs</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>How long is the typical dry period in your herd (n=376)</strong></td>
<td></td>
</tr>
<tr>
<td>Less than 30 days</td>
<td>1</td>
</tr>
<tr>
<td>Between 30 and 45 days</td>
<td>28</td>
</tr>
<tr>
<td>Between 45 and 60 days</td>
<td>69</td>
</tr>
<tr>
<td>More than 60 days</td>
<td>3</td>
</tr>
<tr>
<td><strong>Methods/practices at dry-off (n=372)</strong></td>
<td></td>
</tr>
<tr>
<td>Abrupt cessation of milking</td>
<td>73</td>
</tr>
<tr>
<td>Milk the cows once a day until dry-off</td>
<td>26</td>
</tr>
<tr>
<td>Change the ration to reduce energy intake</td>
<td>20</td>
</tr>
<tr>
<td><strong>Housing for Dry Cows (n=374)</strong></td>
<td></td>
</tr>
<tr>
<td>Tie stall</td>
<td>5</td>
</tr>
<tr>
<td>Free Stall</td>
<td>40</td>
</tr>
<tr>
<td>Loose/Manure Pack</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 3.2. Adaptation of different dry-off practices and \textit{Staph. aureus} prevalence in Ohio dairies based on herd size.

<table>
<thead>
<tr>
<th>Practices</th>
<th>Less 50 (n=108)</th>
<th>50-199 (n=230)</th>
<th>More 200 (n=40)</th>
<th>Total</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dry cow therapy (TDCT)</td>
<td>59.8%</td>
<td>88.9%</td>
<td>92.3%</td>
<td>81.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Internal teat sealant</td>
<td>13.3%</td>
<td>40.4%</td>
<td>53.8%</td>
<td>34.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Abrupt cessation of milking</td>
<td>53.9%</td>
<td>77.6%</td>
<td>87.5%</td>
<td>73%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Reduced milking frequency</td>
<td>37.2%</td>
<td>19.2%</td>
<td>10.0%</td>
<td>23.7%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>\textit{Staph. aureus} detected in BTM</td>
<td>71%</td>
<td>66%</td>
<td>72%</td>
<td>69%</td>
<td>0.69</td>
</tr>
</tbody>
</table>
Table 3.3. Summary of proportion (%) regarding calf and heifer management practices implemented by dairy herds in Ohio (n=number of producers that answered specific question).

<table>
<thead>
<tr>
<th>Management Practices</th>
<th>Proportion of Herds %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time most calves receive colostrum (n=373)</strong></td>
<td></td>
</tr>
<tr>
<td>Within 1 hour after birth</td>
<td>20</td>
</tr>
<tr>
<td>Between 1 and 6 hours</td>
<td>68</td>
</tr>
<tr>
<td>Between 6 and 12 hours</td>
<td>12</td>
</tr>
<tr>
<td><strong>Source of colostrum (n=375)</strong></td>
<td></td>
</tr>
<tr>
<td>Own mother’s colostrum</td>
<td>86</td>
</tr>
<tr>
<td>Pool several cows with mother’s colostrum</td>
<td>14</td>
</tr>
<tr>
<td><strong>Age of weaning (n=368)</strong></td>
<td></td>
</tr>
<tr>
<td>4-5 weeks of age</td>
<td>8</td>
</tr>
<tr>
<td>6-7 weeks of age</td>
<td>43</td>
</tr>
<tr>
<td>8 or more weeks of age</td>
<td>49</td>
</tr>
<tr>
<td><strong>Where calves (birth to weaning) were raised (n=370)</strong></td>
<td></td>
</tr>
<tr>
<td>On-site at the home farm</td>
<td>95</td>
</tr>
<tr>
<td>Calf raiser</td>
<td>4</td>
</tr>
<tr>
<td>All heifers are sold</td>
<td>1</td>
</tr>
<tr>
<td><strong>Where pre-weaned heifers housed (n=371)</strong></td>
<td></td>
</tr>
<tr>
<td>Individually</td>
<td>79</td>
</tr>
<tr>
<td>Group hutch</td>
<td>21</td>
</tr>
<tr>
<td><strong>Where weaned heifers housed (n=370)</strong></td>
<td></td>
</tr>
<tr>
<td>Individuals pens</td>
<td>10</td>
</tr>
<tr>
<td>In group pens</td>
<td>90</td>
</tr>
<tr>
<td><strong>Where springing heifers housed (n=369)</strong></td>
<td></td>
</tr>
<tr>
<td>With mature cows</td>
<td>15</td>
</tr>
<tr>
<td>With dry cows</td>
<td>52</td>
</tr>
<tr>
<td>Separate pen</td>
<td>35</td>
</tr>
<tr>
<td><strong>Cross- suckling (n=369)</strong></td>
<td></td>
</tr>
<tr>
<td>Never noticed</td>
<td>30</td>
</tr>
<tr>
<td>Occasionally</td>
<td>66</td>
</tr>
<tr>
<td>Frequently</td>
<td>4</td>
</tr>
<tr>
<td><strong>Use of anti-suckling device (n=370)</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>64</td>
</tr>
<tr>
<td>Yes, routinely</td>
<td>13</td>
</tr>
<tr>
<td>Occasionally</td>
<td>24</td>
</tr>
</tbody>
</table>
Table 3.4. Different calf raising practices or calf habits and their association with presence of *Staph. aureus* in Ohio dairies. *P*-value were based on Chi-square or Fisher’s exact test.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Proportion of Farms (%)</th>
<th>Staph. aureus %</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Allow to nurse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>40</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>No</td>
<td>60</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Cross-suckling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>70</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Use of anti-suckle devices **</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>No</td>
<td>64</td>
<td>65</td>
<td>35</td>
</tr>
</tbody>
</table>

*p*-values refer to association of practice and being positive for *Staph. aureus*

** comparison was made between herds that use or not use anti-suckle devices (option of use occasionally was not included.)
Chapter 4

Genetic relatedness and virulence factors of bovine Staphylococcus aureus isolated from teat skin and milk

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4.1. Introduction

Staphylococcus aureus (Staph. aureus) is a major pathogen that can cause a wide variety of diseases in humans and animals. In cattle, Staph. aureus is often associated with intramammary infections (IMI) and is considered a contagious pathogen that is mostly transmitted from cow to cow during the milking process. Staphylococcus aureus usually causes subclinical infections with negative impacts on milk quality and production, which also increases risk of culling as well as labor, treatment, and replacement costs. A solid understanding of
epidemiology of *Staph. aureus* infections, e.g. sources and transmission of the organism, is crucial for an effective control program.

Results from published literature regarding the role of teat skin as a source of *Staph. aureus* IMI are conflicting and involvement of teat skin in *Staph. aureus* epidemiology is not fully understood (Zadoks et al., 2002; Haveri et al., 2008; Capurro et al., 2010). The use of molecular techniques, such as pulsed-field gel electrophoresis (PFGE) (Haveri et al., 2007), binary typing (Zadoks et al., 2000), and multilocus sequence typing (MLST) (Enright et al., 2000), has brought up new insights but also more questions regarding this issue. Zadoks et al. (2002) concluded that most *Staph. aureus* mastitis cases are caused by strains highly adapted to the mammary gland and different from skin isolates. However, another study suggested that most *Staph. aureus* isolates from teat skin and teat canal were genetically indistinguishable from those isolated from infected mammary glands (Haveri et al., 2008). It has also been reported that isolates from extra-mammary sites (such as vagina, muzzles/nares, hock skin) were indistinguishable from isolates found in milk (Capurro et al., 2010; Mork et al., 2012). Thus, it remains unclear if isolates originating from different sources belong to a same strain and trigger similar inflammatory response in the mammary gland and if they carry same virulence factors that contribute to the severity of IMI.

A limited number of *Staph. aureus* strains are typically detected within a herd with one predominant strain causing the majority of IMI (Joo et al., 2001;
Tenhagen et al., 2007; Haveri et al., 2008). It has been suggested that low prevalence strains act similarly to environmental pathogens and could simply be colonizers of the skin and contaminants in the milk, and thus no or only mild response would be detected in the mammary gland (Sommerhäuser et al., 2003; Fournier et al., 2008). It could be assumed that the predominant strains possess certain characteristics that have allowed them to become prevalent in a herd and to cause IMI. The capability of *Staph. aureus* to cause disease is related to a number of virulence factors that allow the organism to adhere to a surface, invade or avoid the immune system, and cause harmful effects to its host (Bien et al., 2011). *Staph. aureus* is able to produce large array of toxins and other virulence factors that contribute to the manifestation and severity of staphylococcal infections or pathogenesis of mastitis (Sutra and Poutrel, 1994). There is a paucity of studies comparing carriage of virulence factors in bovine *Staph. aureus* isolates from different sources. The overall objective of the current study was to assess the association between teat skin colonization by *Staph. aureus* and *Staph. aureus* IMI by 1) evaluating genotypic relatedness of *Staph. aureus* isolates from milk and teat skin of dairy cows using PFGE and 2) characterizing the isolates based on the carriage of virulence factors. The hypothesis was that *Staph. aureus* IMI is associated with teat skin colonized by *Staph. aureus*. 
4.2. Materials and Methods

4.2.1. Selection of herds

Four Ohio dairy herds previously involved in other studies and known to have *Staph. aureus* IMI were included in the study. A whole herd sampling collecting composite milk samples from each lactating cow in these herds had been conducted two to six months prior to when cows were sampled for this study. These milk culture results had revealed *Staph. aureus* IMI prevalence on a cow level between 6 and 15% in these herds. Sample size calculation for the current study was based on an assumption that quarters colonized by *Staph. aureus* on teat skin would be two times more likely to have a *Staph. aureus* IMI than quarters not colonized (Rajala-Schultz, unpublished data). Based on this and on the results of the whole herd samplings, five known *Staph. aureus* positive cows and five cows with no previous *Staph. aureus* positive cultures and five cows with unknown status (not sampled in the herd testing) from each herd were randomly selected (www.random.org) to be enrolled in the study, with the exception of Herd 3 where 4, 4 and 4 cows were sampled, respectively. Altogether 57 cows in their first to third lactation were sampled and included in the study. Information about herd size and *Staph. aureus* herd prevalence is presented in Table 1. All herds practiced pre- and post-milking teat dipping, treated clinical mastitis cases with antibiotics, applied blanket dry cow therapy and had their milking equipment regularly serviced and properly maintained.
4.2.2. Bacteriological procedures and identification of *Staph. aureus*

4.2.2.1. Milk samples

A total of 228 quarter milk samples were aseptically collected immediately before routine milking following procedures described by National Mastitis Council (Hogan et al., 1999). Milk samples and teat skin swabs from each quarter were collected using disposable latex gloves that were changed between each animal. Milk samples were transported in ice to the laboratory, and kept frozen, up to a week, until further processing. Milk samples were thawed at room temperature and ten microliters (10 µl) of milk was plated on blood agar containing 5% sheep blood (Remel Inc., Lenexa, KS, USA) and on *Staph. aureus* selective BBL Chromagar plates (BD Diagnostic Systems; Sparks, MD, USA). Plates were incubated at 37°C and checked for growth at 24 and 48 hours. *Staph. aureus* was phenotypically identified based on colony morphology (rose to mauve colonies on Chromagar plates) and hemolysis, Gram stain, positive catalase test, and positive tube coagulase rabbit plasma test which was read at 4 and 24 hours. Colony counts on each plate were recorded and a sample with at least 1 CFU/10µl of milk was considered positive. From each positive sample, a representative single colony was subcultured on a blood agar plate and the 24-h growth was stored frozen at -80°C until further processing. Somatic cell count (SCC) in each quarter milk was determined in the local Dairy Herd Improvement (DHI) laboratory using another set of fresh milk samples collected at the same time as the aseptic samples.
4.2.2.2. Teat skin swabs

A total of 228 teat skin swabs were collected from the same cows and quarters at the same milking as the milk samples, but before teat preparation. If teats were visually soiled, dry paper towels were used to clean the teats before collecting the swab samples. In order to cover the entire surface of the teat barrel, a cotton swab soaked in tryptic soy broth (TBS; BD Diagnostic Systems; Sparks, MD, USA) was rolled from teat base over the teat end to opposite teat base, followed by rotation of the swab in a manner that the whole teat barrel surface would be covered with ten such rotations. An individual swab was used for each teat and gloves were changed between animals. After collection, swabs were immediately placed into sterile tubes filled with 5 ml of TSB and transported in ice to the laboratory for processing. In the laboratory, TSB tubes containing the sampled swabs were incubated at 37°C overnight and ten microliters of the solution was plated on Chromagar and blood agar plates containing 5% sheep blood. All plates were incubated at 37°C, checked for growth at 24 and 48 hours and colonies were identified as Staph. aureus using the same criteria as for milk. From each positive sample, a single representative colony was subcultured on a blood agar plate and the 24-h growth was stored frozen at -80°C until further processing. For the purpose of the study, an isolate was defined as "a pure culture of bacteria obtained by subculture of a single colony from a primary
isolation plate, presumed to be derived from a single organism, for which no information is available aside from its genus and species” (Tenover et al. 1995).

4.2.2.3. Pulsed Field Gel Electrophoresis (PFGE)

One milk and skin isolate from each positive quarter was selected for PFGE analysis. The procedure was performed according to a Canadian standardized PFGE method for \textit{Staph. aureus} subtyping as previously described (Mulvey et al., 2001). Briefly, PFGE assays were performed on overnight bacterial cultures. Concentrations of bacterial cell suspensions were adjusted by diluting with sterile cell suspension buffer (CSB: 10 mM Tris-HCl, 20 mM NaCl, and 50 mM Tris EDTA) to an optical density of 1.35 by spectrophotometer using 610 nm wavelength. Lysostaphin (2 µl of 1 mg/ml) was added to 150 µl bacterial cell suspensions and gently mixed; followed with the addition of 150 µl 2% low-melting-point agarose and gently mixed by pipetting. Approximately 100 µl was dispensed into molds to form agarose plugs. Agarose plugs with embedded bacterial cells were then lysed using lysis buffer (500 µl, 10 mM Tris-HCl, 50 mM NaCl, 50 mM EDTA 0.2% Deoxycholate, and 0.5% N-lauryl sarcosine), and proteinase K (20 mg/ml) in PK buffer (250 mM EDTA and 1% N-lauryl sarcosine) and washed vigorously using wash buffer (1.4 ml, 10 mM Tris-HCl and 0.1 mM EDTA) at least 3 times on a shaker at 37°C. Restriction enzyme buffer (REB) for \textit{SmaI} (New England Biolab, Ipswich, MA, USA) was diluted to 1×, added to the tube containing one-fourth of a plug and incubated at 25°C for 10 min. REB was
removed from the tube without damaging the plug slice and 150 μl of 1× REB containing 25 U of Smal was added and incubated at 25°C for at least 2 hours. The digested plugs were embedded in a special agarose (Nusieve Agarose for PFGE, Rockland, ME) and then separated using a CHEF-DRIII (Biorad, CA) apparatus under the following conditions: voltage (6 V/cm), initial switch time of 5.3 seconds and final switch time of 34.9 seconds for 19 hours. One plug of Salmonella serovar Braenderup H9812 was used as a standard reference.

4.2.3. Virulence factors in milk and teat skin isolates

Forty-two virulence factors, including 14 cytotoxins (hla, hlb, hld, hlgA, hlgB, hlgC, lukA, lukB, lukD, lukE, lukM, lukF, ’lukF-pv, lukS-pv), 9 adhesion factors (chp, clfA, clfB, ear, ebh, emp, fnbA, fnbB, fbpA) and 19 enterotoxins (sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, seu, and tsst-1) were screened for by using several multiplex PCR assays as described elsewhere (Jarraud et al., 2001; Tristan et al., 2003; Park et al., 2011), with minor modifications.

Briefly, multiplex PCRs were performed with AmpliTaq Gold polymerase system (Applied Biosystems). Each reaction mixture (50 μL) contained 5 μL 10× PCR reaction buffer, 200 μM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Applied Biosystems), 3 mM MgCl₂, 200 nM each primer, 2.0 U AmpliTaq Gold polymerase, and 10 ng of chromosomal DNA. The cycling conditions consisted of initial denaturation at 94°C for 10 min, followed by 35
cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec and ending with a final extension at 72°C for 10 min. The PCR products were resolved by electrophoresis in 2.0% agarose gels with 0.5× Tris-acetate-EDTA buffer and stained with ethidium bromide, and visualized under UV light.

4.3. Genotypic and statistical analysis

PFGE results for all isolates used in this study were analyzed with Bionumerics software (Applied Math, Saint-Marten, Belgium). Cluster analysis was performed by the unweighted pair-group method with arithmetic averages (UPGMA) to generate a dendrogram. Banding patterns were compared by using Dice coefficient with a position tolerance of 2.0%. Cut-off value of 80% was applied to interpretation of the banding patterns. Two or more isolates were considered to form a cluster if they had over 80% similarity; isolates were considered a pulsotype if they were 100% similar.

Strength of association between finding Staph. aureus on teat skin and Staph. aureus IMI in the same quarter was assessed by calculating relative risk (RR) and its 95% confidence interval. Association between the source (milk or teat skin) and carriage of different virulence factors was tested using chi-square test of independence or Fisher’s exact test, as appropriate. Similarly, proportions of isolates carrying virulence factors across different PFGE clusters and from Staph. aureus infected quarters with high and low SCC (categorized into two
groups, using 400,000 cells/ml cut-off) were compared using chi-square test of independence or Fisher's exact test, as appropriate, using Stata 10.0 (StataCorp, College Station, TX, USA). This relatively high cut-off of 400,000 cells/mL was used to classify the quarters as high and low based on somatic cells, because only three infected quarters had SCC below 400,000 cells/m. Somatic cell count was transformed to linear score (SCS) and non-parametric Wilcoxon Signed rank test was used to compare SCS’s in quarters which gave rise to isolates in different clusters.

4.4. Results
4.4.1. *Staphylococcus aureus* prevalence in milk and teat skin

*Staph. aureus* was detected in 28 (12%) of the 228 milk samples collected and in 25 (11%) of the 228 teat skin samples. Number of *Staph. aureus* positive samples from milk and teat skin in the study herds as well as basic information about the herds are presented in Table 4.1. In total, *Staph. aureus* was detected in 20 cows and 43 quarters. Of these quarters, ten (23%) were positive both for milk and skin, 18 only for milk and 15 only for teat skin. Thus, 38% (20/53) of the isolates originated from quarters found to be positive both in milk and teat skin, 34% (18/53) from quarters positive in milk but negative in skin and 28% (15/53) from quarters positive in teat skin but negative in milk. All but one cow with *Staph aureus* IMI in the current study had also previously been positive for *Staph. aureus*. Infection status of the one additional cow identified in this study with two
Staph aureus positive quarters was previously unknown. Among the uninfected cows identified during the earlier whole herd sampling (i.e., cows with no previous diagnosis of Staph aureus IMI), only teat skin samples were found to be positive for Staph. aureus in the current study.

Without considering genotypic relatedness of the isolates (i.e., identifying the organism only at species level), colonization of teat skin with Staph. aureus was highly significantly associated with finding the organism also in milk (P<0.0001): quarters colonized with Staph. aureus on teat skin had 4.5 times higher risk of being diagnosed with Staph. aureus IMI compared with quarters negative on teat skin (relative risk, RR=4.51, 95% CI for RR 2.35 to 8.66).

4.4.2. Distribution of milk and teat skin isolates across PFGE clusters

One isolate from each positive milk and teat skin sample was typed using PFGE to assess the genotypic relatedness of the isolates. Altogether 25 pulsotypes were recognized (Figure 4.1). Each pulsotype contained between one and eight isolates, which often originated from both milk and teat skin. Isolates with ≥ 80% similarity were grouped into a cluster; three clusters named A, B and C were identified. All three clusters contained isolates from both milk and teat skin. Clusters A and B had isolates from all herds, while cluster C contained isolates exclusively from Herd 1. Distribution of the 53 Staph. aureus isolates (milk and teat skin) across the clusters was as follows; eight isolates (15.1%) were grouped in cluster A, 38 (71.7%) in cluster B and seven (13.2%) in cluster
C. Of the 38 isolates in the predominant cluster B, 60.5% originated from milk and 39.5% from teat skin. Clusters A and C, on the other hand, contained more teat skin than milk isolates (Figure 4.2). Majority (60%) of the teat skin isolates (15/25) came from quarters without Staph aureus IMI. Teat skin isolates from uninfected quarters/cows were present in all three clusters.

Some cows contributed several isolates if they were infected in more than one quarter and their teat skin was also positive. Isolates originating from milk and teat skin of a same quarter of a cow were labeled as pairs (Table 4.2). A total of ten pairs of isolates were found and they came from Herd 1 (three cows; four quarters) and Herd 2 (three cows; six quarters). No pairs were found in Herds 3 and 4. Genotypic variability was observed within the pairs, as isolates in five out of the ten pairs belonged to different clusters while isoaltes in the other five pairs were identified in a same cluster. Also, within cows quarters could be infected with isolates belonging to different clusters (Table 4.2 and Figure 4.1).

4.4.3. Distribution of virulence factors in bovine teat skin and milk isolates

Of the 42 virulence factors, 14 (hla, hlb, hld, hlgA, hlgB, hlgC, lukA, lukD, lukE, emp, ebh, fbpA, clfA, and clfB) appeared highly conserved in both milk and skin isolates, as at least 80% of the isolates carried them (Table 4.3). Virulence factor ear was identified only in one milk isolate and three factors (lukF-pv, lukS-pv and chp) were not found at all in the study. Most enterotoxin genes were found in a relatively low frequency with teat skin isolates carrying them in a
higher proportion than milk isolates. When the proportion of milk and teat skin isolates harboring different virulence factors were compared, a statistically significant difference was found for lukF', seh, sei and sej (Table 4.3). All these virulence factors were found more frequently \( (P<0.05) \) in teat skin isolates than in those originating from milk. When comparing the proportion of teat skin isolates carrying different virulence factors from quarters with or without Staph. aureus IMI, no significant differences were noticeable.

Virulence factors clfA \( (P=0.005) \), clfB \( (P=0.004) \) and fbpA \( (P=0.054) \) were more commonly harbored by isolates in the predominant cluster B, whereas fnpB, sei, sem and sen \( (P<0.01) \) were more common in isolates belonging to the minor clusters A and C (Table 4.4).

### 4.4.4. SCC and CFU in Staphylococcus aureus positive milk samples

In 89% of the Staph. aureus positive milk samples more than 1000 CFU/ml (10 colonies/0.01ml of milk) was found and the remaining samples had at least 500 CFU/ml, suggesting moderate to abundant growth in all positive samples. More concisely, 57% of the samples had more than 5000 CFU/ml, 32% had between 1000 – 5000 CFU/ml, and 11% had 500 to 900 CFU/ml.

Overall, SCC in milk from the infected quarters was high; only three quarters had SCC lower than 400,000 cells/ml (the reason for using such a high cut-off for categorizing quarters as low and high based on SCC), and the isolates from these quarters belonged to clusters A and C. The median SCC in quarters
from which the isolates in the clusters A, B and C originated was 3.55, 3.43 and 1.13 \( \times 10^6 \) cells/ml, respectively and no statistically significant differences were found when comparing SCC between clusters \((P=0.80)\). Three virulence factors \((hlgB, clfA, \text{ and } clfB)\) were significantly more common among isolates that originated from quarters with SCC higher or equal to 400,000 cells/ml than among isolates from quarters with SCC less than 400,000 cells/ml (Table 4.5). On the other hand, factors sei, sem, sen and seu were significantly more common in isolates from quarters with SCC < 400,000cells/ml. SCC results were missing for two infected quarters.

4.5. Discussion

The purpose of this study was to assess the role of teat skin colonization with \textit{Staph. aureus} in \textit{Staph. aureus} IMI by evaluating genotypic relatedness and virulence factors of \textit{Staph. aureus} isolates from teat skin and milk. The results from this study showed that colonization of teat skin with \textit{Staph. aureus} was significantly associated with \textit{Staph. aureus} IMI as quarters colonized with this organism on teat skin had 4.5 times higher risk of being diagnosed with \textit{Staph. aureus} IMI than quarters negative on teat skin. In addition, results from the current study suggested that \textit{Staph. aureus} isolates from milk were closely related to those on teat skin, as isolates from all three clusters were found both on teat skin and in milk from the infected mammary glands. These observations are in agreement with the results of Haveri et al. (2008) who also reported that
*Staph. aureus* isolates from teat skin and teat canal were genetically indistinguishable from those isolated from infected mammary glands and that these sites could act as reservoirs for IMI. Also other studies have reported that *Staph. aureus* isolates detected from extra-mammary sites or from environment are genotypically the same as isolates from milk (Capurro et al., 2010; Mork et al., 2012). However, since the current study was cross-sectional in nature, no directionality could be inferred.

Even though isolates from all three clusters were shared between milk and teat skin, only a low number of milk-teat skin isolate pairs originating from a same quarter of a cow was found. In half of these pairs, the *Staph. aureus* isolates from teat skin were closely related (belonged to the same cluster) and in half of the pairs they were genetically different from those isolated from milk of the same quarter. In two occasions, isolates from different clusters caused infections in different quarters of the same cow, with similar observation reported also by Sabour et al (2004). The finding of dissimilar milk-teat skin pairs may be due to the fact that only one isolate per positive sample was saved and genotyped using PFGE. Haveri et al. (2008) reported finding different pulsotypes from a single sample when more than one isolate was chosen for PFGE typing, so it is possible that more strains and thus more matching pairs within quarters may have been found had several isolates been genotyped. This is supported by the fact that *Staph. aureus* IMI was highly significantly associated with teat skin being colonized with the same organism, even if isolates were not shown to belong to
the same cluster. Herds 3 and 4 had the lowest number of positive samples in total and no milk-teat skin pairs within a quarter were found in them, thus the number of *Staph. aureus* positive cows available to be sampled at the time of the study may have limited the possibility of finding more pairs. Recent studies have shown that *Staph. aureus* is shed consistently in milk from naturally infected cows (Walker et al., 2011; Walker et al., 2013) but longitudinal studies on colonization of teat skin of dairy cows are lacking; thus it is possible that if cows and quarters had been sampled repeatedly, a more detailed picture of *Staph aureus* epidemiology, on a molecular level, may have emerged (Capurro et al, 2010).

*Staph. aureus* can be found on teat skin of cows without an IMI as reported by Haveri et al. (2008) and Piccinini et al. (2009) as well as observed in the current study. Without any indication of inflammation in the mammary gland (low SCC) and with low CFU/ml of the organism in the sample, an isolate could simply be a contaminant from teat skin and not a cause of a true IMI (Piccinini et al 2009; Middleton et al, 2002; Fournier et al., 2008), potentially impacting a correct diagnosis of an IMI. However, when using careful aseptic sampling procedures, contamination of a milk sample with *Staph aureus* from teat skin appears unlikely. In the current study, the number of CFUs was high in all milk samples found positive for *Staph. aureus* and almost all quarters also had high SCC. This is in agreement with Walker et al. (2013) who studied *Staph. aureus* shedding patterns throughout a lactation and also found at least 1000 CFU/ml of
*Staph. aureus* in majority of the samples. Additionally, all except two *Staph aureus* positive quarters (from the same cow) in the current study were from cows identified positive for *Staph aureus* in earlier samplings, further diminishing the likelihood of contamination of milk samples from teat skin.

*Staphylococcus. aureus* strains found on teat skin of cows without *Staph aureus* IMI were genetically closely related to strains found in milk or skin of infected quarters. Zadoks et al. (2002) reported that *Staph. aureus* isolates from teat skin and milk can be found on milking unit liners implying that liners can be fomites for transmission of *Staph. aureus*. Especially among high producing cows contamination of bedding by milk leakage from *Staph. aureus* infected quarters is possible (Capurro et al., 2010). Thus, the possibility of contamination of teat skin with *Staph aureus* isolates from milk cannot be totally dismissed. In the current study over half of all teat skin isolates came from uninfected quarters suggesting that *Staph. aureus* on teat skin is not a result of direct contamination from milk of that same quarter.

A higher proportion of isolates in cluster B harboured *clfA* and *clfB* genes than isolates from the other clusters. Presence of these adherence genes, which are involved in the initial attachment of *Staphylococcus aureus* to epithelial cells of the teat canal, may have contributed to the ability of these isolates to become the predominant strain and to get establish in these herds. This agrees with Haveri et al (2008), who also reported the adhesins *fnbA* and *fnbB* in their predominant PFGE types. Similarly, significantly higher proportion of isolates
from quarters with SCC greater or equal to 400,000cells/mL carried these genes, corroborating with Capurro et al. (2010a) and Fournier et al. (2008) who had suggested that the dominance and pathogenicity of some Staph. aureus strains is probably due to differences between virulence factors of the strains.

High percentage of Staph. aureus isolated from milk of cows with IMI has been reported to harbor different enterotoxin genes, which imply that these toxins may be important in development of mastitis (Srinivasan et al., 2006; Rall et al., 2014). On the other hand, Larsen et al. (2000) questioned the role of these toxins in mastitis pathogenesis since only one of 414 isolates from cows with mastitis carried toxic genes in their study. In the current study, higher proportion of teat skin isolates compared to milk isolates carried some virulence factor genes suggesting that presence of these factors may provide some advantage to endure in an adverse environment. The practices used to control mastitis such as pre- and post-milking teat dipping or potential abrasion caused by use of cloth/paper towels for teat cleaning before milking could be some examples of these environmental challenges.

The current study found different enterotoxin genes in varying frequency, ranging from 0 to 25% in milk isolates and from 0 to 56% in teat skin isolates. In general, the prevalence of enterotoxin genes in Staph. aureus strains from bovine milk with clinical or subclinical mastitis varies greatly as discussed in Oliveira et al. (2011) and Srinivasan et al. (2006). These researchers suggested that this may be due to variable environmental and management factors in
different geographic regions. Occurrence of enterotoxin and tsst-1 genes in Staph. aureus may be a concern to raw milk consumers since Staph. aureus is a common foodborne pathogen isolated from milk (Oliver et al., 2009).

The present study had some limitations that need to be considered when interpreting the results. A small number of herds was enrolled and only portion of the cows in these herds were sampled for the purpose of the study. The sampling within the herds, however, was systematically stratified to identify known infected and uninfected cows and those whose Staph. aureus status was not known. Only one isolate from each positive sample was genotyped using PFGE, which may have limited the ability to find different Staph. aureus strains within a cow and a quarter and within a sample. Also, since this was a cross-sectional study, no inferences regarding causality or directionality can be made.

### 4.6 Conclusion

Results from PFGE genotyping demonstrated that some Staph. aureus isolates from milk and teat skin were closely related. Additionally, quarters colonized by Staph. aureus on teat skin were at a significantly higher risk of also having a Staph. aureus IMI compared to quarters negative on teat skin. The presence of particular virulence factors (e.g. clfA, clfB) may have contributed to the ability of certain isolates to become the predominant strain and to get established in those herds. Further investigation relating strain characteristics
and presence of combinations of virulence factors to severity of infection will be of clinical interest.

4.7 References


Table 4.1. Characteristics of the study herds (herd size and prevalence of bovine *Staph. aureus* based on earlier sampling), number of cows and quarters sampled in this study and number of cows from which isolates originated (n), number of positive milk and teat skin samples and average SCC value (x1000cells/ml) in quarters infected with *Staph. aureus* in the study herds.

<table>
<thead>
<tr>
<th>Herd ID</th>
<th>Herd size¹</th>
<th>Herd <em>Staph. aureus</em> prevalence %</th>
<th>Number of cows/quarters sampled</th>
<th>Source cows (n)</th>
<th>Positive samples Milk</th>
<th>Positive samples Teat skin</th>
<th>Positive samples SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>140</td>
<td>15</td>
<td>15/60</td>
<td>8</td>
<td>5</td>
<td>15</td>
<td>601</td>
</tr>
<tr>
<td>2</td>
<td>201</td>
<td>8</td>
<td>15/60</td>
<td>4</td>
<td>11</td>
<td>6</td>
<td>3714</td>
</tr>
<tr>
<td>3</td>
<td>97</td>
<td>7</td>
<td>12/48</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>7410</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
<td>6</td>
<td>15/60</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>4183</td>
</tr>
</tbody>
</table>

¹ herd size, includes both lactating and dry cows
Table 4.2. Distribution of *Staph. aureus* isolates from milk and teat skin of a same quarter of a cow across different PFGE clusters by the herd of origin.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Cow ID</th>
<th>Cluster (Milk/teat skin isolates; quarter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>182$^1$</td>
<td>A/B; RF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B/C; LF</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>C/B; RR</td>
</tr>
<tr>
<td></td>
<td>205</td>
<td>C/B; RF</td>
</tr>
<tr>
<td>2</td>
<td>452$^1$</td>
<td>B/B; LF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B/B; LR</td>
</tr>
<tr>
<td></td>
<td>429$^1$</td>
<td>B/B; LR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/B; RF</td>
</tr>
<tr>
<td></td>
<td>496</td>
<td>B/B; RF</td>
</tr>
</tbody>
</table>

$^1$ Cows 182 and 429 had two quarters infected and Cow 4 had three quarters infected. Herds 3 and 4 did not have any pairs of milk and teat skin isolates originating from the same quarter of a cow. LF-left front, LR-left rear, RF- right front, RR-right rear quarter.
Table 4.3. Number and percentage of *Staphylococcus aureus* isolates from bovine milk and teat skin that carried different virulence factors. *P*-value indicates whether the proportion of milk and teat skin isolates carrying virulence factors differed significantly (based on Chi-square or Fisher’s exact test, as appropriate).

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Number (%) of isolates carrying particular gene in:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk (n=28)</td>
<td>Teat Skin (n=25)</td>
</tr>
<tr>
<td>lukA</td>
<td>28 (100%)</td>
<td>25 (100%)</td>
</tr>
<tr>
<td>hlgA, hlgC, hla, hlb, emp, fbpA</td>
<td>28 (100%)</td>
<td>24 (96%)</td>
</tr>
<tr>
<td>lukD, lukE, ebh</td>
<td>27 (96%)</td>
<td>25 (100%)</td>
</tr>
<tr>
<td>hld</td>
<td>26 (93%)</td>
<td>25 (100%)</td>
</tr>
<tr>
<td>clfA</td>
<td>25 (89%)</td>
<td>24 (96%)</td>
</tr>
<tr>
<td>hlgB, clfB</td>
<td>24 (86%)</td>
<td>21 (84%)</td>
</tr>
<tr>
<td>lukB</td>
<td>20 (71%)</td>
<td>17 (68%)</td>
</tr>
<tr>
<td>fnbA</td>
<td>12 (43%)</td>
<td>7 (28%)</td>
</tr>
<tr>
<td>fnbB</td>
<td>4 (14%)</td>
<td>8 (32%)</td>
</tr>
<tr>
<td>lukM</td>
<td>5 (18%)</td>
<td>10 (40%)</td>
</tr>
<tr>
<td>lukF'</td>
<td>4 (14%)</td>
<td>11 (44%)</td>
</tr>
<tr>
<td>ear</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>lukF-pv, lukS-pv, chp</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sea, ser, sec, see</td>
<td>0</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>seb, seo, sep</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sed</td>
<td>5 (18%)</td>
<td>9 (36%)</td>
</tr>
<tr>
<td>seg</td>
<td>4 (14%)</td>
<td>5 (20%)</td>
</tr>
<tr>
<td>seh</td>
<td>0</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>sei</td>
<td>7 (25%)</td>
<td>14 (56%)</td>
</tr>
<tr>
<td>sej</td>
<td>2 (7%)</td>
<td>9 (36%)</td>
</tr>
<tr>
<td>sek</td>
<td>0</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>sel</td>
<td>2 (7%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>sem</td>
<td>2 (7%)</td>
<td>6 (24%)</td>
</tr>
<tr>
<td>sen</td>
<td>2 (7%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>seq</td>
<td>2 (7%)</td>
<td>0</td>
</tr>
<tr>
<td>seu</td>
<td>3 (11%)</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>tsst-1</td>
<td>1 (4%)</td>
<td>2 (8%)</td>
</tr>
</tbody>
</table>
Table 4.4. Distribution of virulence factors in bovine *Staph. aureus* isolates (from milk and teat skin) across different PFGE clusters (predominant cluster B compared to minor clusters A&C). Only factors whose proportions differed statistically significantly between the clusters are presented. N=total number of isolates in the cluster, n= number of isolates with a specific factor.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Cluster (n, %)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A&amp;C (N=15)</td>
<td>B (N=38)</td>
</tr>
<tr>
<td><em>clfA</em></td>
<td>11 (73%)</td>
<td>38 (100%)</td>
</tr>
<tr>
<td><em>clfB</em></td>
<td>9 (60%)</td>
<td>36 (95%)</td>
</tr>
<tr>
<td><em>fnbB</em></td>
<td>7 (47%)</td>
<td>5 (13%)</td>
</tr>
<tr>
<td><em>fbpA</em></td>
<td>4 (27%)</td>
<td>14 (37%)</td>
</tr>
<tr>
<td><em>sei</em></td>
<td>11 (73%)</td>
<td>10 (26%)</td>
</tr>
<tr>
<td><em>sem</em></td>
<td>6 (40%)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td><em>sen</em></td>
<td>4 (27%)</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>
Table 4.5. Carriage of virulence factors in bovine *Staph. aureus* milk isolates originating from quarters with somatic cell count (SCC) above (n=23) or below 400,000 cells/ml (n=3). Only factors where statistically significant differences were observed are presented.

<table>
<thead>
<tr>
<th>Factors</th>
<th>SCC &lt;400 (x1000 cells/ml)</th>
<th>SCC &gt;400 (x1000 cells/ml)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hlgB</em></td>
<td>1 (33%)</td>
<td>21 (91%)</td>
<td>0.05</td>
</tr>
<tr>
<td><em>clfA</em></td>
<td>1 (33%)</td>
<td>22 (96%)</td>
<td>0.02</td>
</tr>
<tr>
<td><em>clfB</em></td>
<td>0</td>
<td>22 (96%)</td>
<td>0.002</td>
</tr>
<tr>
<td><em>sei</em></td>
<td>3 (100%)</td>
<td>4 (17%)</td>
<td>0.013</td>
</tr>
<tr>
<td><em>sem</em></td>
<td>2 (66%)</td>
<td>0</td>
<td>0.009</td>
</tr>
<tr>
<td><em>sen</em></td>
<td>2 (66%)</td>
<td>0</td>
<td>0.009</td>
</tr>
<tr>
<td><em>seu</em></td>
<td>2 (66%)</td>
<td>1 (4%)</td>
<td>0.027</td>
</tr>
</tbody>
</table>
Figure 4.1. Dendrogram showing genetic relatedness of *Staph. aureus* isolates from bovine milk and teat skin. Numbers at the upper left indicate the percentage of similarity. Dotted vertical line indicates 80% similarity cut-off and horizontal lines indicates the three different clusters identified (A, B, C). Site refers to different mammary quarters: LF-left front, LR-left rear, RF-right front, RR-right rear. Dairy ID refers to Herds 1-4.
Figure 4.2. Distribution of bovine *Staph. aureus* isolates from milk and teat skin across different PFGE clusters detected in four Ohio dairy herds.
Chapter 5

Molecular characterization of Methicillin-resistant *Staphylococcus aureus* (MRSA) from bulk tank milk in Ohio dairy herds

5.1. Introduction

The use of antimicrobial drugs in both human and veterinary medicine has led to concerns about the development of antimicrobial resistance and its potential impact to public health (Aarestrup, 1999; Sefton, 2002). Antimicrobials are used in livestock production to improve feed efficiency and enhance growth, as well as for treatment, control, and/or prevention of diseases. In dairy farms the leading reason for antimicrobial use is treatment of clinical mastitis, one of the most common and economically important disease in dairy cattle (Pol and Ruegg, 2007; Saini et al., 2012).

The National Health Monitoring System (NAHMS), 2007 study which was conducted in 17 major dairy states indicated that about 85.4% of dairy operations treated mastitis in cows with antibiotics, with β-lactams the primary antibiotic class used. Over 90% of dairy operations used intramammary antibiotics to treat cows at dry-off, and, approximately 80% of farms that practiced antibiotic dry cow therapy treated all cows on the farm (USDA, 2008).
Despite the fact that much research and effort has been dedicated to mastitis control, it remains a persistent problem and is the most expensive disease of dairy cows (Schepers J.A., 1991; Barkema et al., 1999). *Staphylococcus aureus* (*Staph. aureus*) is one of the most prevalent pathogens causing intramammary infection (IMI) in dairy cows with an estimated herd prevalence of 43% in US dairy herds (USDA, 2008). This pathogen has many characteristics that have helped its existence over the years. Among these are its virulence, capacity to form biofilm and ability to acquire new exogenous genes allowing it to adapt to a variety of environmental conditions (Melchior et al., 2006; Moellering, 2012).

Historically, soon after introduction of penicillin, the first penicillin resistant bacteria were detected. In the late 1940s and throughout the 1950s, *Staph. aureus* developed resistance to penicillin. Then, methicillin, a synthetic form of penicillin, was introduced to counter the increasing problem of penicillin-resistant *Staph. aureus*. In 1961, British scientist Patricia Jevons and Mary Barber identified the first strains of *Staphylococci* resistant to methicillin (Barber, 1961; Jevons, 1961).

Methicillin resistance is conferred by the acquisition of *mecA* gene which encodes a unique penicillin-binding protein (PBP2a or the recently discovered alternative PBP2 encoded by mecC) which render the isolate resistant to all β-lactams except for the novel class of cephalosporins, which have sufficiently high
affinity to PBP2a, to be active against MRSA (Chambers and Deleo, 2009). Methicillin resistant *Staph. aureus* emerged as a major public health problem first causing health care-associated infections (HA-MRSA) then expanding broadly, causing also community-acquired infections (CA-MRSA), posing a challenge for infectious disease medicine. Lately, a new category has been added to describe MRSA cases associated with exposure to livestock; these MRSA strains are referred as livestock-associated MRSA (LA-MRSA; Leonard and Markey, 2008).

In recent years, an increased number of reports have been published on prevalence of MRSA in companion and livestock animals, as well as in humans who are in close contact with these species (van Loo et al., 2007; Wulf et al., 2008; Denis et al., 2009; Catry et al., 2010). An increased risk for colonization and infection with MRSA strains is expected among livestock producers, farm managers and veterinarians as suggested by some authors (Denis et al., 2009; Spohr et al., 2011; Garcia-Graells et al., 2012).

Food products of animal origin have also been found to be contaminated with MRSA. Recently attention has focused on reports of MRSA-contaminated retail meat where the prevalence can range from 0.5 to 12% (de Boer et al., 2009; Pu et al., 2009; Hanson et al., 2011). A study in Italy found 3.0-3.75% of raw milk samples testing positive for *mecA* gene (Wendlandt et al., 2012). In Japan, out of 363 *Staph. aureus* isolates from milk, only 4 (0.01%) were MRSA (Hata et al., 2010). On the other hand, among 93 *Staph. aureus* isolates from mastitic bovine milk in Turkey, 17% were positive for *mecA* (Turkyilmaz et al.,
Twenty-seven MRSA were detected from 375 *Staph. aureus* isolates from cows with subclinical mastitis on a farm in Hungary and these strains were indistinguishable by genotyping methods from a strain isolated from a person who worked with these cows. This study suggested the first documented case of transmission between humans and cattle, however, the direction of transmission could not be proven (Juhasz-Kaszanyitzky et al., 2007).

The first report of MRSA from farm animals was described in Belgium in 1972 from a cow with mastitis (Devriese et al., 1972). During the following four decades, only a few cases have been published in cattle. Bulk tank milk (BTM) has been used to estimate the prevalence of MRSA in some countries: 36 MRSA were found in 635 BTM (about 5% prevalence) in Germany during a study conducted between 2009-2010 (Kreausukon et al., 2012) and 10 of 465 BTM sampled in England and Wales during 2011-2012 (Paterson et al., 2013). Similarly, out of 3047 bovine mastitic milk samples from herds with bulk tank milk SCC >200,000 cells/mL in Korea between 1997 and 2004, 835 (27%) were identified as *Staph. aureus* and 21 (2.5%) of these *Staph. aureus* isolates were resistant to methicillin (MIC≥4µg/mL) (Moon et al., 2007).

Contrary to Asia and Europe, only a few studies have examined and reported the presence of MRSA in BTM in United States. Using samples from a USDA Dairy study of 2007, *nuc* and *meca* genes (identification of *Staph. aureus* and methicillin resistance, respectively) were found in seven out of 218 BTM, but due to the methodology used it was not possible to distinguish if the gene
originated from *Staph. aureus* or other *Staphylococcus* spp. The authors then concluded that MRSA could not be detected in samples from milk (Virgin et al., 2009). A study from Minnesota found two MRSA isolates from BTM of 50 farms studied (from two different farms), one isolate characterized as belonging to MLST-5 USA100 unknown spa-type related to hospital-associated MRSA, and the second isolate as MLST-8, USA300, spa-type t121, identified as community-associated MRSA (Haran et al., 2012). A third study from a farm in New Mexico, USA identified 40 *Staph. aureus* isolates from 29 raw milk samples. Included in these isolates, were seven from hospital cows and 33 from healthy cows. Analysis using PCR found that all hospital isolates were mecA positive with no further description of spa-typing or *Staphylococcus* cassette chromosome (SCCmec; Matyi et al., 2013).

The objective of the study was to estimate the prevalence of methicillin resistant *Staph. aureus* from bulk tank milk in Ohio, to characterize the isolates with respect to their protein A (spa type), staphylococcal cassette chromosome mec (SCCmec) type and to assess the phenotypic antimicrobial resistance pattern for *Staph. aureus* isolates from bovine bulk tank milk. The hypothesis was that MRSA will be detected in BTM samples in low prevalence.
5.2. Materials and Methods

5.2.1. Questionnaire description and identification of Staphylococcus aureus isolates

A total of 210 Staph. aureus isolates used in this study were obtained from bulk tank milk across Ohio as a part of another study (DaCosta et al., 2014 unpublished). Briefly, a survey about mastitis control, udder health, drying-off and heifer raising practices, was mailed to 780 dairy producers across Ohio in collaboration with their milk testing laboratory, and 307 farms agreed to have their BTM tested and collected up to 3 different time points (in a monthly interval).

Milk samples were submitted to Ohio State University Mastitis laboratory frozen and were kept at -20⁰C until further processing. Milk was thawed at room temperature and one hundred microliters were plated on Baird Parker media. The plates were incubated at 37⁰C, examined for growth at 24 and 48 hours and suspect colonies were phenotypically further identified as Staph. aureus based on Gram stain, positive catalase test, and a positive tube coagulase rabbit plasma test read at 4 and 24 hours.

A total of 210 Staph. aureus isolates were screened for the presence of MRSA. All isolates were epidemiologically independent (i.e., from different herds) except for two isolates that belonged to the same farm (210 isolates from 209 farms).
5.2.2. Antimicrobial susceptibility test

A total of 210 selected *Staph. aureus* isolates were screened for their antimicrobial susceptibility using the Kirby-Bauer disc diffusion method and incubated at 35°C. The antimicrobials tested included cephalotin (30 µg), cefoxitin (30 µg), ceftiofur (30 µg), penicillin (10 µg), oxacillin (1 µg), cloxacillin (1 µg), ampicillin (10 µg), tetracycline (30 µg), streptomycin (10 µg), erythromycin (15 µg) and vancomycin (30 µg). The procedure and interpretation followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). Quality control strains included *Staph. aureus* ATCC 29213 and MRSA ATCC 43300. Each plate was evaluated by the same person three times to confirm values. After the first measurement of the diameter of the zone of inhibition for each set of plates (15 to 20 plates), second and third measurements were repeatedly performed by the same person for the same set of plates in a random order. An average of the three inhibitory zone diameter measurements was used to categorize isolates as susceptible or non-susceptible, this latter category including intermediate and resistant isolates. Details on zone diameter from different antimicrobials used in the current study are presented in Table 5.1. Multiresistance was defined as resistance to three or more classes of antimicrobial agents. Antimicrobials tested were included for their relevance in treatment of mastitis in cows and also for their importance in human medicine.

With studies showing inducible clindamycin resistance in *Staph. aureus* (Afridi et al., 2014; Lall and Sahni, 2014; Yusuf et al., 2014), a series of dilution
using 2 μg, 4 μg and 6 μg of oxacillin was prepared to confirm possible inducible oxacillin resistance. Mueller-Hinton (MH) broth containing the suspected isolates that harboured mecA gene, positive controls for MRSA (ATCC 43300) and MSSA (ATCC 29213) were incubated for 24 hours at 35°C. After this period, using a swab, solution was individually streaked in four sets of MH plates (the three different concentrations of oxacillin and no antibiotic for control). Plates were incubated at 35°C for 48 hours and evaluated for growth.

5.2.3. Genotypic characterization

A duplex PCR was performed following the modified version of Jonas et al. (2002) using femB and mecA genes on the 210 selected isolates. Primers used for detection of mecA gene were MecA1 (5’ – GTA GAA ATG ACT GAA CGT CCG ATAA) and MecA2 (5’ –CCA ATT CCA CAT TGT TTC GGT CTA A), yielding a 310bp amplicon and for detection of femB, primers FemB1 (5’- TTA CAG AGT TAA CTG TTA CC) and FemB2 (5’- ATA CAA ATC CAG CAC GCT CT) producing an S.aureus -specific 651-bp PCR product. The DNA was extracted following the modified version of Kilic et al. (2006).

Pulsed-field gel electrophoresis (PFGE) was performed following the Centers of Disease Control (CDC) protocol described at http://www.cdc.gov/HAI/pdfs/labSettings/ar_mras_PFGE_s_aureus.pdf.

Typing of the polymorphic region of the protein A gene (Spa typing) was performed according to a previously described procedures (Hallin et al., 2009).
SCCmec typing was performed as described by Milheirico et al. (2007), and identified according to the method of Zhang et al. (2005).

5.3. Results

5.3.1. Antimicrobial resistance for Staphylococcus aureus

A total of 210 Staph. aureus isolates from BTM from different farms were selected for this study. Of these, 76% (160/210) were pansusceptible; 16% (33/210) were resistant to one class, 5.7% (12/210) resistant to two classes and 2.4% (5/210) multi-resistant (Table 5.1). Proportion of resistant organisms ranged from zero for vancomycin to 13% (27/210) for penicillin and 13.4% (28/210) for ampicillin. When resistance to two antimicrobials was considered, the most common combinations were resistance to penicillin/ampicillin with 9% (18/210) and to tetracycline/erythromycin with 2% (4/210) of isolates.

With the finding of mecA positive isolates being susceptible for oxacillin in Kirby-Bauer, Mueller-Hinton (MH) broth containing a successive dilution of 2 μg, 4 μg and 6 μg of oxacillin was incubated with the two positives isolates (harboured mecA gene). No growth was seen in any of the plates containing the two isolates that carried the mecA gene.

5.3.2. MRSA characterization

Initially only one isolate per farm was screened for the presence of mecA gene. For the one farm that yielded a mecA positive isolate, another isolate was selected for additional testing from the same farm. This second isolate was also
confirmed positive for mecA gene. The two positive isolates were from BTM collected with a 30-day interval. Both isolates were resistant only to penicillin and ampicillin and susceptible to oxacillin and cloxacillin. The isolates with mecA gene were considered MRSA as shown in Figures 5.1 and 5.2.

Based on PFGE, both isolates were indistinguishable, and presented 85.7% of similarity with strain USA 200. This strain (USA 200) is most frequently associated with MRSA infections in health care settings (Klevens et al., 2007). Subtyping was performed in both mecA positive isolates. These isolates harbored staphylococcal chromosomal cassette type IV, which is characteristic of a community-associated MRSA. According to spa typing, the isolates were grouped as spa type ST021 from the Ridom Spa server website (www.spaserver.ridom.de).

5.4. Discussion

This is the first study assessing the presence of MRSA in BTM from Ohio dairies. Based on a survey for herd characteristics and management practices (e.g., size, production, milking routine, calf and heifer raising), these dairies can be considered typical of Ohio dairy farms. Limited information has been published about prevalence of MRSA in the US dairies using standardized procedures. Due to diverse methodology used in different laboratories, it is difficult to estimate an overall prevalence of MRSA in animals (Weese, 2005). A concurrent investigation of MRSA prevalence using routine methods and
enrichment media to increase the probability of identifying MRSA was used in a recent study in Germany (Spohr et al., 2011). The authors found that the proportion of MRSA positive samples was three times higher after using selective agar. However, the volume used was different, 0.01 mL of milk, when using blood agar plates and 1 mL when using selective method, making a true comparison of methods difficult. A limitation of the present study was that no enrichment media was used. This was due to the fact that the isolates were derived from another study, the purpose of which was to determine the prevalence of Staph. aureus in bulk tank milk. This limitation would likely lead to an underestimation of actual prevalence of MRSA in this population. In spite of this, the present study remains one of the first reports identifying MRSA isolates in BTM in the US.

A study from BTM samples in the US did not show the presence of mecA gene in Staph. aureus (Virgin et al., 2009). Currently, to the best of the authors' knowledge only two US reports on MRSA in milk have found positive isolates (Haran et al., 2012; Matyi et al., 2013) in contrast to several reports from Europe. There are some hypotheses that may explain the differences in the low prevalence of MRSA in the US compared to higher prevalence in Europe (Weese, 2010; Ferreira et al., 2012). Some of these factors include close contact between food animals and humans and coexistence of animals of different species within farm or that can be an emerging disease with later onset when compared to Europe (Weese, 2010).
In the Matyi et al. (2013) study, out of 40 samples the authors found five *Staph. aureus* isolates positive for *mecA*, (prevalence of 12.5% MRSA). Unfortunately, information about the farm (e.g., size, animal density), cows (mastitic or not), the reason for the cows being in the hospital pen (that required a veterinarian visit) or whether the milkers were positive for MRSA was not given. This high prevalence can be partially explained by the fact that all *mecA* positive isolates were from hospital cows, and likely undergoing antimicrobial treatment. One meta-analysis study had demonstrated that humans exposed to antibiotic therapy have almost 2-fold chance of acquiring MRSA as opposed to non-exposed subjects (Tacconelli et al., 2008).

A study by van Griethuysen et al. (2005) raised a concern of the potential loss of *mecA* gene during storage of isolates. In that study, the *mecA* gene was lost in over 14% of MRSA isolates after 2 years of storage at -80\(^\circ\) C. These authors hypothesized that MRSA isolates consist of heterogeneous populations – *mecA* positive and *mecA* negative cells, with the *mecA* negative cells resisting storage conditions better. The *Staph. aureus* isolates in the present study had not been tested for the presence of the *mecA* gene before storage at - 80\(^\circ\)C, making it therefore difficult to predict the influence of freezing temperature in the current study samples. Phenotypically, however, all isolates were susceptible for oxacillin and cloxacillin.

*Staph. aureus* causes mastitis in ruminants and studies have reported MRSA in milk samples from cows worldwide, in Europe (Vanderhaeghen et al.,
MRSA in milk can be considered as minor food safety issue due to pasteurization, but the increasing consumption of raw milk or cheese made of unpasteurized milk by general population, dairy producers and their families might expose individuals to MRSA.

Besides the possibility of food contamination, close contact between humans and animals is also an important risk factor for transmission of \textit{Staph. aureus} (MRSA or MSSA). It is known that \textit{Staph. aureus} is primarily spread during milking process via milkers hands, towels and milking clusters (Hoedemaker et al., 2001).

Mastitis continues to be the most frequent reason for antibiotic usage in dairy herds (Pol and Ruegg, 2007; Oliver and Murinda, 2012). Among various antimicrobials tested in the current study, resistance to penicillin and ampicillin was most commonly observed, as is reported before (Erskine et al., 2002). Overall, in the present study, percentage of \textit{Staph. aureus} resistant to ampicillin, penicillin and erythromycin, was higher than in a Canadian study that reported resistant proportion estimates at 2.6, 8.8 and 0.7 %, respectively (Saini et al., 2012). Resistance to oxacillin (2.4%) was higher in the present study when compared to studies reporting 0.6% (Erskine et al., 2002).

Interestingly, both \textit{mecA} positive isolates in the current study were phenotypically susceptible for oxacillin in Kirby-Bauer. In an experiment to
assess potential inducible resistance, these isolates remained susceptible to oxacillin and resistance could not be induced under increasing oxacillin concentrations. Therefore, *mecA* gene, although present, was not functional and therefore the isolates did not express any phenotypically detectable response. Other studies have also reported *Staph. aureus* isolates that are phenotypically susceptible to oxacillin but carry *mecA* gene, named oxacillin-susceptible *mecA* positive *Staph. aureus* (Hososaka et al., 2007; Jean et al. 2011; Pu et al., 2014). Out of 480 strains of *Staph. aureus* from 11 hospitals in Japan, 6 strains matched the criteria for oxacillin-susceptible *mecA* positive *Staph. aureus* (Hososaka et al., 2007). Researchers in China have found high percentage of MRSA (47.6%, 49/103) in milk samples from clinical cases of mastitis in cows. Among these *mecA*-positive isolates, 76% were phenotypically susceptible to oxacillin and were thus classified as oxacillin-susceptible *mecA* positive *Staph. aureus* (Pu et al., 2014).

When performing only Kirby-Bauer test the possibility of some MRSA isolates being misdiagnosed as negative (MSSA) exist, making “parallel testing” using both phenotypic and molecular methods necessary for a MRSA accurate diagnosis. False negative isolate can have a public health implications as evidence exist that MRSA can be transmitted from human to animal - reverse zoonotic, and from animal to human - zoonotic (Juhasz-Kaszanyitzky, et al., 2007; Spoor et al., 2013). MRSA has also been detected in retail meat and raw
milk samples indicating a potential risk for food-borne transmission of MRSA (Doyle et al., 2012).

Kirby-Bauer disk diffusion has been validated for common bovine mastitis pathogens and was found to have a moderate-to-high accuracy in comparison to the manual broth microdilution test (Saini et al., 2011); however, recommendationed procedures should be followed carefully to obtain to reliable findings. The final result can be influenced by large number of factors that can vary between laboratories and technicians. Examples of these factors are inoculum density, knowledgment of organism requirements to growth, appropriate disk storage and manipulation, and number and placement of disks. Other factors such as quality variation between different batches of commercial medium or changes in antimicrobial contents of the discs over time, require a constant attention to yield optimal results. Antimicrobial testing is a complex procedure and guidelines providing instructions not only in how to perform the test but also how to interpret the results are necessary. Following the Clinical Laboratory Standards Institute guidelines and standards help to ensure uniformity of techniques and reproducibility of results, making comparisons between studies possible (CLSI 2013).

In the present study, proportion of resistance for cephalosporins (cephalotin 1.4 %, cefoxitin 0.9 % and ceftiofur 1.4 %) commonly used for dry cow therapy and intramammary clinical mastitis treatment, was higher compared to other studies where resistance to cephalotin equal to 0.1% (Makovec and
Ruegg, 2003), and 0.2% (Erskine et al., 2002). The higher observed rate of cephalosporin resistance in these isolates is most likely due to inconsistency in laboratory technique, as methicillin-susceptible Staphylococci are regarded as predictably susceptible to all cepahlosporins (CLSI, 2013). In general, results of this study suggest that most antimicrobials are still effective for treatment and control of bovine mastitis despite of being extensively used, at least based on in-vitro susceptibility testing performed in a laboratory. Furthermore, establishing prevalence of MRSA in milk samples is essential due to its potential zoonotic transmission and role as a reservoir of antimicrobial resistance factors.

5.5. Conclusion

Based on the current study, the prevalence of methicillin-resistant *Staphylococcus aureus* in dairy herds in Ohio at the present is low. The results from this study revealed new type of MRSA with characteristics distinct from hospital- or community-acquired MRSA classified as oxacillin-susceptible *mecA* positive *Staph. aureus*. This findings underscore the need for genetic methods (in addition to phenotypic tests) to accurately identify MRSA in cows.

5.6. References


CLSI, 2013 Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals; Approved Standard-Fourth Edition and Supplement, VET01A4E and VET01S2E.


Hoedemaker, M., B. Korff, B. Edler, M. Emmert and E. Bleckmann. 2001. Dynamics of Staphylococcus aureus infections during vaccination with an


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Weese, J. S. 2010. Methicillin-resistant *Staphylococcus aureus* in animals. ILAR J. 51:233-244.


Table 5.1. Antimicrobial drugs included in each plate for Kirby-Bauer disk diffusion and corresponding zone diameters from *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin - (Am)</td>
<td>10 μg</td>
<td>≤ 28</td>
</tr>
<tr>
<td>Ceftiofur - (XNL)</td>
<td>30 μg</td>
<td>≤ 17</td>
</tr>
<tr>
<td>Cephalotin - (CF)</td>
<td>30 μg</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Cephoxitin - (FOX)</td>
<td>30 μg</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Cloxacillin - (CX)</td>
<td>1 μg</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Erytromycin - (E)</td>
<td>15 μg</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Oxacillin - (OX)</td>
<td>1 ug</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Penicillin - (P)</td>
<td>10 μg</td>
<td>≤ 28</td>
</tr>
<tr>
<td>Streptomycin - (S)</td>
<td>10 μg</td>
<td>≤ 11</td>
</tr>
<tr>
<td>Tetracycline - (TE)</td>
<td>30 μg</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Vancomycin - (VA)</td>
<td>30 μg</td>
<td>.</td>
</tr>
</tbody>
</table>
Table 5.2. Distribution of resistance patterns in *Staphylococcus aureus* isolates from bulk tank milk tested by Kirby-Bauer.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number (%) of resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pansusceptible</td>
<td>159 (76%)</td>
</tr>
<tr>
<td>Cephalotin (CF)</td>
<td>3 (1.4%)</td>
</tr>
<tr>
<td>Cefoxitin (Fox)</td>
<td>2 (0.95%)</td>
</tr>
<tr>
<td>Ceftiofur (XNL)</td>
<td>3 (1.4%)</td>
</tr>
<tr>
<td>Penicillin (P)</td>
<td>27 (12.9%)</td>
</tr>
<tr>
<td>Oxacillin (Ox)</td>
<td>5 (2.4%)</td>
</tr>
<tr>
<td>Cloxacillin (Cx)</td>
<td>6 (2.85%)</td>
</tr>
<tr>
<td>Ampicillin (AM)</td>
<td>28 (13.3%)</td>
</tr>
<tr>
<td>Tetracycline (Te)</td>
<td>11 (5.2%)</td>
</tr>
<tr>
<td>Streptomycin (S)</td>
<td>14 (6.7%)</td>
</tr>
<tr>
<td>Erytromycin (E)</td>
<td>11 (5.2%)</td>
</tr>
</tbody>
</table>
Figure 5.1. Duplex PCR performed in *Staphylococcus aureus* isolates to confirm the presence of *femB* and *mecA* genes (ATCC 43300). Positive isolate for *mecA* gene (ref 298) is identified with an arrow.
Figure 5.2. Picture of two positives, identical Methicillin-resistant *Staphylococcus aureus* isolates from same farm showed by pulse field gel electrophoresis.
Chapter 6
Discussion and Conclusions

*Staph. aureus* is categorized as a contagious pathogen. With the advance of molecular studies it has been shown that the boundaries of contagious and environmental is shrinking to the point that some strains of *Staph. aureus* are called by some researchers also environmental pathogens (Sommerhäuser et al, 2003). This is mainly because of the way that it behaves, having teat skin or fomites playing an important role in transmission of this organism. Although the five point mastitis plan had been successful in controlling contagious mastitis, it appears that with the shift of dairy production to a more modern dairy industry, a more herd specific approach to control mastitis is necessary contrary to same “one size fits all” recommendation.

*Staph. aureus* is an important cause of mastitis in dairy herds. In the current study, a questionnaire was developed and sent to 780 Ohio dairy producers to evaluate which management practices applied in the farms are potentially associated with the presence of Staph aureus in the BTM. With a response rate of 49.2%, total of 384 surveys were returned. The estimated prevalence of this organism (48%) was in accordance with the reported national prevalence (43%) when culturing only one bulk tank milk (BTM). When two or
three samples of BTM were considered, cumulative prevalence increased to 64% and 69%, respectively.

*Staph. aureus* is known to cause subclinical mastitis and persistent long-term infections with damage to the milk secretory cells, resulting in reduced milk production. Also, increase in somatic cell count (SCC) at the individual quarter and cow level and/or ultimately in the BTM occurs as cow’s immune system fights mastitis pathogens. Higher SCC levels in bulk tank milk reduce quality and yield of dairy products and consequently, are not desirable by milk processors, that offer premiums (financial incentives) for higher quality milk. In the current study, probability of finding *Staph. aureus* from BTM in herds with BTM SCC < 150,000 cells/mL was significantly lower than in herds with higher BTM SCC. Controlling subclinical mastitis and producing low SCC milk therefore represents a potential profit opportunity to producers associated with both increased production and increased milk price.

Even though recommended milking procedures were reported to be applied for majority of producers in this study, *Staph. aureus* was frequently found in Ohio farms with somatic cell count higher than 300,000 cells/mL. Due to the fact that producers were anonymous to investigators it was not possible to verify how milking practices were executed in those 307 herds that provided milk to be cultured during the study. However, some questions can be raised. Do milkers know and practice the principles for mastitis control. Do those milkers receive any training? Who trains the employees? How often are they re-trained?
Are they ensuring proper coverage of teats with a germicide after milking? Questions that when appropriately addressed can decrease the number of infections.

The purpose of biosecurity practices is to prevent introduction of pathogens to the dairy farm. It is also important to limit the transmission of these pathogens within an infected dairy operation. Information from the National Animal Health Monitoring System (NAHMS) dairy study in 2007 shows that biosecurity practices such as having knowledge of the disease status of the source operation, testing new cattle for specific diseases before commingling with farm, implementing a quarantine period, and vaccinating for specific diseases have not been regularly adopted on US dairy operations. Of farms that introduced new cattle to their operation 23% required testing, and 20% quarantined recently introduced animal. Numbers presented in the current study were similar than NAHMS study with only 20% of herds practicing quarantine and 13% testing animals for Johne’s and contagious pathogens before introducing purchased animals into the herd. This represents not only a risk of disease introduction to herds, but also an opportunity for animal health improvement if appropriate attention is given to this topic among dairy producers.

The relevance of the dry period has long been recognized in the production cycle of a dairy cow for the acquisition and elimination of intramammary infection (Bradley and Green, 2000). None of the drying-off practices surveyed in this study (use of antimicrobial intramammary dry cow
therapy, use of internal teat sealants nor method of drying cows off (abrupt or gradual) were significantly associated with *Staph. aureus* prevalence in the current study.

Blanket dry-cow therapy or the use of antimicrobials in all cows has been largely recommended and findings from this study corroborate the wide adoption of this practice with 81% of responding herds reporting to treat all cows with antimicrobials at dry-off. An alternative to this practice is the selected use of dry cow treatment (10% of herds in this study choose to use antimicrobial in only selected cows) which is a topic of great debate but of great value especially with concerns about antimicrobial residues in the food chain and possible emergence of antibiotic resistant organisms. The surveyed dry-off practices were significantly associated only with herd size, with larger herds being more likely to use internal teat sealant, blanket dry cow treatment, and to dry cows off abruptly. However, no association with *Staph. aureus* prevalence and the practices were found.

Torres et al. (2008) used measures easily available to dairy producers such as cow SCC and clinical mastitis history, as potential selection criteria for deciding which cows to treat at dry-off. Based on the same study, no significant differences were found between milk yield or SCC of treated and untreated low SCC cows during the subsequent lactation (Rajala-Schultz et al 2011). A recent Canadian study looked at the use of on-farm culture system to select cows for treatment and found selective dry cow treatment as effective in controlling mastitis as blanket dry cow treatment (Cameron et al, 2014). Findings for these
studies can be an indication that with practical and effective selection criteria mastitis can be controlled with use of selective dry treatment without an increased risk of infections or clinical mastitis in the next lactation.

*Staph. aureus* colonization of the teat skin of dairy cows has been recognized as an important risk factor for *Staph. aureus* IMI. Findings from this study comparing quarters colonized by *Staph. aureus* and those not colonized showed that quarters with teat skin colonized with *Staph. aureus* were 4.5 times more likely to be diagnosed with *Staph. aureus* IMI than quarters not colonized on teat skin. Furthermore, evaluation of genetic relatedness of *Staph. aureus* isolates from milk and teat skin of dairy cows using pulsed-field gel electrophoresis (PFGE) showed that isolates from these two sources were closely related, suggesting that teat skin colonization with *Staph. aureus* can be an important factor involved in *Staph. aureus* IMI. Virulence factors were screened and teat skin isolates had a higher proportion of enterotoxins compared to milk suggesting that presence of these factors may provide some advantage to endure in an adverse environment. The practices used to control mastitis such as pre- and post-milking teat dipping or potential abrasion caused by use of cloth/paper towels for teat cleaning before milking could be some examples of these environmental challenges.

Mastitis is a very common disease occurring in most herds worldwide and it remains as the largest single cause of antibiotic use in adult dairy cattle in the United States. The necessity for dairy industry provide milk supply that is safe
and wholesome requires a commitment from all involved in this area. Prudent use of antibiotic drugs on dairy farms needs to be a priority for the industry also to keep the consumer trust. In the current study MRSA in cattle did not appear to be a huge problem in US herds, but as new genes emerge and new technologies evolve more discoveries can be in a way and industry should be prepared.

6.1. References


Blowey, R. and P. Edmondson. 2010. Mastitis Control in Dairy Herds. Chapter 4 -


CLSI, 2013 Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals; Approved Standard-Fourth Edition and Supplement, VET01A4E and VET01S2E


Odensten, M. O., B. Berglund, K. Persson Waller and K. Holtenius. 2007. Metabolism and udder health at dry-off in cows of different breeds and production levels. J. Dairy Sci. 90:1417-1428.


(MRSA) in three dairy herds in southwest Germany. Zoonoses Public Hlth. 58:252-261.


Wilson, D. J., P. M. Sears and L. J. Hutchinson. 1998. Dairy producer attitudes and farm practices used to reduce the likelihood of antibiotic residues in milk and dairy beef: A five state survey. Large Animal Practice.


www.nmconline.org/docs/NMCchecklistNA.pdf


Appendix

Ohio Dairy Producers Survey

The objective of this study is to survey Ohio dairy producers about their mastitis control and heifer raising practices. Thank you for taking the time to complete and return this survey.

Herd characteristics

1. What is the size of your herd (including milking and dry cows):
   a. 1-49
   b. 50-199
   c. 200-499
   d. 500-999
   e. 1000 cows or more

2. What breed are cows in your herd?
   a. Holsteins
   b. Jerseys
   c. Brown Swiss
   d. Other, please specify_______________________
   e. We have cows of more than one breed – which breeds? ________

3. What was your rolling herd average milk production during the last 12 months?
   a. Less than 15,000 lbs
   b. 15,000 to 20,000 lbs
   c. 20,001 to 30,000 lbs
   d. Over 30,000 lbs

4. How would you characterize your herd? Circle all that apply
   a. Conventional
   b. Organic
   c. Grazing
   d. Other special herd characteristics you would like to share__________________________
5. How often do you test milk of your cows (through DHIA or owner test)?
   a. Once a month
   b. Every 1.5-2 months
   c. Less frequently than every other month
   d. We don’t test

6. If you test, what do you test for? Circle all that apply
   a. Milk production
   b. Milk components (fat, protein)
   c. Somatic cell count (SCC)
   d. Milk urea nitrogen

7. What was the average somatic cell count in your bulk tank milk during the past three months?
   a. Less than 150,000 cell/ml
   b. 150,001 – 300,000 cells/ml
   c. 300,001 – 500,000 cells/ml
   d. Over 500,000 cells/ml

8. What type of housing is provided for milking cows?
   a. Tie stall barn
   b. Free stall barn
   c. Loose housing/manure pack
   d. Other Please, specify_________________________

9. What type of housing is provided for dry cows?
   a. Tie stall barn
   b. Free stall barn
   c. Loose housing/manure pack
   d. Other Please, specify_________________________

10. Do cows have access to pasture at any time during the year?
    a. No, none of our cows have access to pasture
    b. Yes, all cows are on pasture when available
    c. Only dry cows have access to pasture
    d. Only milking cows have access to pasture
    e. Cows have access to an outside exercise lot
11. How many times a day do you milk your cows?
   a. two times/day
   b. three times/day
   c. four times/day or more
   d. some cows are milked twice, some more often

12. What type of milking parlor do you have?
   a. Flatbarn
   b. Herringbone
   c. Parallel
   d. Rotary
   e. Other __________________________
   f. No parlor, we milk cows in tie-stalls

13. What type of bedding do you use? Circle all that apply
   a. Straw
   b. Sand
   c. Sawdust
   d. Other. Please, specify_____________________________

Biosecurity practices in the herd

14. How would you characterize your herd?
   a. Closed herd (we raise all replacement heifers on farm and do not buy outside animals, including breeding bulls) Jump to Questions 17
   b. Open herd (we purchase animals from outside sources, some or all replacement heifers are raised outside the home farm)
   c. Open herd (we take animals from our herd to fairs and shows and bring them back to our farm)

15. If you purchase outside animals, do you quarantine them before comingling them with your own animals?
   a. No
   b. Yes. For how long and where?_____________________________
16. If you purchase animals, do you test them for any diseases prior to bringing them in? Circle all that apply.
   a. No testing done
   b. Purchased animals tested for Johne’s
   c. Purchased animals tested for Mycoplasma mastitis
   d. Purchased animals tested for *Staphylococcus aureus* mastitis
   e. Purchased animals tested for *Streptococcus agalactiae* mastitis
   f. I ask whether the source herd has any diseases, but do not test
   g. Other. Please, specify________________________________

**Udder health, mastitis control and milking procedures**

17. Who is responsible for milking the cows in your herd? Circle all that apply
   a. Owner / family members, male
   b. Owner / family members, female
   c. Hired labor, male
   d. Hired labor, female

18. Please circle all that apply to your milking routine.
   a. We pre-strip
   b. We pre-dip
   c. We post-dip
   d. We use disposable single-use towels
   e. We use cloth single-use towels which are washed between milkings
   f. Known infected cows (e.g. *Staph aureus* cows) are milked last or separately

19. Do you use antibiotic intramammary dry cow therapy?
   a. Yes, all cows are routinely treated at dry-off
   b. Yes, but only selected cows are treated with antibiotics at dry-off
   c. We don’t treat any cows at dry-off
20. If you treat cows selectively at dry-off, how do you decide who gets treatment?
   a. Cows with high somatic cell count (SCC) based on DHI records
      i. What SCC cut-off do you use?____________________
   b. High SCC, based on CMT testing before dry-off
   c. A history of a cow having clinical mastitis during the lactation
   d. We culture some or all cows prior to dry-off to decide
   e. Other reason, please, explain
      __________________________________________________________________________

21. Do you use internal teat sealants (Orbeseal) at dry-off?
   a. No
   b. Yes, all cows are treated with internal teat sealant
   c. Yes, selected cows are treated with internal teat sealant

22. How do you decide when to dry cows off?
   a. Based on expected calving date
   b. Based on milk yield
   c. Other. Please, specify____________________

23. How much do cows typically milk at the time of drying them off?
   a. Less than 20 lbs
   b. 21-40 lbs
   c. 41-60 lbs
   d. More than 60 lbs

24. How long is a typical dry period in your herd?
   a. Less than 30 d
   b. 30-45 d
   c. 46-60 d
   d. More than 60 d
25. How do you dry cows off. Circle all that apply?
   a. Abrupt cessation of milking
   b. We milk the cows once a day before dry-off – for how long? __________________
   c. We change the ration to reduce the energy intake
   d. Other, please, specify____________

26. Do you collect milk samples for culture from cows with clinical mastitis?
   a. Never
   b. Occasionally
   c. From all clinical cases
   d. Only from chronic cows that have already been treated earlier

27. Have any of the following organisms ever been cultured from milk of the cows in your herd? (Circle all that apply).
   a. *Staphylococcus aureus*
   b. *Streptococcus agalactiae*
   c. *Mycoplasma* spp.
   d. No, as far as I can remember

28. Do you (your veterinarian or your co-op) monitor the herd udder health via culture of bulk tank milk samples?
   a. No, we do not culture the bulk tank milk
   b. Bulk tank milk has been cultured occasionally
   c. Yes, we culture the bulk tank milk regularly – how often?

   **Calf and heifer raising practices**

29. When do most calves receive colostrum on your farm?
   a. Within 1 hour after birth
   b. Between 1 and 6 hours
   c. Between 6 and 12 hours after birth
   d. Between 12 and 24 hours after birth

30. Check all that applies to colostrum feeding in your herd?
   a. Colostrum is fed from bottles
   b. Colostrum is “tubed” using eosophageal feeder
   c. Calves are fed their own mother’s colostrum
d. We pool colostrum from several cows
e. Colostrum is pasteurized
f. Other, please specify__________________

31. How soon are calves moved from their mother?
   a. Immediately, no nursing allowed
   b. Calves are allowed to stay with their mothers until they nurse
   c. Calves stay with their mother for at least a day

32. What milk products are calves fed on your farm? Circle all that apply
   a. Medicated milk replacer
   b. Nonmedicated milk replacer
   c. Pasteurized salable milk (from bulk tank)
   d. Unpasteurized salable milk (from bulk tank)
   e. Pasteurized mastitic milk and/or milk from treated cows
   f. Unpasteurized mastitic milk and/or milk from treated cows

33. How are calves fed milk/milk replacer in your farm?
   a. Exclusively from bottles
   b. Exclusively from open buckets
   c. We have group feeders (multiple nipples on a large milk bucket/barrel with multiple calves feeding simultaneously
   d. Other, please specify __________________________

34. Milk bottle/bucket sanitation process on your farm:
   a. Buckets and bottles washed after feeding each calf
   b. Buckets and bottles rinsed after feeding each calf
   c. Buckets and bottles washed after each feeding
   d. Buckets and bottles rinsed after each feeding
35. How often are calves fed per day?
   a. 1X
   b. 2X
   c. 3X
   d. 4X or more frequently

36. When calves are normally weaned?
   a. 4-5 weeks of age
   b. 6-7 weeks of age
   c. 8 weeks of age
   d. later than 8 weeks of age

37. Where heifer calves (from birth to weaning) are raised? Circle all that apply
   a. On-site at the home farm
   b. At a calf raiser who only raises our calves
   c. At a calf raiser who gets calves from several farms
   d. All heifer calves are sold

38. How are pre-weaned heifers housed? Circle all that apply
   a. In individual hutches
   b. Individual pens
   c. In stalls
   d. In group hutches / pens
   e. Other, please,
      specify_____________________________________

39. How are weaned heifers housed? Circle all that apply
   a. In individual pens
   b. In stalls
   c. In group pens
   d. All weaned heifers are raised off-site with only our heifers
   e. All weaned heifers are raised off-site with animals from several sources
   f. Other, please,
      specify_____________________________________
40. Do heifer calves suckle each other?
   a. No, I have never noticed it
   b. Have noticed it occasionally
   c. Yes, it occurs frequently

41. Do you use anti-suckling devices?
   a. No
   b. Yes
   c. Occasionally, on some heifers

42. How are springing heifers housed?
   a. With mature cows in the same pen
   b. With dry cows
   c. In their own separate pen

43. Would you be willing to provide us to a sample of your bulk tank milk through your marketing organization and milk testing laboratory, so that we can study the possible association between management practices and likelihood of finding contagious pathogens in the bulk tank milk? (Results from this survey will be presented only as summaries, and the identity of NO individual farm will be revealed at any point.)
   a. Yes
   b. No

44. Would you like to receive information regarding the culture results of your own bulk tank milk and the overall results of the survey?
   a. Yes
   b. No
45. In order for the OSU Veterinary Extension to better serve you, please rate the importance of the following topics to you based on the current needs of your dairy herd and your interest on these topics

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<th>The following topics are:</th>
<th>High</th>
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<td>Economics of alternative feeds</td>
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<td>Record-keeping system and monitoring</td>
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<td>Mastitis control &amp; troubleshooting high SCC</td>
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<td>Dairy facilities and cow comfort</td>
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<td>Best management practices for animal welfare</td>
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<td>Economics of dairy business &amp; transitioning</td>
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<td>On-farm biosecurity protocols</td>
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<td>Buying sound animals (testing &amp; procedures)</td>
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We greatly appreciate your assistance with this study.

Please return this survey in the enclosed postage-paid envelope.