THESIS

PRESENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AMONG DAIRY FARM WORKERS IN COLORADO

Submitted by

Jessica Nuñez

Department of Environmental and Radiological Health Sciences

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Master's Committee:

Advisor: Joshua Schaeffer

William Brazile
Claudia Gentry-Weeks

ABSTRACT

PRESENCE OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS AMONG DAIRY FARM WORKERS IN COLORADO

Dairy farmers experience a heavy burden of bioaerosol-related respiratory ailments. Bioaerosols are known to contain inflammagens (specifically endotoxin), and a diverse bacterial community that is associated with upper respiratory inflammation and pulmonary decrement among workers. However, identifying casual agents (beyond endotoxin) is still an area that warrants further research. Industrialization and modernization of the dairy industry have led to dramatic changes to production, work organization and tasks. Consequently, exposure patterns have been altered. It has been demonstrated that the mass of dairy bioaerosols is predominantly present in particle size ranges that span 10-100 µm in aerodynamic diameter; these are known to deposit in the upper respiratory system (i.e., the nasopharyngeal region) (Schaeffer et al., 2017). The nose contains complex bacterial communities, and this *microbiome* may play a role in the inflammatory response to bioaerosols. Recently, the nasal microbiome in dairy farmers was shown to contain over two times the bacterial diversity and abundance, as compared to non- farmers. It is believed that this diversity is protective against the colonization of methicillin- resistant *Staphylococcus aureus* (MRSA). In contrast, persistent nasal carriage of MRSA, specifically livestock-associated strain, has been seen in swine production workers. Recent evidence shows an increase in soft tissue infections (SSTI) caused by large animal-

derived *S. aureus* (LA-MRSA) among swine and livestock workers. The objective of this research was to characterize the presence and carriage of *Staphylococcus spp.* with a focus on livestock associated MRSA in the nasal passages of dairy workers.

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TABLE OF CONTENTS

INTRODUCTION

Dairy workers are at an increased risk for adverse respiratory outcomes due to their exposure to harmful bioaerosols, which is a significant concern given that approximately 130,000 workers are employed across 31,657 licensed dairy farms in the United States (USDA National Agriculture Statistical Service; USDA Economic Research Service, 2020). The economic impact of respiratory illnesses in this sector can be substantial, affecting not only the health and productivity of workers but also the overall efficiency and profitability of dairy operations. As the industry continues to evolve with larger herd sizes and advanced technologies, ensuring the health and safety of dairy workers becomes increasingly critical.

Dairy farm herd sizes vary significantly, according to the National Dairy FARM program herd sizes are broken down as small-scale-farms (typically <100 cows), medium-scale farms (100-499 cows), and large-scale farms (>500 cows). Although larger farms may have newly designed milking parlors and advanced technology to improve production (e.g., rotary parlors, herringbone parlors, and automatic robotic milking systems), the overall tasks and bioaerosol burdens remain the same. While rotary milking parlors enhance the efficiency of the milking process for larger herds, these newer configurations have not substantially reduced the burden of bioaerosols. Regardless of parlor design, dairy farm tasks largely involve moving cows, milking, mixing feed, providing medical care, reproduction, raising calves, general housekeeping, and maintaining equipment.

The typical workday of ~~a~~ dairy farm workers consists of eight-to-twelve-hour shifts with continuous milk production. Many of them are expected to perform multiple tasks or rotate

through various duties, with core operations taking place near animals (e.g. milking & reproduction). These tasks are carried out in an outdoor environment (no shelter), or a mix of outdoor and partially indoor (enclosure with open walls, e.g. milking parlor). Accompanying natural ventilation, milking parlors often use large fans to circulate air. The use of large fans, cross ventilation, heavy machinery, area washing, as well as movement of the large animals can resuspend the organic dust. Milking parlors use high-pressure washing to remove manure, urine, milk residues, to maintain strict sanitation standards. According to a study performed in 2012, these practices were shown to significantly reduce respirable dust concentrations among dairy parlor workers, decreasing levels from 0.25 mg/m^3 to 0.12 mg/m^3 (Choudry et. al., 2012). However, the study found no significant changes in the levels of aerosolized endotoxins, which remained relatively unchanged despite the cleaning intervention. This suggests that while parlor washing is effective in controlling respirable dust, in part due to the increase in relative humidity that accompanies the washing process, it may not adequately address the potential risks associated with endotoxin exposure. Additionally, this process can create wet environments rich in bacteria, which, when disturbed, can lead to increased aerosolization of these potentially harmful biological agents. Exposure to airborne particles, namely aerosols containing material of biological origin, is of great concern as the dairy industry employs approximately 130,000 workers in the United States. Hereinafter, these aerosols will be referred to as bioaerosols.

Bioaerosols

Bioaerosols are known to harbor diverse microbial communities, including opportunistic pathogens, allergens, and proinflammatory agents (e.g., endotoxins and other components that

elicit inflammatory cellular responses). Prior research has predominantly focused on aerosols with aerodynamic diameters smaller than 10 μm, as these particles are believed to pose the highest health risk due to their deposition in the thoracic and pulmonary regions of the respiratory tract, where gas exchange occurs (Poole et al., 2019; Schaeffer et al., 2017). However, a 2017 study characterizing dairy bioaerosols by size, source, and composition reported a previously undocumented bimodal size distribution at < 3 μm and > 30 μm (Schaeffer et al., 2017b). These results suggest that the bioaerosols generated on dairy farms extend well above 10 μm. Moreover, these larger particles represent the dominant fraction of total aerosol mass and persist across seasons and dairy operations (Schaeffer et al., 2017b). Particles between 10 μm and 100 μm tend to accumulate in the upper airways, often resulting in symptoms such as rhinitis and sinusitis. Notably, altered breathing patterns, such as oral breathing induced by strenuous activity or nasal congestion, may allow these larger particles to bypass initial filtration and reach the oropharynx area with some entering the tracheobronchial and alveolar regions (Madl AK et al., 2010). This is particularly concerning for dairy workers, who exhibit elevated rates of respiratory conditions including bronchitis, occupational asthma, hypersensitivity pneumonitis, and organic dust toxic syndrome (Reynolds et al., 2013; Schaeffer et al., 2017). Supporting this concern, a study conducted by Garcia et al. (2013) found that bioaerosol concentrations were significantly higher in personal breathing zone samples compared to area samples, highlighting the potential for elevated worker exposure during routine tasks (Garcia et al., 2013).

Given that the majority of bioaerosol mass comprises larger particles (10–100 μm), impaction in the nasopharyngeal region is due to their aerodynamic properties. As a result, the

nasal cavity may serve as a reservoir for microbial agents and associated inflammatory constituents contained within the bioaerosol matrix. Due to the distinct environmental exposures present on a dairy farm, the circulating bioaerosols exhibit a very diverse bacterial community. A study performed in 2017, which examined bacterial abundance, endotoxin presence, gram positive bacteria, and their respective origins, concluded that the primary source of bacterial communities were animal sources, followed by bird and human sources (respectively) (Schaeffer et al., 2017). These were the predominant sources regardless of milking parlor configurations. Notably, among the key constituents of the bioaerosols, *Staphylococcus, Streptococcus, and Pseudomonas* spp. were found, all of which possess potential pathogenic properties. Past studies have predominantly focused on the presence of gram-negative bacteria and the presence of endotoxins. Recent studies finding correlation between *Staphylococcus* with a rise in clinical infection cases has made *Staphylococcus aureus* a focal point of interest.

Endotoxins

Endotoxins are lipopolysaccharides (LPS) that are embedded within the cell wall of gram-negative bacteria, such as *Pseudomonas* species. Although *S. aureus* lacks LPS, it has lipoteichoic Acid (LTA) which serves as the counterpart to LPS in Gram-positive bacteria. These molecules are released when the bacterial cell is damaged, replicates, or experiences death. LPS and LTA are biologically active and are widely recognized to elicit a strong immune response resulting in significant cellular inflammation, cellular changes, and are implicated in the etiopathogenesis of numerous pulmonary diseases (Garcia et al., 2013). When these molecules enter the body's bloodstream or tissues, they activate numerous immune cells such

as macrophages, dendritic cells, which in turn initiate pro- inflammatory cytokines (TNF-α, IL-6, IL-1). LPS and LTA can easily bind to dust and human impact is closely related to dose, location, and route of exposure. Entry into the respiratory tract was observed to decrease lung diffusion capacity, fever, inflammation, dyspnea, and not limited to, bronchial obstruction (Kim et al., 2018).

Staphylococcus

Staphylococcus is a gram positive and facultative anaerobe that can survive in both aerobic and anaerobic environments. Staphylococcus is responsible for frequent infections in hospital and community settings. Several research articles have confirmed that presence of Staphylococcus, specifically *Staphylococcus aureus* (SA), in the nasal passages of 30-80% of the population (A. F. Brown et al., 2013; Sakr et al., 2018; Wertheim et al., 2005). Both *staphylococcus* and *streptococcus* are considered human commensal bacteria, and both are responsible for significant clinical infection cases. *S. aureus* is responsible for 20-40% mortality rate of all bacteremia cases in hospital settings (A. F. Brown et al., 2013). In addition, there has been an increase in chronic skin and soft tissue infections (SSTIs), osteomyelitis, endocarditis, and septic arthritis due to this pathogen (A. F. Brown et al., 2013). Given emerging information and implications of infections, *S. aureus* is of public health concern because dairy cows are principal reservoirs for *S. aureus* (Schaeffer et al., 2017; Spoor et al., 2013).

Recent studies examining industrial hog operation worker exposure identified employment to be a risk factor for soft tissue and skin infections, as well as the colonization of Livestock Associated Methicillin Resistant *Staphylococcus aureus (LA-MRSA).* This is due to their direct contact with animals, decomposing waste aerosolization, and overall aerosols produced

from natural animal and human movement (Nadimpalli et al., 2015, 2016). Livestock associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) is a particular family of strains under the umbrella of the Methicillin Resistant *Staphylococcus aureus (MRSA)*. Within this umbrella exists two more commonly known human pathogens: Hospital Associated Methicillin Resistant *S. aureus* (HA-MRSA) and Community Associated Methicillin Resistant *S. aureus* (CA-MRSA). These strains have certain genetic factors that can adapt to overcome the human immune system and evade antimicrobial attacks, in turn creating a public health concern over spread and management. In contrast, LA-MRSA lacks certain genetic characteristics, typically found in CA-MRSA and HA-MRSA (Figure1). Despite this, cases are on the rise among at-risk populations and their families (Cuny et al., 2015; Nadimpalli et al., 2016; Tegegne et al., 2017). Several studies have set out to characterize the composition of the bioaerosols present on dairy operations, but few studies exist that investigate specifically the presence of LA-MRSA among dairy workers.

While the harmful effects related to the respiratory system and lung function are widely recognized with smaller particle sizes, there is little information on larger particle size in bioaerosols' impact on respiratory health, and little information on infection with zoonotic pathogens and the associated risk factors for workers. This research aims to detect the presence of *S. aureus*, MRSA and LA-MRSA among the sample population using culture-based methods and molecular and phenotypic characterization techniques (PCR and MALDI-TOF MS). These findings can inform the identification of both current and emerging public health threats, highlight potential barriers to effective treatment—such as vancomycin resistance, which poses a critical concern given that vancomycin is often considered a last-line therapy for multidrug-

resistant infections—and contribute to a better understanding of prevalence (Table 1) (Enright et al., n.d.; Mcguinness et al., 2017; Wertheim et al., 2005). It is hypothesized that dairy workers will demonstrate comparable *S. aureus* and MRSA colonization rates and characteristics to those observed in industrial hog operations.

REVIEW OF LITERATURE

Bioaerosols on Dairy Farms

Bioaerosols generated on dairy farms contain living organisms such as bacteria, viruses, fungi, and inorganic particulates that can originate from dry and wet bedding material, feed, animal mucus, freshwater from agriculture/crop operations, feces, animal dander etc. (Schaeffer et al., 2017). Based on this diverse profile, dairy farmers may be exposed to various zoonotic pathogens, in addition to other constituents which can elicit a wide range of health outcomes, including those involving inflammatory and immunogenic pathways. (Garcia et al., 2013; Reynolds et al., 2013; Schaeffer et al., 2017; Shukla et al., 2017; Vandendriessche et al., 2013). Most research to date has focused on exposure and health outcomes specific to endotoxins. Consequently, knowledge gaps persist in the understanding of pathogen exposure. Continued investigations into these exposures can help characterize contributions to health risk by examining deposition, genetic factors and elicited immune response, especially those pathogens that can persist on the human body (e.g., *S. aureus*).

Livestock operations have undergone significant modernization to enhance efficiency, resulting in changes to both animal management and worker practices aimed at maximizing productivity. These modernizations generate larger plumes of bioaerosols (caused by animal

traffic, job task and soil disturbance) and consequently increase the potential occupational and public health risk to those similarly found on swine and poultry farms (Reynolds et al., 2013). Among these developments, certain dairy parlor designs now position workers' breathing zones near areas of high animal traffic and wet environments, thereby increasing potential exposure to airborne contaminants. Workers are responsible for washing areas periodically while ventilation is active and traffic is constant, and standing water is common. Although bioaerosol contaminants from livestock operations have been linked to increased rates of respiratory ailments such as asthma, Chronic Obstructive Pulmonary Disease (COPD) and overall interstitial lung disease (pulmonary fibrosis and inflammation, among others), the identification of causal factors is still an existing gap in knowledge (Reynolds et al., 2013; Schaeffer et al., 2017).

Gram positive bacteria were the most abundant in air samples across all tested dairies. Primary gram-positive organisms were attributed to predominantly *Staphylococcus (*methicillin susceptible *Staphylococcus aureus*- MSSA) and *Corynebacterium* (A. F. Brown et al., 2013; Schaeffer et al., 2017; Shukla et al., 2017).These findings are important in identifying size distribution of targeted organisms, and where they deposit within the respiratory tract. The first line of defense to bioaerosol and particulate matter (PM) is the upper airway consisting of the nose, nasal passages, sinuses and upper larynx. The nasal passages are where bioaerosol and local nasal microorganisms (microbiota) interact to establish colonization.

Nasal Colonization

The nasal cavity is host to a diverse community of microbiota. The interactions within these microbial communities may play a key role in health and disease. The nasal microbial profile can serve as an important barrier to invading organisms. It also functions as an immune

defense for potentially pathogenic organisms using competitive interference, mechanical clearance mechanisms, physical barrier (mucous), and an innate antibacterial response (Libeler et al., 2016; Quinn & Cole, 2007; Sakr et al., 2018; Siegel & Weiser, 2015). The long-term establishment of a microbial community within the nasal passages (colonization) is multifactorial with factors depending on existence or depletion of existing microbiota, extrinsic and intrinsic environment such as humidity, bioaerosol load, immune response, health status, cilia presence, host bacterial interaction, and underlying health conditions of the host (Sakr et al., 2018).

Researchers have reported varying rates of *S. aureus* prevalence among the general human population with persistent or continuous (using 'culture rule' of two nasal swabs taken with a one week interval) colonization reported up to 30%, while non-persistent nasal carriage prevalence rate among the general population is estimated to be 20%-80% (A. F. Brown et al., 2013b; Quinn & Cole, 2007a; Sakr et al., 2018). Dairy farmers have shown to have 2.1 times greater nasal microbiota diversity than that of non-dairy farmers (Shukla et al., 2017). This diversity is also believed to inhibit colonization of *S. aureus* in the nasal cavity via competitive inhibition. In addition, *S. aureus* directly competes with methicillin resistant *Staph aureus* and inhibits the colonization of MRSA (Leibler et al., 2016; Sakr et al., 2018). With the wide bacterial diversity and the inhibition of Staph aureus, dairy workers may be more vulnerable to the colonization of LA-MRSA.

Methicillin Susceptible *Staphylococcus aureus* (MSSA)

Two types of *S. aureus* of major concern in the community, hospital and public health realms are based on their susceptibility to a β-lactam antibiotic, i.e., methicillin susceptible *S.*

aureus (MSSA), and methicillin resistant *S. aureus* (MRSA). Methicillin resistant infections cannot be treated with beta-lactam antibiotics, makes treatment more challenging. *S. aureus* is a gram-positive, commensal organism to the human nasal mucosa, and skin. Depending on the genetic components, it can also be the underlying factor to serious infections with high morbidity and mortality rate. The *vestibulum nasi*, or anterior nares, is the most common reservoir site for *S. aureus* (within the upper respiratory system), but the skin, axilla and perineum area are also common (A. F. Brown et al., 2013; Nadimpalli et al., 2016; Nakano et al., 2022; Reynolds et al., 2013; Sakr et al., 2018). *Staphylococcus aureus* has evolved to express complex mechanisms that overcome the host immune system, which include antibody apoptosis, and targeted adhesion (A. F. Brown et al., 2013; Sieber et al., 2020; Siegel & Weiser, 2015; Spoor et al., 2013). The epithelial cells of the nasal cavity secrete antimicrobial peptides that are the hosts first immune defense against the colonization of *Staphylococcus aureus.* To colonize, *Staphylococcus aureus* must overcome nasal cilia, the mechanical clearance provided by nasal secretion, and it must evade the chemical and cellular clearance provided by antimicrobial and inflammatory proteins (A. F. Brown et al., 2013; Quinn & Cole, 2007; Sakr et al., 2018). *Staphylococcus aureus* has adapted to overcome these barriers in the following ways: the ability to produce proteases which bypass the antimicrobial peptides; the ability to produce binding proteins; as well as physiological changes to its cell wall (A. F. Brown et al., 2013; Quinn & Cole, 2007; Sakr et al., 2018).

Host health status and the composition of the nasal microbiota influence immune responses. Microbiota diversity is thought to be protective against the colonization of *S. aureus* due to colonization competition (A. F. Brown et al., 2013; Ghasemzadeh-Moghaddam et al.,

2015; Sakr et al., 2018; Shukla et al., 2017). This competition is a protective mechanism that helps prevent potential pathogenic organisms from dominating a space and can play a role in preventing pathogenic organisms from even establishing residency and preventing further infection.

Methicillin Resistant *Staphylococcus aureus (*MRSA)/ Livestock Associated Methicillin Resistant *Staphylococcus aureus (*LA-MRSA)

Previously, MRSA was only seen in a hospital setting. However, in recent years the MRSA burden on public health has been shared among three primary strains: hospital-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA). The first detected MRSA specimen was in the 1960s. By the 1980s, MRSA had spread globally and its impact on public health erupted. MRSA distinguished itself from MSSA by its antibiotic resistance properties. The most common gene to encode for this is the *mecA* gene located on the staphylococcal cassette chromosome *mec* (*SCCmec*). It is believed that *S. aureus* developed resistance to methicillin via horizontal transfer of genetic material from an unknown related species (Boakes et al., 2011; Enright et al., 2002). Horizontal transfer can be described as a lateral exchange of genetic material between organisms, and exchanged DNA is incorporated without a mating process or a vertical parent to offspring transfer. This transfer allowed *S. aureus* to acquire the *mecA* gene, which encodes a modified Penicillin-Binding Protein (PBP2a) (Enright et al., 2002).

Penicillin-Binding Proteins are responsible for building the bacterial cell wall, and β-lactam antibiotics, such as penicillin, target these proteins to inhibit cell wall synthesis which in turn destroys the bacterial cell. A modified PBP2a has a low affinity for β-lactam antibiotics allowing

cell wall synthesis to continue uninterrupted. In addition to the *mecA* gene, the genetic element *mecC* has been recently discovered. MRSA strains carrying *mecC* have been linked to cattle and other animals, with evidence suggesting they can be transmitted from livestock-associated MRSA to humans.

MRSA strains are typically identified by genes within the staphylococcal cassette chromosome *mec* (SCC*mec*) (i.e., antibiotic resistance *mecA* gene) and by multilocus sequence typing (MLST), where isolates are characterized by internal fragment matching of core (housekeeping) MRSA genes (97% conserved) and identified as Clonal Complex (CC). Clonal complexes can provide insight into transmission pathways and host adaptation (Table 1) (Larsen et al., 2017; Leibler et al., 2016; Spoor et al., 2013; Stefani et al., 2012). However, phenotypic characteristics can also be used to help identify the unique variations amongst species of MRSA, which are typically carried on mobile genetic elements 'MGE' (Maiden et al., 1998; Nadimpalli et al., 2015; Stefani et al., 2012). Because of the difficulty in comparison across laboratories, instead of testing for enzymes for which these genes encode, MLST identifies alleles directly from the nucleotides of housekeeping genes within the chromosome to identify clonal characteristics (Maiden et al., 1998). These housekeeping genes are highly conserved regions and are core genes shared amongst all strains. In contrast, carriage of varied MGEs, such as *tet(M)* (tetracycline resistance gene) and Panton-Valentine Leukocidin cytotoxin gene (*pvl*), help identify species specificity (Stefani et al., 2012). These mobile elements result in rapidly adapting organisms which readily share information via horizontal transfer, overcome host specificity, and are crucial in the continual evolution of MRSA (Stefani et al., 2012). CA-MRSA strains often carry mobile genetic elements encoding virulence factors, including the

Panton–Valentine Leukocidin cytotoxin. This is associated with enhanced virulence compared to many hospital-associated and livestock-associated MRSA strains. The Panton-Valentine Leukocidin cytotoxin functions by releasing cytotoxic proteins (*LukS-PV and LukF-PV*) that form pores in the membrane of host immune cells making them vulnerable to lysis (Melles et al., 2006). In contrast, LA-MRSA lineage is found to originate from pigs and cattle (CC398) and has developed resistance to tetracycline antibiotics via the *tet(M)* gene (Nadimpalli et al., 2015). In the early 2000s, the first human case with LA-MRSA infection was identified, and since then prevalence has increased within higher livestock density populations (Stefani et al., 2012).

Table 1

Antibiotic Characterization Strategy

Antibiotic Class	**Example Antibiotic**	**Resistance Marker / Mechanism**	**Relevance to MRSA/LA-MRSA Identification**	**Study Application**
β-lactams	Methicillin (reference drug)	*mecA* → PBP2a	Differentiates MSSA from MRSA	PCR confirmation of MRSA isolates
Tetracyclines	Tetracycline	*tet(M)* gene	Common in livestock-associated strains	Supports LA-MRSA classification
Glycopeptides	Vancomycin	Reduced susceptibility concern	Last-line MRSA therapy for humans	Indicates An Increased Public Health Risk

In the 1990s through the early 2000s, the CA-MRSA case load on the healthcare system rapidly increased with many cases being skin and soft tissue infections (Stefani et al., 2012). A survey study performed in 2008 looked at emergency room cases of cellulitis, abscesses, folliculitis, mastitis and additional diagnoses from 1993-2005 found that 97% of cultured strains were genetically identified as CA-MRSA and had the Panton-Valentine Leukocidin gene (Boakes

et al., 201; Melles et al., 2006; Stefani et al., 2012). The Panton-Valentine Leukocidin gene belongs to a family of cytotoxins (compounds toxic to cells) called synergohymenotropic toxins (Melles et al., 2006). Toxins within this category are very effective evaders of the host immune response, primarily through the destruction of immune cells, specifically neutrophils (DeLeo et al., 2005; Melles et al., 2006). Secretion of these leukocidal proteins allows it to circumvent destruction by the body's neutrophils. This enables *Staphylococcus aureus's* (CA-MRSA) ability to cause further damage to the human body, such as necrotizing skin lesions and necrotizing pneumonia (DeLeo et al., 2005; Stefani et al., 2012).

Both HA-MRSA and CA-MRSA encode a *staphylococcal* complement inhibitor (SCIN or *scn* gene) which evades the human immune system, namely neutrophils (van Wamel et al., 2006). SCIN is a C3 convertase inhibitor which arrests the C3 and C3C5 convertase complex, a signal prerequisite to immune complement activation. Cleavage of C3 molecules allows for downstream activation of C3 enzyme complexes. These C3 complex enzymes covalently bond with the bacterial cell wall signaling for membrane destruction and for neutrophils to phagocytose. Phagocytosis is the process by which neutrophils ingest or engulf bacteria creating a membrane-bound organelle that neutralizes the foreign invader (Rooijakkers et al., 2005; van Wamel et al., 2006). The interruption of the complement cascade allows the bacterial cell to avoid targeted destruction by the human immune system. In contrast to HA-MRSA, where neutrophils are able to attack and perform phagocytosis, CA-MRSA's virulence factors (including *pvl*) release toxins that create holes within the neutrophils' cellular membrane causing it to self-destruct, a process called apoptosis; This process results in cellular death or necrosis (Melles et al., 2006; Nordenfelt & Tapper, 2010). While HA-MRSA lacks *pvl* gene, it has

a larger SCC*mec* cassette and typically is expresses multi-drug (antibiotic) resistance. CA-MRSA is specifically known to predominantly affect healthy humans and doing so without needing a typical contamination source (i.e., a hospital setting and HA-MRSA), making it a growing public health concern (Boakes et al., 2011).

LA-MRSA

LA-MRSA CC398 was first confirmed as a human disease in 2003 and since then population incidence has increased worldwide (Price et al, 2012). Researchers investigating laboratory MRSA results across Europe found that LA-MRSA was responsible for up to 25% of submitted laboratory isolates. This range was associated with livestock density and proximity of population (Cuny et al., 2015; Stefani et al., 2012). Most countries reported results under 5%, but laboratory rates in the border region between Germany (11%-20%) and The Netherlands (11.9%-25%) were high due to the concentration of livestock farming, specifically pig operations (Stefani et al., 2012). Transmission to humans typically occurs by direct animal interaction and inhalation of contaminated dust, feed, excrements etc. (Cuny et al., 2015).

Previous studies have shown occupational exposure to industrial hog operations resulted in up to 77%-86% of workers testing positive for nasal carriage of LA-MRSA at some point in their career (Cuny et al., 2015). No evidence was found indicating LA-MRSA was spreading amongst healthcare settings, but risk factors (e.g., veterinary attendance and direct contact with animals) for carriage of LA-MRSA and SSTIs were identified amongst occupational livestock farmers (Cuny et al., 2015; Stefani et al., 2012). Similar findings were discovered in a study performed in the United States, with the first case of LA-MRSA detected among swine workers (Hatcher et al., 2017; Nadimpalli et al., 2016; Rinsky et al., 2013). A study investigating the

prevalence of MRSA within industrial hog workers, their families and the general community found that occupational exposure to swine was a risk factor for *S. aureus* and MRSA nasal colonization (Nadimpalli et al., 2016; Rinsky et al., 2013). Nasal prevalence of *S. aureus* amongst occupational swine workers was 22% higher than community reference adults (Hatcher et al., 2017). Nasal carriage of *S. aureus* among children was 18% higher in those living with swine industry workers than the community reference population (Hatcher et al., 2017). Researchers also found multidrug resistant *S. aureus* (MDRSA) was four percent higher for swine workers than for the reference community population – posing a risk to the workers, community health and for the healthcare system that provides care for these individuals (Cuny et al., 2015b; Hatcher et al., 2017; Rinsky et al., 2013). Higher prevalence among industrial workers and their families can be attributed to their proximity to livestock animals and their daily activities.

Prior to 2017, the industrial livestock industry commonly used low dose antibiotics, zinc and antiparasitic medication within the animal feed to control pathogens within the livestock population, often administered for both prophylactic (disease prevention) and therapeutic (treatment of active infection) purposes. Many of these were also used among the human healthcare system leading to public and animal health concerns due to the overuse within the farming systems (Cuny et al., 2015; Love et al., 2011; Rinsky et al., 2013). The Food and Drug Administration (FDA) had previously approved over 685 medications for livestock that free feed, a practice where the animals would have access to eating as much or as little as they desire (Love et al., 2011). The inconsistent feeding, and thereby under/overdosing, lead to several problems including bacterial resistance and further pathogenic evolution (Love et al., 2011).

In 2017, the FDA introduced more protective measures to prevent the overuse of antibiotics and banned the addition of their use in livestock feed for growth promotion. Nonetheless, the past overuse of antibiotics has led to LA isolates to develop a resistance to tetracycline via the *tet(M)* gene, which is currently used as a marker for LA-MRSA (Figure 1) (Nadimpalli et al., 2016). Along with *tet(m)* gene, LA-MRSA is also identified by the absence of the *scn* gene (a highly conserved virulence factor of human *S. aureus* infections) which is also an indicator for multidrug resistance (Leibler et al., 2016). Although LA-MRSA lacks the *scn* gene, it has capability to adopt an immune evasion gene known as immune evasion cluster (IEC), a gene previously only seen in human origin MRSA. This has been reported in approximately 19% of LA-MRSA infections (Cuny et al., 2015). This adaptation is a growing concern to overall public health, especially communities near industrial food animal operations.

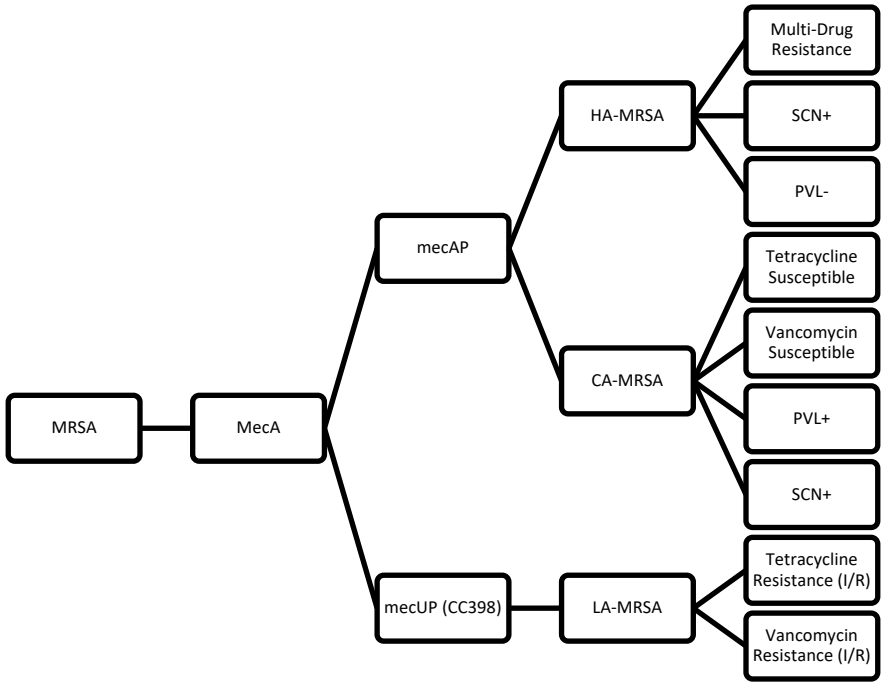

Figure 1 Species Characteristics

Note: figure demonstrates characteristics typically found for each MRSA lineage

Public Health Impact

The increase in LA-MRSA cases among humans has led to investigations into the prevalence and risk posed to public health (Cuny et al., 2015; Nadimpalli et al., 2016). Staphylococcus is responsible for a vast variety of infections globally due to its adaptability, virulence factors, resistance mechanisms, immune evasion capabilities, and its ubiquitous presence in most environments, making it a formidable microorganism. Persistent nasal carriage is common and from the anterior nares, *S. aureus* can disseminate to other parts of the body and cause potentially lethal consequences (Shukla et al., 2017). Nasal colonization has been linked, and is often considered a prerequisite, to the increased pathogenicity of *S. aureus*, and its role in secondary infections such as osteomyelitis, pneumonia, endocarditis, various post-operative infections as well as SSTIs (Brown et al., 2013; Larsen et al., 2017b; Sakr et al., 2018; Weiser, 2015). A series of recent studies found that approximately 39% of occupational hog workers' families carried *S. aureus* in their nasal passages, and 16% of workers were carriers of multidrug resistant *S. aureus* (MDRSA), while their families tested at 10% (Nadimpalli et al., 2016; Rinsky et al., 2013). Among these carriers, 13% of *S. aureus* carriers reported recent SSTIs versus only 3% of non-carriers (Nadimpalli et al., 2016). For MDRSA, 16% reported recent SSTIs versus 5% non-MDRSA carriers (Nadimpalli et al., 2016). This study highlights the prevalence of MSSA and MDRSA, but limitations exist in identifying LA-MRSA among these strains. Researchers noted that characteristics of LA-MRSA were noted among MDRSA (e.g. tetracycline resistance).

Methods

Sample collection from the nasal cavity is typically accomplished using cotton tip applicators swirled in each nostril (nasal swabs), and stored in a buffer solution, or enrichment solution (i.e. PBS) (Hatcher et al., 2017; Nadimpalli et al., 2016; Shukla et al., 2017; Ye et al., 2016). The use of nasal swabs has limitations both from qualitative and quantitative perspectives. A good sample collection is based on technique, location, and shedding/transfer of bacteria from nasal wall/cells. In addition to these variations, the nasal swab procedure is typically more uncomfortable than that of the lavage (Gritzfeld et al., 2011). The Naclerio nasal lavage method was used with this study's participants and has been shown to better detect bacterial and viral presence compared to nasal swabbing (using culture method) (Gritzfeld et al., 2011). With the Naclerio method, participants' heads are tilted back (~30 degrees), they are instructed to take a deep breath and hold it while the researcher inserted 5mls of solution into each nostril. They were then instructed to count to 10 seconds and expel the solution into a sterile sample cup.

Presumptive and confirmatory methods are typical when determining the presence of Methicillin Susceptible *Staphylococcus aureus*. Presumptive presence is typically conducted with culture-based analyses, while confirmatory presence is typically concluded with polymerase chain reaction (PCR) or by matrix-assisted laser desorption/ionization (MALDI-TOF). *Staphylococcus aureus* growth has been proven on variety of solid media including, Brain Heart Infusion Agar, Tryptic Soy Agar, Mueller-Hinton Agar, and most recently (selective growth), CHROMagar™ *Staph aureus* (Vitko & Richardson, 2013). Since robust growth is described on non-selective media, further isolation can be accomplished with the addition of a

Staphylococcus aureus selective media. The use of selective media provides direct differentiation of the desired organism and has become increasingly common in the detection process (Gaillot et al., 2000; Nadimpalli et al., 2016; Rinsky et al., 2013).

Objective of Research

The objective of this study is to identify the presence and prevalence of MRSA in the nasal cavity among dairy farm workers, and to differentiate the detected MRSA by the presence and absence of specific genes. From this information, the hope is to gain insight into possible current and future public health concerns.

METHODS

Participant Recruitment

Participants were recruited from five large dairy farms (each housing over 2,000 cows) in the Southwestern United States between May 2019 and January 2020. A chain-referral sampling and pre-sampling informational meetings were both methods used to reach potential participants. Dairy workers across various roles, including milking, feeding, animal care, maintenance, operations, and administrative duties, were invited to take part in the study. Prior to sample collection, participants were required to fill out a pre-study questionnaire to establish prior health history, demographic information and to assess eligibility for study participation. Participants filled out daily questionnaires to assess daily health status changes, shift work activities, and environmental conditions.

Individuals were excluded if they had recently undergone surgery, sustained chest injuries, or had a history of stroke or heart disease. Additionally, those taking

immunosuppressive, anti-autoimmune, or chemotherapy medications were not eligible to participate. All participants provided written consent in either English or Spanish, and the study protocols were approved by the Colorado State University Institutional Review Board.

This study was part of a larger intervention project in which participants were enrolled for five consecutive workdays examining pulmonary function testing, nasal microbiota and cytokine analysis. However, workers who were unable to commit to the full five-day period were still included for the nasal microbiota study (*S. aureus carriage).* Participants were instructed not to perform a nasal rinse before their work shift and to avoid smoking tobacco or using e-cigarettes within 15 minutes before sample collection.

Sample Collection

A total of thirty-one participants were recruited from five dairy operations located in Northern Colorado and Texas. Exposure data, including air sampling and pulmonary function testing, were available for one hundred nineteen work shifts. Participants were enrolled in the study for multiple days across a workweek, ranging from 2-5 days; of these, 65% of the participants enrolled for 4 or 5 days. Samples were collected during the fall, winter and spring seasons. Two hundred thirty-eight lavage samples were collected from varying job tasks each day ranging from office workers, milkers, reproduction workers, veterinarians etc.

Nasal lavage samples were collected both before and after each shift, resulting in two samples per participant per day. For sample collection, participants were briefed on the Naclerio Method where they would be required to tilt their head back while a trained researcher administered 5 mL of lavage fluid into each nostril over approximately 10 seconds

(Figure 2) (Gritzfeld et al., 2011). After the saline was administered, volunteers were instructed to lean forward and allow the saline to drain from their nose into a sterile sample cup.

Figure 2 Example of Nasal Lavage Administration

After the collection of the nasal lavage, investigators measured the return with a serological pipette for an accurate quantification. The collected lavage sample was then transferred into sterile 15 mL conical vials. Protease Cocktail inhibitor (1% v/v) was added in the field to the lavage for the purpose of preserving cellular information for a study investigating inflammatory response. Samples were stored on ice until arrival at Colorado State University and stored at 6°C in the laboratory. A 1 mL aliquot was taken from the lavage sample 12 hours post collection for *S. aureus* detection and transferred to a cryogenic vial. Samples were mixed in a 1:1 ratio with 40% glycol stock to achieve a 20% solution for long-term preservation. This step was taken to prevent ice crystal formation, which could damage cell membranes and degrade proteins. Samples were then placed for long term preservation in a -80°C freezer. Preserved samples were thawed to room temperature prior to sample analyses.

As part of the intervention study design, participants were divided into two groups. One group received a hypertonic saline nasal lavage ([400 milliosmoles]), while the other group

received a normotonic saline lavage ([308 milliosmoles]). Samples collected for *S. aureus* detection were not impacted by this variation in sampling methods.

Sample Analysis

Media

Methicillin susceptible *S. aureus* (MSSA) analysis of the nasal lavages was performed at Colorado State University via culture-based methods. Preserved nasal lavage samples were allowed to slowly defrost (to room temperature) prior to media inoculation. All sample handling and media inoculation occurred in a biosecurity level II cabinet (Type II A), within an enclosed room, to protect researcher and samples from contamination. A sterile 10µL stainless steel inoculation loop was used to inoculate 100mm plates using lawn streaking technique (total of 30-40µL/ plate). A Fisherbrand ™ Bacti-Loop ™ Micro-Sterilizer was used to sterilize the inoculation loop between each streak and sample; the inoculation loop was allowed to cool prior to sample collection. Quality control was accomplished using a positive control with every sample batch (*Staphylococcus aureus ATCC 25923- MSSA)*. Inoculated media was incubated at 37°C for 24 hours in aerobic conditions and up to 48 hours for delayed growth. Procedure was repeated for all phase I media (TSA, CHROMagar™ Staph aureus, CHROMagar™ MRSA II, and Mannitol Salt Agar). After incubation, growth for each media was recorded “growth” vs “no-growth” for the non-selective media, and “positive” versus “negative” for selective and differential media (Table 2). Two to four positive colonies were collected and transferred to Brain Heart Infusion Broth and preserved with 20% glycerol solution and stored in -80°C freezer in preparation for later steps.

Isolated colonies preserved in BHI broth were later propagated on Tryptic Soy Agar (TSA), which is a general growth medium used for the cultivation, isolation, and storage of a variety of fastidious and non-fastidious microorganisms. Colonies propagated on TSA allowed for researcher to achieve 0.5 McFarland Standard required prior Antibiotic Sensitivity Testing. TSA Media plates were stored at room temperature per manufacturer recommendation prior to analysis.

CHROMagar™ Staph aureus is a chromogenic selective medium with high selectivity (>99.0%) and specificity results (Gaillot et al., 2000). *Staphylococcus aureus* growth is indicated by mauve colonies. Media plates were stored at 4°C and were allowed to reach room temperature prior to inoculation per manufacturer's recommendations. Pre-made media was ordered from DRG International Inc.

CHROMagar™ MRSA II is both a selective and differential medium for the qualitative detection of MRSA from the anterior nares (BBL CHROMagar™ MRSA II, 2017). The medium incorporates cefoxitin within the agar to select for methicillin resistant *Staphylococcus aureus*, and chromogenic substrates that indicate MRSA by chromatic changes (mauve colonies). Media plates were stored at 4°C and were allowed to reach room temperature prior to inoculation per manufacturer's recommendations. Pre-made media was ordered from DRG International Inc.

Mannitol Salt Agar (MSA) is a selective and differential medium for *Staphylococcus aureus* that contains a high concentration of salt (7.5%). The medium includes the food source mannitol, and phenol red (pH indicator). Very few organisms can tolerate the high salt levels of the media, and beyond that few can ferment mannitol (Brown et al., 2005). The fermentation of the mannitol releases an acidic byproduct that triggers the phenol red to change colors from

red to yellow (Brown et al., 2005). This is further selective for *Staphylococcus aureus*. Media plates were stored at 4°C and were allowed to reach room temperature prior to inoculation per manufacturer's recommendations.

Mannitol Salt Agar was used as the primary source of colonies for downstream PCR confirmation because it selectively enriches salt-tolerant *Staphylococcus* species while differentiating mannitol-fermenting organisms, allowing broader recovery of presumptive *Staphylococcus aureus* isolates prior to molecular confirmation. Unlike CHROMagar™ Staphylococcus aureus, which is optimized for rapid chromogenic identification and may preferentially select for strongly expressing phenotypes, MSA permits growth independent of color-dependent differentiation. Additionally, CHROMagar™ MRSA II contains cefoxitin as a selective agent, which may suppress growth of isolates with variable or low-level mecA expression. By subculturing colonies from MSA rather than relying exclusively on chromogenic media, this approach minimized potential bias introduced by antibiotic selection pressure and allowed recovery of a broader range of *S. aureus* strains, including those with heterogeneous or variable *mecA* expression. Molecular confirmation was therefore performed on isolates recovered under non–β-lactam selective conditions to enable more comprehensive detection of *mecA* carriage.

Antibiotic sensitivity/susceptibility testing (AST) is a technique used to determine if an organism is susceptible to an antibiotic or resistant. AST is commonly carried out via the Kirby Bauer disc diffusion protocol (Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, 2009; Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Edition., Standard—Eleventh, 2012; Nadimpalli et al., 2016). Cefoxitin, vancomycin, and tetracycline

were used to presumptively identify MRSA species. Samples were thawed and allowed to come to room temperature prior to testing. Samples were cultivated on TSA or Mueller Hinton agar 24-48 hours prior to AST. Two to four isolates were collected from the plates and mixed into sterile NaCl solution until a turbidity matching 0.5 McFarland Standard was achieved. With the new sample solutions, samples were lawn streaked onto Mueller Hinton agar, plates were allowed to dry prior to antibiotic disc placement. Antibiotic discs were impregnated with 30µg of each corresponding antibiotic. Once plates were dry (but no more than 15 min after streaking), antibiotic discs were placed equidistant from one another onto the plate. Plates were stored at 37°C for 18 hours (tetracycline) and 24 hours (cefoxitin and vancomycin).

Mueller Hinton agar II is a non-selective and non-differential media to grow a variety of organisms. This general media is also used as the recommended media to perform antibiotic sensitivity testing according to the Clinical and Laboratory Standards Institute (CLSI). The high starch concentration within the media helps absorb the released toxins from growing and dying bacteria, and in turn prevents them from interfering with the AST. Mueller Hinton media also contains a looser agar allowing for better antibiotic diffusion.

Table 2

Culture Media Used for Presumptive Identification of Staphylococcus aureus and MRSA

Culture Medium	Type	Purpose in Study	Positive Result Indicator
Tryptic Soy Agar (TSA)	Non-selective	Propagation of isolates and preparation for AST	General bacterial growth

CHROMagar™ Staphylococcus aureus	Selective / chromogenic	Presumptive identification of *Staphylococcus aureus*	Mauve colonies
CHROMagar™ MRSA II	Selective / differential	Presumptive MRSA detection	Mauve colonies in presence of cefoxitin
Mannitol Salt Agar	Selective / differential	Differentiation via mannitol fermentation	Yellow colonies
Mueller-Hinton Agar	Non-selective (AST standard media)	AST	Zone of inhibition measurement

PCR & Electrophoresis

DNA Extraction and purification was accomplished using Lucigen MasterPure™ Gram Positive DNA Purification Kit in preparation for PCR. Prior to PCR amplification, purified DNA samples were tested for purity/concentration via a NanoDrop. DNA samples had to achieve a ratio of absorbance (at A260/A280) of 1.7-1.8 to continue onto PCR amplification. PCR was accomplished using GoTaq® Green Master Mix for DNA amplification in preparation for gel electrophoresis. MRSA control strain ATCC 700699 (*mecA*-positive, SCC*mec* type II, reduced vancomycin susceptibility, *PVL*-negative) was used as a positive control for PCR amplification. Gene primers (forward and reverse) were purchased through Integrated DNA Technologies (IDT). Genes tested included *mecUP1-mecA, mecUP2-mecA, mecAP4, mecAP7, pvl1, pvl2, scn-1,* and *scn-2* (Table 3). UltraPure™ Agarose gel was used for electrophoresis.

Table 3

Study Workflow

Identification Stage	Method	Indicator	Interpretation
Culture isolation and screening	Selective and non-selective culture media	Colony growth and morphology	Presumptive identification of *Staphylococcus aureus* or MRSA
Phenotypic resistance screening	Antibiotic susceptibility testing (Kirby-Bauer)	β-lactam (cefoxitin) resistance	Suggests MRSA candidate isolate
Molecular confirmation	PCR	*mecA / mecA* from LA-MRSA (CC398) gene detection	Confirms MRSA classification
Lineage indicator	PCR	*scn, pvl* presence	Suggests human-adapted MRSA lineage
Species confirmation	MALDI-TOF MS	Protein fingerprint match	Confirms MRSA isolate species

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI- TOF-MS)

Fresh colonies of nasal lavage-derived isolates and positive controls were submitted for species identification and MRSA subtyping via MALDI Biotyper. A representative colony from each sample was harvested from the plate using a toothpick and then applied to two spots on the target plate. Spots were then overlaid with 1µl 70% formic acid to promote cell lysis and allowed to dry. Each lysed sample or bacterial test standard was overlaid with 1 µl of α-Cyano-4-hydroxycinnamic acid (HCCA, 10 mg/ml in 50% CAN, 2.5% TFA) and allowed to air dry. The sample was analyzed on a Microflex-TOF mass spectrometer (Bruker Daltonics, Billerica,

MA) in positive ion, linear mode using an ion source voltage of 20 kV. External calibration was performed using a protein calibration mixture (Bacterial Test Standard, Bruker Daltonics) on a spot adjacent to the samples. Data was collected and automatically processed using MALDI Biotyper Compass software (version 4.1.100.10, Bruker Daltonics). Spectra were searched against BDAL, IVD, Mycobacterium and Filamentous Fungi libraries as well as subtyping diagnostic peaks.

STATISTICAL ANALYSIS

RStudio Version 1.4.1103 was used to perform all statistical analyses. Because sample sizes were small, Fisher's exact test and McNemar's Test was used rather than chi-square testing. Fisher's Exact test was used to evaluate the relationship between mecUP detection and presence of the *scn* gene (Table 9). While Exact McNemar's Test was used to assess agreement between molecular detection of *mecA* (positive by either primer set) and phenotypic cefoxitin resistance.

Twenty-five samples underwent PCR testing. Genes of interest were *scn*, pvl and variations of *mecA*. Because PCR testing was performed on a subset of isolates rather than all nasal lavage samples, molecular detection frequencies are reported based only on the PCR-tested isolates, not the full two hundred thirty-six nasal lavages. We identified the profile indicative of LA-MRSA to be *mecA* positive (mecUP), *scn* negative, *pvl* negative, tetracycline intermediately resistant or resistant, and with any vancomycin resistance variability. In contrast, we identified CA-MRSA genetic profile to be *mecA* positive (mecAP), *scn* positive, *pvl* positive or negative, tetracycline susceptible, and vancomycin susceptible.

Presence of MSSA and MRSA was calculated as the proportion of culture-positive samples among all nasal lavage samples included in analysis (n = 236). Genetic confirmation of MRSA was defined by any isolate that was positive for mecA gene.

RESULTS

The average nasal lavage sample volume was 6.6mL. Participants occasionally swallowed some or all the saline, while it can also be assumed that some saline would be retained in the nasal passages due to investigator instructions to not blow their nose during sample collection process. Following sample preparation, two hundred thirty-eight nasal lavage samples (pre- and post-shift) were collected. Two samples did not produce enough volume to be divided between this study and the study investigating inflammatory response; the latter was prioritized for sample analysis. Two hundred thirty-six nasal lavage samples were included in this study. Demographics of the workers are summarized in Table 4, and the working tasks participants reported during their shift are in Table 5.

Table 4

Participant Demographics

Characteristic	**Percentage (%)**
Working at the dairy one year or longer	71.0
Smoker (current or former)	16.1
Male	77.4
Reported milking	9.7
Reported direct animal contact	54.8

Reported feeding or maintenance in parlor and stalls	29.0
Reported administrative work	9.7
Age Group	**Percentage (%)**
20-29 years old	32.3
30-39 years old	48.4
40-49 years old	9.7
50+ years old	9.7

Table 5

Participant Tasks Reported During Study Period

Category	**Task**
Production	Milking
Maintenance	Maintenance
	Milking parlor maintenance
	Lagoon/waste maintenance
	Repairing pens and gates in corral
Animal Care	Regular medical care
	Tending to sick or injured animals
	Hoof trimming
	Moving animals
	Mixing feed

	Feeding
	Re-bedding and scraping stalls in corral
Reproduction	Birthing
	Calving
	Breeding
	Husbandry
Administrative	Office work

Media testing

A total of 93 samples tested positive for MSSA (39%) using CHROMagar™ Staph aureus and Mannitol Salt Agar. Specifically, 22 samples were positive on MSA alone (9%), eight samples were positive on MSA and CHROMagar™ Staph aureus (3%), and one sample was positive on both MSA and CHROMagar™ MRSA II. Four samples (2%) tested positive for MRSA using CHROMagar™ MRSA II (Table 6).

Table 6

Prevalence of MSSA and MRSA in Media (n=236)

Outcome	Positive Samples (n)	Prevalence (%)
MSSA	93	39%
MRSA	4	2%

Antibiotic Sensitivity Testing

Results for vancomycin susceptibility indicated that 17 of 25 isolates (68%) were susceptible, seven (28%) demonstrated intermediate susceptibility, and one isolate (4%) was resistant, representing a potential public health concern. For tetracycline, 23 isolates (92%) were susceptible, while one isolate (4%) demonstrated intermediate susceptibility, and one isolate (4%) was resistant. Cefoxitin susceptibility testing showed that three isolates (12%) were resistant, while 22 isolates (88%) were susceptible (Figure 3).

Among the MRSA isolates identified on selective media (n = 4), all were susceptible to vancomycin. One isolate demonstrated cefoxitin resistance, and two isolates exhibited reduced susceptibility to tetracycline, including one intermediate and one resistant isolate.

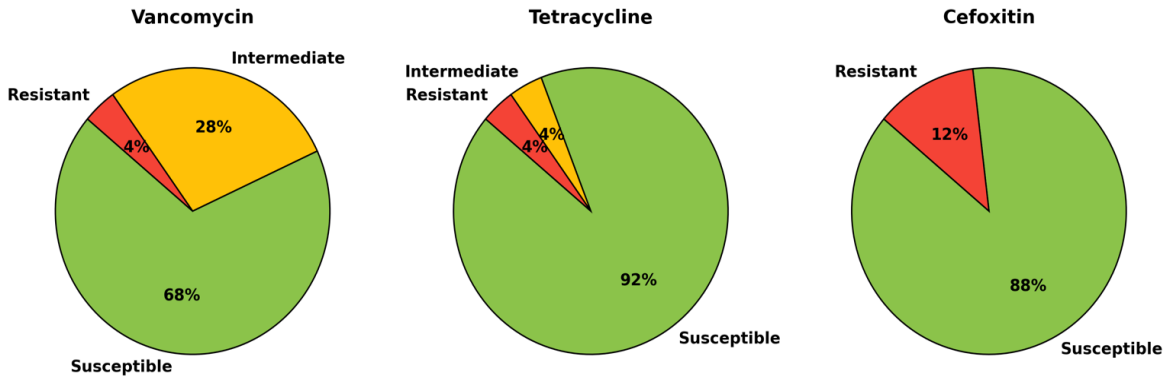

Figure 3 Antibiotic Susceptibility Results

PCR Testing

A subset of isolates (n = 25) underwent PCR testing targeting *mecA* gene variants, *scn*, and *pvl*. Fourteen samples tested positive for the presence of *scn* gene (56%). One sample tested positive for the *pvl* gene (4%) (Table 7). When results are organized by job category, lack

of *scn* gene detection was observed to be highest amongst reproduction, followed by administration (Figure 4).

Detection of the *mecA* gene varied by primer set. The mecUP primer set detected the *mecA* gene in 17 isolates (68%), while the mecAP primer set detected *mecA* in 10 isolates (40%). A total of 9 isolates (36%) tested positive for *mecA* using both primer sets. A summary of results can be found in Table 7. When results are organized by job category, mecA gene detection was observed to be highest amongst reproduction, followed by animal care and administrative duties (Figure 5). These findings indicate variability in *mecA* detection between primer sets (Table 8).

Table 7

PCR Gene Detection Results

<table>
<tr><th colspan="4">Gene(s) Detected</th><th>Positive Samples</th><th>Percentage (%)</th></tr>
<tr><td colspan="4">Scn +</td><td>14</td><td>56%</td></tr>
<tr><td colspan="4">PVL +</td><td>1</td><td>4%</td></tr>
<tr><td rowspan="5">mecA+</td><td rowspan="2">CC398 (LA-MRSA)</td><td>mecUP1</td><td rowspan="2">17 (68%)</td><td rowspan="5">18</td><td rowspan="5">72%</td></tr>
<tr><td>mecUP2</td></tr>
<tr><td rowspan="2">Classical/Clinical</td><td>mecAP4</td><td rowspan="2">10 (40%)</td></tr>
<tr><td>mecAP7</td></tr>
<tr><td colspan="2">Both</td><td>9 (36%)</td></tr>
<tr><td colspan="2">Neither primer set detected mecA</td><td>7 (28%</td><td></td><td></td></tr>
</table>

*Note***:** mecUP and mecAP represent two primer sets targeting the mecA gene. Percentages are calculated using the total number of PCR-tested isolates (n = 25).

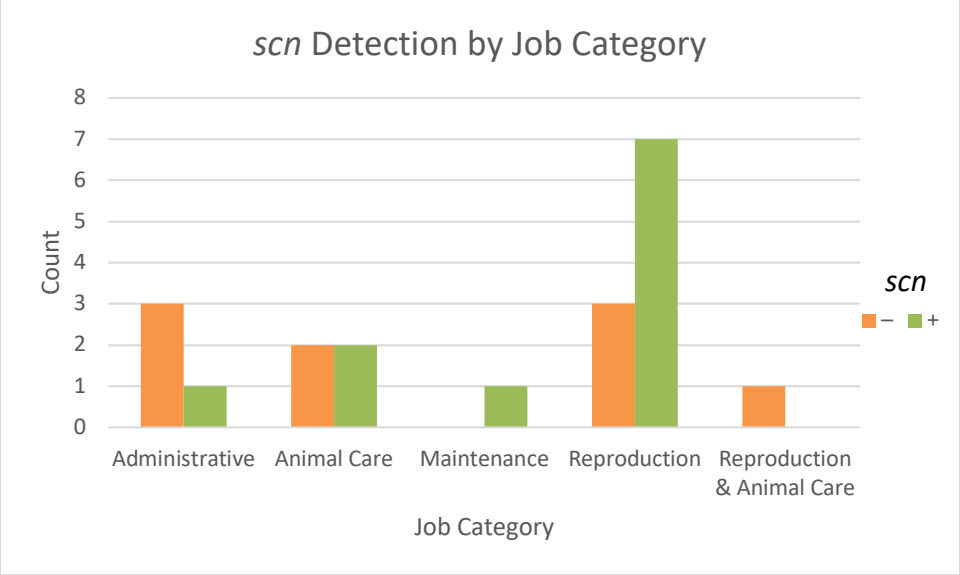

Figure 4 scn Detection by Job Category

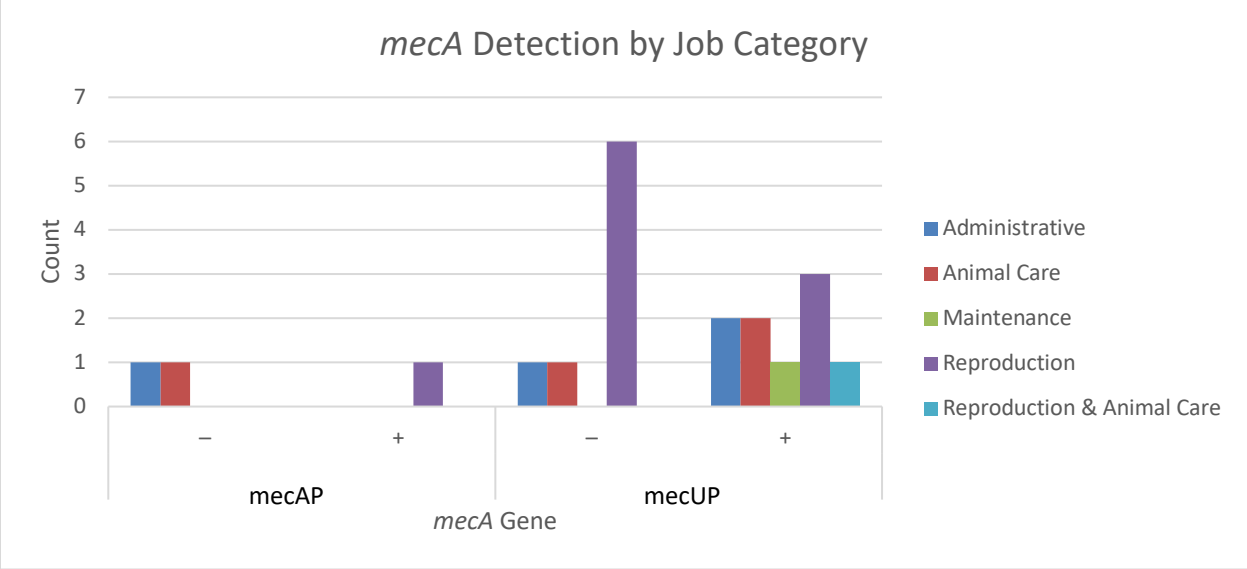

Figure 5 mecA Detection by Job Category

Table 8

Molecular and Antimicrobial Profiles of Presumed MRSA samples (n = 20)

Category	Task	Sample ID	scn	pvl	mecAP	mecUP	Vancomycin	Cefoxitin	Tetracycline
Reproduction	Birthing	3068 *	+	–	+	–	Susceptible	Susceptible	Susceptible
Animal Care	Medical/ Feeding/ Moving	3019	+	–	+	+	Susceptible	Susceptible	Susceptible
Maintenance	Maintenance	2042 *	+	–	+	+	Susceptible	Susceptible	Intermediate
Administrative	Office Manger	2002	–	–	+	+	Susceptible	Resistant	Resistant
Reproduction	Birthing	2074	+	–	+	+	Intermediate	Resistant	Susceptible
Administrative	Supervisor/ Office	3017	–	–	+	+	Susceptible	Susceptible	Susceptible
Reproduction	Birthing	2090	+	–	+	+	Susceptible	Susceptible	Susceptible
Reproduction	Supervisor/ Birthing	3097	–	–	+	+	Intermediate	Susceptible	Susceptible
Animal Care	Medical/ Mover	3016	–	–	+	+	Susceptible	Susceptible	Susceptible
Reproduction & Animal Care	Birthing/ Stalls/ Feeding	2089	–	–	+	+	Susceptible	Susceptible	Susceptible
Reproduction	Birthing	2085	+	–	–	+	Intermediate	Susceptible	Susceptible
Reproduction	Birthing	2092	+	–	–	+	Susceptible	Susceptible	Susceptible
Reproduction	Birthing	2076	+	–	–	+	Susceptible	Susceptible	Susceptible
Reproduction	Birthing	3098	+	–	–	+	Susceptible	Susceptible	Susceptible
Animal Care	Mover/ Hoof Trimming	3094	+	–	–	+	Intermediate	Susceptible	Susceptible
Reproduction	Birthing	3076	–	–	–	+	Intermediate	Susceptible	Susceptible
Reproduction	Birthing	3085	–	–	–	+	Intermediate	Susceptible	Susceptible
Administrative	Supervisor	2087	–	–	–	+	Intermediate	Susceptible	Susceptible

Administrative	Office Manger	3005 *	+	–	–	–	Susceptible	Resistant	Resistant
Animal Care	Medical/ Mover	2016 *	–	–	–	–	Susceptible	Susceptible	Susceptible

Note: *Among the CHROMagar™ MRSA II isolates (n = 4), three isolates (75%) were positive for the *scn* gene, two isolates (50%) were positive for mecAP, and one isolate (25%) was positive for mecUP. No MRSA isolates were positive for the *pvl* gene.

Detection of MRSA

Presence of MRSA was identified using phenotypic and genotypic methods. Eighteen isolates (72%) were *mecA* positive using at least one primer set. Cefoxitin resistance was identified in 4 isolates on selective media (16%). Considering both methods together 20 individual isolates were presumptive MRSA. These findings indicate that molecular markers of methicillin resistance were detected more frequently than phenotypic MRSA identified through selective media alone (Table 9).

Table 9

Detection of MRSA among PCR Tested Samples

Classification	n	%
mecA positive (any primer)	18	72%
Media-confirmed MRSA	4	16%
Total isolates with phenotypic or genotypic evidence of methicillin resistance	20	80%

Using Exact McNemar's test, agreement between the detection of the *mecA* gene and phenotypic cefoxitin resistance was assessed using the contingency table shown in Table 10. Out of the 25 isolates tested, 2 isolates were *mecA* positive and cefoxitin resistant, while 6

isolates were *mecA* negative and cefoxitin susceptible; Indicating significant discrepancy between methods (p =0.0002747; 95% CI: 0.0015- 0.29).

Table 10

Agreement between PCR mecA detection and cefoxitin resistance

	PCR mecA +	PCR mecA –	Total
Cefoxitin Resistant	2	1	**3**
Cefoxitin Susceptible	16	6	**22**
Total	**18**	**7**	**25**

LA-MRSA

Fisher's exact test identified no statistically significant association between markers associated with LA-MRSA (mecUP presence and *scn* absence) (p = 1). The estimated odds ratio was 0.68 (95% CI: 0.08–5.0), indicating that mecUP positive isolates had lower odds of being *scn*-negative compared to mecUP negative isolates (Table 11). However, this association was not statistically significant. This suggests that these markers were not clearly segregated within the isolate population and supports the presence of mixed molecular profiles (CA-MRSA) rather than a distinct livestock-associated strain.

Sample 2002 exhibited both genotypic and phenotypic characteristics consistent with MRSA. The isolate was mecUP positive (as well mecAP positive) and demonstrated cefoxitin-resistance, confirming methicillin resistance. The isolate was also *scn*-negative, *pvl*-negative and demonstrated tetracycline resistance, a profile in line with expected livestock-associated MRSA strains (CC398). Additionally, several other isolates demonstrated both mecUP presence and *scn* absence, a molecular profile indicating potential Livestock lineages. Isolates were examined across job categories, and reproduction accounted for most potential LA-MRSA isolates

followed by administration (Figure 6). Although confirmatory lineage typing was not conducted (MLST or spa typing), the combined molecular and antimicrobial resistance profile is suggestive of a livestock-associated MRSA strain.

Table 11

Contingency Table for Presumptive LA-MRSA (n=25)

	mecUP		
scn	Positive	Negative	total
Positive	9	5	14
Negative	8	3	11
total	17	8	25

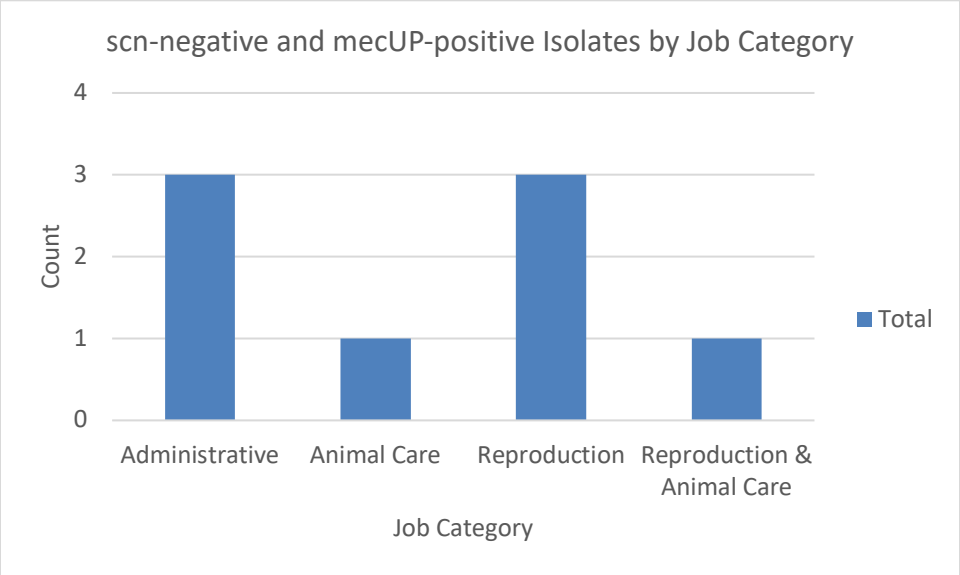

Figure 6 Potential Livestock-Associated Markers by Job Category

DISCUSSION

This study provides evidence that nasal carriage of *Staphylococcus aureus* was common among dairy workers represented in this study, with MSSA identified in 39% of nasal lavage samples. The recovery of MSSA from approximately one-third of samples is consistent with

background colonization rates reported in the general population and aligns with dairy farm studies documenting high relative abundances of gram-positive organisms, including *Staphylococcus*, within bioaerosols (Schaeffer et al., 2017; Shukla et al., 2017). In contrast, media culture confirmed MRSA prevalence was low (2%), lower than that reported among swine production workers, where occupational carriage has been documented at considerably higher levels (Nadimpalli et al., 2016; Rinsky et al., 2013).

Although culture-based MRSA detection was limited, molecular characterization revealed a more complex picture. Within the subset of isolates that underwent PCR testing, *mecA* was detected by at least one primer set in 72% of isolates. However, these molecular findings were not directly predicted in phenotypic cefoxitin resistance testing, and agreement between genotypic and phenotypic classification was poor. This discrepancy suggests variability in *mecA* expression, potential primer target differences, or heterogeneous bacterial populations within samples. The frequent detection of *mecA* in the absence of phenotypic cefoxitin resistance highlights the importance of distinguishing molecular carriage from phenotypic expressed resistance.

Assessment of lineage associated markers further demonstrated overlapping molecular characteristics. The mecUP primer set, targeting mecA variant associated with CC398, was detected in 68% of PCR-tested isolates. The *scn* gene, commonly present in human-adapted strains, and typically absent in classic livestock-associated MRSA, was identified in over half of isolates. A combined mecUP-positive and *scn*-negative profile was observed in 32% of isolates. However, these markers were not clearly segregated, and no statistically significant association was observed between mecUP detection and absence of *scn* using Fisher's exact test. This

pattern suggests molecular heterogeneity rather than dominance of a classic livestock-associated lineage (CC398).

In contrast to reports from swine operations, where CC398 isolates are often characterized by consistent absence of the immune-evasion cluster and frequent tetracycline resistance (Nadimpalli et al., 2016; Rinsky et al., 2013; Cuny et al.), tetracycline resistance was uncommon in this cohort. Together, these findings indicate that while markers consistent with potential livestock association were present, they were not sufficiently distinct to support definitive classification as classic (CC398) livestock-associated MRSA.

Taken collectively, the results support a heterogeneous *S. aureus* presence among dairy workers, characterized by overlapping human-adapted and livestock-associated genes. In addition, the presence of *mecA* gene variants and mixed lineage markers highlights the need for continued surveillance and more detailed sequence-based characterization to better understand transmission dynamics in dairy environments.

When analyzed by job category, mecA detection, and isolates demonstrating both mecUP presence and scn absence were observed across all categories. While reproduction roles accounted for the highest number of isolates demonstrating these characteristics, these markers were also identified in administrative categories, indicating they were not limited to high animal-contact roles. Indirect exposure pathways should be investigated for office workers to help identify risks. Overall, these findings highlight the complexity of Staphylococcus aureus colonization in this setting and suggest that both direct and indirect occupational exposures likely contribute to the observed patterns.

Public Health Relevance

From a clinical standpoint, the detection of vancomycin resistant and intermediately susceptible isolates is of concern, given the reliance on vancomycin as one of the last treatment options for multidrug-resistant staphylococcal infections. Even at relatively low presence, vancomycin resistant MRSA carriage among dairy workers has potential implications for secondary transmission to household contacts and for complicating therapy decisions for healthcare providers. Distinguishing between human adapted and livestock-associated MRSA has important prevention and community health implications; the mixed *scn* profile in this study underscores the need for further genetic characterization to guide targeted interventions.

Current interventions to control bioaerosols in the dairy setting is still a major challenge for the industry. Bioaerosol sources are vast while dairies operate on a 24-hour production schedule. Although engineering controls would be the most effective strategy, application may not be feasible for smaller operations. A study looking at the effectiveness of dairy parlor washing to reduce bioaerosols was effective in limiting bioaerosols, endotoxins were not statistically affected by the administrative control (Choudry et al.). Personal protective equipment is a common form of protection (e.g. N95 respirator) but are heavily reliant on proper use and maintenance to be effective forms of protection. Effectiveness of controls on dairy worker exposure to dairy bioaerosols is still an understudied area.

Limitations

Limitations include the sample size, and the cross-sectional sampling approach, which does not distinguish between transient and persistent carriage, in field processing, and media limitations. The limited sample size reduced the statistical power and prevented the ability to make statistically significant associations. $GPower_{50}$ was used to estimate a sample size of fifty-two per group (hypertonic vs isotonic saline lavage; N=104) for the main aim of HICAHS grant number 1U01OH010840, which was to evaluate the effectiveness of hypertonic saline nasal lavage for reducing inflammatory responses in dairy workers exposed to bioaerosols. Required sample size was not calculated for this study. Similar cross-sectional approaches reported samples sizes ranging from fifty participants up to one hundred and seventy participants.

The 12-hour holding time before aliquoting for freezing may have influenced culture yield. Also, a limitation of CHROMagar™ media is that, when challenged with a high microbial burden, it may produce false-negative results due to the heavy microbial load of a nasal lavage sample. In contrast, Mannitol Salt Agar (MSA) is less selective than CHROMagar™ *Staph aureus* and may be more likely to yield false-positive presumptive colonies. For this study, MSA was selected as the primary medium from which *S. aureus* colonies were propagated to capture potential MRSA isolates with variability in mecA expression that may not have been detected by CHROMagar™ MRSA II, which relies on cefoxitin-based selection. However, a limitation of this approach is that antimicrobial susceptibility testing was performed only on colonies isolated from MSA and CHROMagar™ MRSA II, rather than including colonies that grew exclusively on CHROMagar™ *Staph aureus*. As a result, susceptibility profiles may not fully represent all MSSA isolates identified during culture-based screening.

PCR testing was performed on a subset of isolates (n = 25) rather than all nasal lavage samples (n = 236). As a result, detection frequencies for *mecA* and other molecular markers cannot be interpreted as true prevalence estimates. While 72% of PCR-tested isolates were *mecA*-positive, this percentage reflects only the tested subset and cannot be extrapolated to the entire cohort. Therefore, molecular findings should be interpreted descriptively rather than as population level prevalence estimates.

Definitive lineage attribution was not possible, as sequence-based methods such as MLST, spa typing, or SCC*mec* subtyping were not performed. Although mecUP and *scn* markers were used to approximate potential livestock association, these markers alone are insufficient to confirm classic CC398 lineage or other LA-MRSA strains. The absence of additional livestock-associated markers, such as *tet(M)*, further limits definitive classification.

An additional limitation of this study relates to the use of MALDI-TOF mass spectrometry for MRSA subtyping. The subtyping module used in this system relies on detection of the PSM-Mec protein as a diagnostic marker. However, this protein is identified in European MRSA strains and is estimated to detect only a small proportion of MRSA strains circulating in the United States. Therefore, absence of a detectable PSM-mec peak cannot be interpreted as absence of methicillin resistance or used to definitively classify lineage among U.S. derived isolates. Because this diagnostic marker is not universally expressed, reliance on MALDI-TOF subtyping alone may underestimate strain diversity. As a result, definitive lineage attribution was not possible within this sample population, and more comprehensive sequence-based typing methods would be required for confirmation.

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