CARRIAGE OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS AUREUS* BY LIVESTOCK WORKERS AND HOUSEHOLD MEMBERS IN NORTH CAROLINA

Maya Nadimpalli

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Approved by:

Dr. Jill Stewart

Dr. Christopher Heaney

Dr. Rebecca Fry

Abstract

MAYA NADIMPALLI: Carriage of Antibiotic-Resistant *Staphylococcus aureus* by Livestock Workers and Household Members in North Carolina
(Under the direction of Dr. Jill Stewart)

The growing animal reservoir of antibiotic-resistant *Staphylococcus aureus* is of public health concern. Industrial livestock operations are a potential source of exposure to these bacteria, which may be transmitted from animals to workers, and then to the community. To increase our understanding of occupational exposures to antibiotic-resistant *S. aureus*, we assessed nasal carriage of *S. aureus*, including multidrug-resistant *S. aureus* (MDRSA) and methicillin-resistant *S. aureus* (MRSA), among workers and household members from industrial livestock operations, antibiotic-free livestock farms, and meat processing plants in North Carolina. We found a higher prevalence of MDRSA in industrial and processing plant participants compared with antibiotic-free participants, and comparable prevalence of MRSA. *S. aureus* belonging to clonal complex 398 was also discovered for the first time in North Carolina. This study contributes to the growing discourse regarding the public health consequences of large-scale antibiotic use in animal production in the United States.

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List of Common Abbreviations

ABF	Antibiotic-free
BP	Baird-Parker with Egg Yolk Tellurite Enrichment
CA	Community-associated
CAFO	Concentrated animal feeding operation
CC	Clonal complex
CoNS	Coagulase-negative staphylococci
CS	CHROMagar™ Staph aureus
HA	Hospital-associated
IND	Industrial
LA	Livestock-associated
MDRSA	Multidrug-resistant *Staphylococcus aureus*
MGE	Mobile genetic element
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
PBP	Penicillin binding protein
PFGE	Pulse-field gel electrophoresis

PP	Processing plant
pvl	Panton-Valentine leukocidin
REACH	Rural Empowerment for Community Help
SCC*mec*	Staphylococcal cassette chromosome *mec*
ST	Sequence type
spa	Surface protein A
WGST	Whole genome sequence typing

CHAPTER 1: INTRODUCTION

Staphylococcus aureus is a ubiquitous human commensal carried by approximately one third of the American population, primarily in the nostrils (Gorwitz et al., 2008). *S. aureus* is also an adept human pathogen capable of causing a wide range of infections, including skin and soft tissue infections, bacteremia, endocarditis, gastrointestinal illness, necrotizing pneumonia, post-operative infections, and toxic shock syndrome (Loir et al., 2003; Lowy, 1998). Antibiotics have been used to treat *S. aureus* infections for decades, but rapidly disseminating multidrug-resistant strains of *S. aureus* (MDRSA) have made treatment more protracted, more burdensome, and less successful in recent years (Chambers et al., 2009). Methicillin-resistant *S. aureus* (MRSA) has proven particularly difficult to treat.

Emerging in hospitals in the 1970s and rising dramatically in prevalence in the 1990s, MRSA is now established worldwide. These strains are resistant to β-lactam antibiotics, a commonly prescribed class of antibiotics that includes cephalosporins and carbapenems. Methicillin resistance is conferred by the *mecA* gene, which is typically acquired through horizontal gene transfer from another organism (García-Álvarez et al., 2011; Grundmann, 2006). The United States has higher rates of MRSA infection than most other industrialized countries, and American fatalities due to MRSA now surpass the combined annual mortality of HIV/AIDS, tuberculosis, and viral hepatitis (Bordon et al., 2010). Moreover, MRSA is no longer strictly a nosocomial infection. MRSA has evolved independently in the community, with cases occurring in healthy individuals having no connection to hospitals or other long-term care facilities. These highly virulent strains represent different genotypes from hospital-associated strains, and rates

of community-acquired MRSA infections are now rising faster than hospital-acquired MRSA infections in the United States. Community-acquired MRSA is currently considered epidemic in the United States (Kennedy et al., 2009).

Most recently, the evolution of a community-acquired MRSA strain associated with intensive livestock production, primarily swine production, has been discovered. Although this novel strain of MRSA is believed to have originated from human-associated methicillin-susceptible *S. aureus* (MSSA) strains (Price et al., 2012), it now persists in a livestock reservoir and can be transmitted between humans and animals. Multilocus sequence type 398 (ST398) was first detected in association with hog-farming in the Netherlands in 2004, and since then has been identified in several European countries as well as South America, China, Canada, and as of 2009, the United States (Arriola et al., 2011; Khanna et al., 2008; Smith et al., 2009; Wagenaar et al., 2009). Livestock-associated MRSA is transmitted not only to farm workers, with observed carriage rates between 23% and 49%, but their families as well (Huijsdens et al., 2006; R. Köck et al., 2009). MRSA and MSSA ST398 infections have also been documented in the greater community (Jiménez et al., 2011), including in individuals with no clear connection to livestock production (Bhat et al., 2009; Golding et al., 2010; Mediavilla et al., 2012; Skov, 2011; Uhlemann et al., 2012).

Carriage of MDRSA and MRSA by North Carolina farm workers and their household members has not yet been investigated. North Carolina is the second largest producer of pork in the country and the third largest producer of poultry. The vast majority of these animals are housed from birth to slaughter in industry-owned concentrated animal feeding operations (CAFOs). North Carolina CAFOs confine thousands of animals in relatively small spaces, produce an estimated 20 million tons of concentrated animal waste annually, and typically dose livestock with low levels of antibiotics to promote growth and prevent disease (Cochran et al., 2000).

North Carolina, however, is also home to a burgeoning sustainable farming movement (Greene, 2010). These farms also raise pork and poultry, along with other animals and produce, without the use of antibiotics, and with limited reliance on external inputs. Given that the persistent, subtherapeutic dosing of animals with antibiotics could create a selective pressure for antibiotic-resistant bacteria which could then be transmitted to humans (Khachatourians, 1998; Pew Charitable Trusts, 2008), a comparison of MDRSA and MRSA carriage rates among industrial workers and their household members to carriage rates among antibiotic-free farmers and their household members merits investigation.

Objectives

The goals of this research were four-fold:

1) To compare the prevalence of carriage of MDRSA and MRSA in 100 consenting industrial livestock workers and household members to 100 antibiotic-free livestock workers and household members in North Carolina;
2) To compare antibiotic resistance patterns among *S. aureus*-positive individuals in both groups;
3) To use lineage-specific PCR to determine whether CC398 is present in North Carolina; and
4) To compare the sensitivity and specificity of two differential media, Baird Parker and BBL™ CHROMagar™ Staph aureus, in their capacity to detect *S. aureus* from nasal swabs.

The findings contained in this Master's thesis will serve as a foundation for a more comprehensive evaluation of antibiotic resistance related to livestock production in North Carolina.

BACKGROUND

Industrial Animal Production in the United States

Within the past sixty years, animal production in the United States has shifted from many small, diversified family farms to fewer, larger, and highly specialized factory farms. Today, factory farming produces the vast majority of food animals raised and slaughtered in the US, and a small number of swine and poultry corporations oversee every aspect of the animal production process. This vertically integrated system can be characterized by standardization of feed, selective in-breeding of animals, and mechanization of feeding, watering, and other husbandry activities and aims to produce a more uniform meat product for the American consumer. The number of animals that are raised on each animal production operation has also surged – between 1994 and 2008, the number of animals per swine operation increased by 180%, while the number of chickens per broiler operation increased by 130% (Pew Charitable Trusts, 2008). By raising more animals within a concentrated, confined space, industrial operators are able to create "economies of scale," shorten the time between birth and slaughter, and increase their profits. As a result of industrialization, the American consumer pays less for meat - in 1970, the average American spent 4.2% of his or her disposable income to purchase 194 pounds of red meat and poultry, while in 2005, Americans spent only 2.1% of their disposable income to purchase 221 pounds of red meat and poultry (Pew Charitable Trusts, 2008). However, these prices do not reflect the human health and environmental costs of industrial animal production, which are instead externalized to society.

Industrial Animal Production: Public Health Concerns

Environmental and health concerns

Animal feeding operations in the United States produce an estimated 500 million tons of waste per year, three times more than the human population (United States Environmental Protection Agency, 2003). Roughly 47% to 60% of this waste is generated by CAFOs (Greger et al., 2010). Waste is collected and stored in large pits beneath caged animals or flushed into outdoor, open-air lagoons, which may serve as optimal breeding grounds for the bacteria that propagate antimicrobial resistance. In North Carolina, no further treatment is required of CAFO liquid manure before it is eventually sprayed onto fields. Over application of manure can lead to runoff laden with harmful pollutants, including nutrients, pesticides, heavy metals, veterinary pharmaceuticals, resistance genes, and infectious pathogens (Figure 1); these pollutants can also leach through permeable soil into aquifers. Lagoons themselves often leak, overflow, or rupture, which can also contaminate groundwater and surrounding surface waters (Sapkota et al., 2007; Steve Wing et al., 2002). Exposure to waterborne contaminants can be particularly serious for vulnerable populations, including children, pregnant women, and the elderly (Reynolds et al., 2008).

Air quality concerns are equally as pressing. Within confinement buildings, workers can be exposed to several categories of irritants, including bioaerosols, dust, toxic gases, and vapors from decomposing animal waste (Cole et al., 2000). The health effects of some of these toxic gases, including hydrogen sulfide, methane, ammonia, and carbon monoxide, are well-documented (Cole, et al., 2000). However, thousands of gases and vapors are responsible for the malodor of CAFOs, most of which are difficult to quantify and whose health effects are unknown (Cole, et al., 2000). Bioaerosols, including bacteria, viruses, fungi, and endotoxins, and

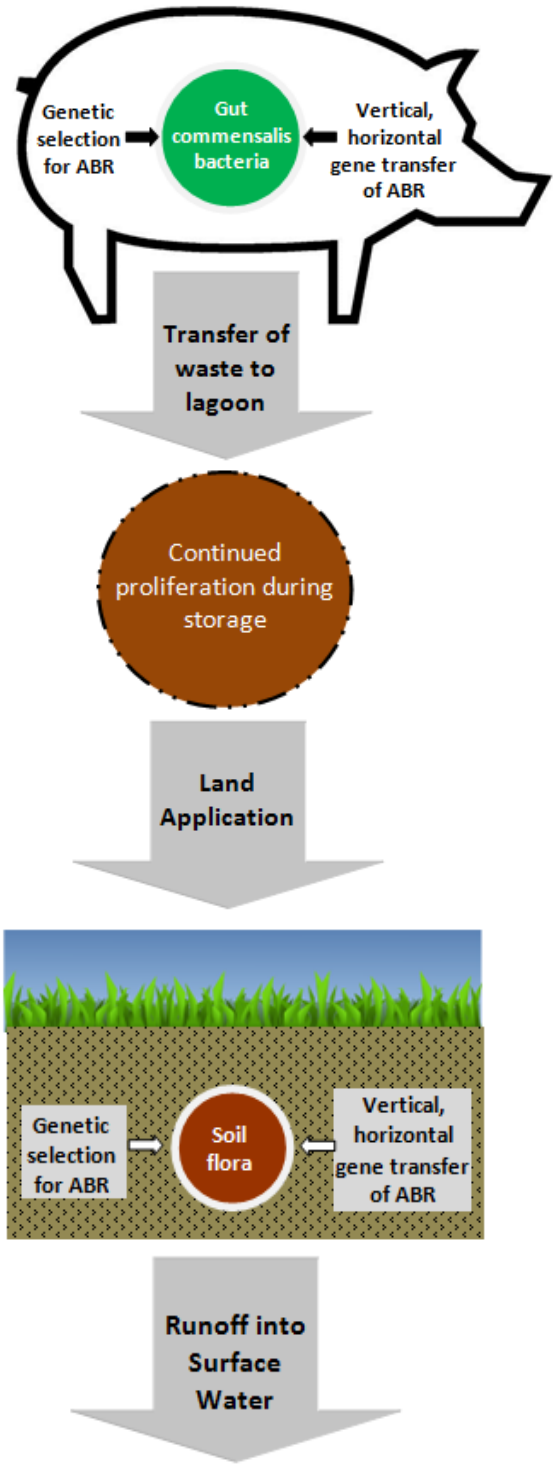

Figure 1. Proliferation and fate of antibiotic resistant bacteria during CAFO waste storage and disposal. ABR = Antibiotic resistance.

dust from animal dander and waste pose further health risks. Acute occupational exposure to any combination of these irritants can result in shortness of breath, coughing, and inflammation of the lungs and mucous membranes; chronic exposures have been linked to bronchitis, asthma, and wheezing (Cole, et al., 2000). These health effects are also observed in the communities that surround CAFOs, due to both direct emissions and aerosolization of particulates during spraying. In combination with degraded mental and social health, lower quality of life measurements are often reported in such communities (S. Wing et al., 2000).

Unfortunately, the environmental and health burdens of industrial animal production are not distributed equally among those who receive the "benefits" of this system. Instead, CAFOs tend to be disproportionately situated in areas of high poverty and a high percentage of non-whites (S. Wing, et al., 2000). These

populations are often most susceptible to the environmental and public health harms produced by CAFO emissions and the land application of swine waste, due to lack of access to medical care, poor nutrition, and other risk factors. Furthermore, these groups often have the least political power to impact policies and practices related to industrial livestock operations in their communities.

Propagation of antimicrobial resistance

Twenty million pounds of antibiotics, or 80% of all antibiotics sold in the United States, are fed each year to industrially-raised animals (Food and Drug Administration, 2009). Antibiotics are used to treat sick or diseased animals, but are also chronically fed to healthy food animals for the purposes of prophylaxis and growth promotion. It is estimated that approximately 85% of the antibiotics given to food animals each year are for these latter two purposes (Pew Charitable Trusts, 2008). However, more than 50% of the types of antibiotics licensed for use in pigs are also used in human medicine (Mellon et al., 2001), and approximately 75% of the nearly 24.6 million pounds of antibiotics consumed for nontherapeutic purposes by livestock each year is excreted (Chee-Sanford et al., 2009). Industrial animal production has thus been linked to an increased animal and environmental reservoir of novel antimicrobial-resistant pathogens (Frank M. Aarestrup, 1995; Mathew et al., 1999). Of serious public health concern is the close contact between workers and livestock in CAFOs, with the potential for transfer of antibiotic-resistant pathogens to workers, and subsequently the surrounding community. Handling or consumption of meat contaminated by antibiotic-resistant bacteria represents another potential exposure route, as multidrug-resistant pathogens, including *S. aureus*, have been repeatedly isolated from US retail meat and poultry (Gilchrist et al., 2007; O'Brien et al., 2012; Waters et al., 2011).

In response to these concerns, leaders in Congress have called for an end to the use of clinically-relevant antibiotics in food animal production in the "Preservation of Antibiotics for Medical Treatment Act" (H.R. 965/S. 1211; PAMTA). In 2012, the FDA announced plans to place controls on the use of cephalosporins in animal production. More recently, the FDA was also court mandated to withdraw approvals for most non-therapeutic uses of penicillins and tetracyclines in food animals. However, PAMTA is still being debated, and any further regulation by the FDA may take years. Unfortunately, legislative and regulatory action could be outpaced by the evolution and dissemination of novel antimicrobial mechanisms.

Food animal production in North Carolina

Industrial animal production has grown rapidly in North Carolina in the past few decades, with the state moving from fifteenth to second in hog production nationally (Table 1), and from fourth to third in poultry production. North Carolina's hog CAFOs are concentrated in the southern Piedmont and in the eastern coastal plain of the state, with the highest densities occurring in Duplin and Sampson counties. Together, hog production in these two counties accounts for nearly half of the state's total production. Growth has leveled off in the last several years due to a moratorium on permitting new hog CAFO liquid waste storage lagoons (North Carolina Department of Agriculture and Consumer Services, 2010). Meanwhile, poultry production in North Carolina has increased in recent years. Turkey production is highest in Duplin and Sampson counties, but egg production occurs in the central part of the state and broiler production is increasing throughout the state.

As it has across the United States, organic agriculture has expanded in North Carolina, particularly in the last decade (Table 2). Organic farms are largely clustered in the central and western regions of the state. The total value of organic sales in 2008 was just above $50 million,

or 1.7% of total US sales (United States Department of Agriculture, 2010). North Carolina is the largest retailer of organic agricultural products in the Southeastern US.

Table 1. Shift in NC hog production from 1982-2007

Year	Number of Operations	Number of hogs
1982	11,390	2.0 million
2007	2,836	10.1 million

Source: (Chee-Sanford, et al., 2009)

Table 2. Growth in NC organic operations from 1997-2007

Year	# of operations	# of cows, pigs, and sheep	# of poultry
1997	4	0	29,700
2007	112	156	1,088,860

Source: (Greene, 2010)

Staphylococcus aureus

Staphylococci are ubiquitous, gram-positive cocci which tend to cluster in grape-like bunches or in short chains. Most staphylococci are 0.5 to 1 μm in diameter, nonmotile, halotolerant to concentrations of 10% w/v NaCl, grow at temperatures between 18° and 40°C, and are aerobic or facultatively anaerobic (Murray et al., 2002). Typically, staphylococci are found in association with the skin, skin glands, and mucous membranes of warm-blooded animals (Crossley et al., 1997). Staphylococci are one of the most common bacteria to cause disease in humans, and in the United States, staphylococci are the most common cause of nosocomial infection (Crossley, et al., 1997). Of at least 32 species, *Staphylococcus aureus* is the most pathogenic and consequently the best researched (Crossley, et al., 1997).

The structure of *S. aureus* and its production of virulence factors and exotoxins are critically related to its fitness as a human pathogen. Like all staphylococci, *S. aureus* has a thick cell wall composed of peptidoglycan, and several serotypes also have a protective polysaccharide capsule that facilitates adherence to surfaces and evasion of the immune system

(Murray, et al., 2002). *S. aureus* can be distinguished from other species of staphylococci by its ability to produce coagulase, a protein which binds fibrinogen in the blood and converts it to insoluble fibrin, thus localizing an infection and protecting the bacteria from immune response cells. *S. aureus* also uniquely produces surface protein A, a cell-wall bound protein which is involved in increased pathogencity of the organism. *S. aureus*' fitness is further enhanced by its production of staphyloxanthin, a carotenoid pigment that acts as a protective antioxidant while imparting a characteristic golden color to *S. aureus* colonies. Several strains of *S. aureus* are capable of producing a variety of exotoxins, including TSST-1 (associated with toxic shock syndrome), exfoliative toxins (associated with staphylococcal scaled skin syndrome), staphylococcal enterotoxin B (associated with food poisoning), and α toxin, β toxin, and Panton-Valentine leukocidin (all associated with necrotizing pneumonia) (Lowy, 1998). The production of these proteins and toxins allows *S. aureus* to cause a wide diversity of benign and lethal infections.

Though humans and animals are its primary reservoir, *S. aureus* has also been detected in air (Bassetti et al., 2005), in drinking and waste waters (LeChevallier et al., 1980; Rosenberg-Goldstein, 2010), and in food (Loir, et al., 2003). It has been demonstrated that *S. aureus* can persist in river and sea water for up to two weeks (Tolba et al., 2008), as well as on dry surfaces for days to months (Kramer et al., 2006). *S. aureus* has also been detected in hog and poultry waste (Dimitracopoulos et al., 1977; Graham et al., 2009).

Epidemiology of S. aureus carriage

Though *S. aureus* can be highly pathogenic, only a fraction of the *S. aureus* that exists in the human population actually causes disease. According to data from the 2001-2004 National Health and Nutrition Examination Survey (NHANES), approximately 1/3 of American men and women harbor *S. aureus* in their nasal passages (Gorwitz, et al., 2008), which is the preferential

site for *S. aureus* colonization. An additional 20% of the population carry it on their skin, hair, or in their throats (Bhatia et al., 2007). Nasal carriage of *S. aureus* is not evenly distributed among the US population. Rather, colonization with *S. aureus* has been found to be significantly more common among males, compared with females ($p<.001$), among non-Hispanic whites and Mexican Americans, compared with non-Hispanic blacks ($p<.001$ and $p<.01$, respectively), and among persons aged <20 years, compared with older persons ($p<.001$) (Gorwitz, et al., 2008). Rates of staphylococcal colonization are higher than average among intravenous drug users, individuals with type 1 diabetes, and AIDS patients (Lowy, 1998). Significant associations between obesity and *S. aureus* carriage have also been described (Gorwitz, et al., 2008). Importantly, carriage of *S. aureus* can be transient. Longitudinal studies have described three patterns of *S. aureus* carriage in the population: persistent carriers, who are thought to comprise 20% of the population, persistent non-carriers, who are thought to comprise an additional 20% of the population, and intermittent carriers, who are thought to comprise the remaining 60% of the population (Peacock et al., 2001). Though concrete population determinants of carriage remain speculative, race, genetics, immune characteristics, strain diversity, and environment may play a role. While nasal carriage may be a risk factor for infection, most individuals who are colonized never become infected (Gorwitz, et al., 2008). Eradication of nasal colonization via mupirocin treatment may or may not prevent subsequent *S. aureus* infection (Wertheim et al., 2005).

Carriage of methicillin-resistant strains of *S. aureus* is far less common than carriage of *S.* a*ureus* in general. As of 2005, MRSA was estimated to be carried by 1.5% of the US population, though rates were steadily increasing (Gorwitz, et al., 2008). Risk factors for MRSA acquisition include hospitalization, use of antibiotics, previous colonization with MRSA, drug use, old age, low socioeconomic status (Charlebois et al., 2002; Gorwitz, et al., 2008),

imprisonment (Aiello et al., 2006), use of work-out gyms (Kirkland et al., 2008), and pig farming (Voss et al., 2005). While hospital-acquired MRSA strains were once predominant in the US, they are largely being replaced by community-acquired strains. Longitudinal studies have demonstrated that increasing rates of MRSA infection in the US in the early 2000s were linked to an expanding reservoir of such community-associated strains (Carleton et al., 2004).

History of antibiotic resistance in *S. aureus*

In the 1940s, penicillin became the first commercially available antibiotic, and its discovery heralded a new age of pathogen control. Almost simultaneously, however, there came reports of certain staphylococci that could destroy the antibiotic through production of an enzyme then termed "penicillinase." Penicillin acts by binding to and inhibiting penicillin binding proteins (PBP), which are integral in catalyzing the peptidoglycan cross-links in bacterial cell walls. As bacteria continue to naturally degrade peptidoglycan links without the capacity to construct new ones, their cell structure weakens, resulting in lysis. Penicillinase, encoded by the *blaZ* gene, hydrolyzes the β-lactam ring of penicillin before it can act, thus protecting bacteria from the antibiotic's mode of action (Lowy, 1998). As early as 1946, it was estimated that 60% of hospital *S. aureus* isolates in the UK were resistant to penicillin; current estimates top 90% (Crossley, et al., 1997). Penicillin-resistance is now nearly ubiquitous in *S. aureus* (Crossley, et al., 1997).

Methicillin was introduced into clinical practice in the early 1960s to combat the surge of penicillin-resistant *S. aureus*. However, only a few years later, the first methicillin-resistant strains of *S. aureus* were isolated. Though the mechanism for the observed resistance was not discovered until 20 years later, it was immediately clear that this mechanism was wholly independent from penicillinase production (Chambers, et al., 2009). While penicillinase production confers only a narrow range of resistance, methicillin-resistance confers resistance

to all β-lactam antibiotics, including carbanepems and cephalosporins. The gene responsible for this resistance, *mecA*, encodes for an altered penicillin-binding protein (PBPa), which has a lower affinity for all β-lactam antibiotics. When bacteria produce PBPa, cell wall synthesis is able to proceed unaffected in the presence of β-lactams. At least 4 distinct waves of *mecA*-encoded methicillin-resistance have been observed, resulting in the global MRSA pandemic occurring today.

Vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA) have emerged in the last decade as a result of the rising use of vancomycin to treat MRSA infections. However, VISA and VRSA infections remain few in number and have been isolated exclusively in the healthcare setting (Chambers, et al., 2009). VRSA carry the *vanA* gene cassette, presumably obtained from vancomycin-resistant *Enterococcus* (Chang et al., 2003). Carriage of this cassette appears to present a significant burden on the bacteria, which may account for the limited spread of VRSA in hospitals and its absence from the community (Foucault et al., 2009). The mechanisms for low-level and intermediate vancomycin resistance remain unclear, though certain morphological changes in VISA, such as cell wall thickening and intensive pigmentation, have been observed (Renzoni et al., 2010).

Presently, *S. aureus* has developed a resistance mechanism for every class of antibiotics ever produced by humans, including many last-line-of-defense antibiotics (Skov, 2011). As is clear from its long history of antibiotic resistance, these organisms have the alarming propensity to rapidly disseminate resistance mechanisms when selective pressures exist. Reducing drug resistance in *S. aureus* will likely require a more judicious use of antibiotics in humans and animals, prevention strategies rather than control, and perhaps the development of alternative treatments, such as vaccines (Daum et al., 2011).

Mechanisms for antibiotic resistance

One of the most important mobile genetic elements (MGEs) encoding resistance in *S. aureus* is the Staphylococcal Cassette Chromosome *mec* (SCC*mec*; see Figure 2). SCC elements are genomic islands that are ubiquitous among staphylococci; SCC*mec* contains the *mecA* gene and is most commonly associated with MRSA (although partial, nonfunctional SCC*mec* sequences – known as "ghost sequences" - have been detected in MSSA) (Corkill et al., 2004). SCC*mec* elements are 20 - 66 kb in length and are integrated at the 3' end of the *orfX* gene, whose function remains unknown. These elements carry two major complexes: the *mec* complex, encoding the *mecA* gene and two regulatory genes (*mecI* and *mecRI*), and the *ccr* complex, which encodes for one or two site-specific cassette chromosome recombinases, allowing the SCC*mec* element to be excised and inserted into new genomes (Deurenberg et al., 2007). Between these complexes and the 3' and 5' ends of the SCC*mec* lie three junkyard, or joining regions: J3, located between *orfX* and *mec*, J2, located between *mec* and *ccr*, and J1, located between *ccr* and the chromosomal region flanking the *SCCmec*. Several allotypes of the *mec and ccr* complexes have been identified, and SCC*mec* elements can be typed based on these differences. SCC*mec* types I-VIII are the most well-known, though several other types likely exist. Subtyping of SCC*mec* is also possible by classifying structural differences in the J1, J2, and J3 regions. In addition to the *mecA* gene, certain SCC*mec* complexes (namely II and III) encode additional drug resistance genes on integrated mobile elements, such as plasmids and transposons (Skov, 2011).

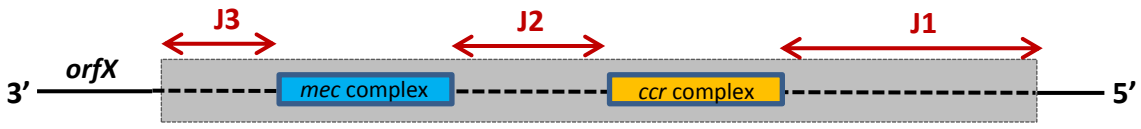

Figure 2. Skeletal representation of the *SCCmec* element.

S. aureus can carry resistance genes inserted at other sites on the chromosome besides SCC*mec*, as well as on plasmids (Deurenberg, et al., 2007). Additionally, *S. aureus* can develop resistance through random mutation of existing genes. Such mutations require selective pressure to maintain themselves in subsequent generations.

Molecular typing of *S. aureus*

Molecular typing originally evolved as an epidemiological tool to track the dissemination of MRSA strains into regional and global populations. However, any strain of *S. aureus* can be typed by any of these same methods. The typing methods that will be discussed here are: pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), typing of the variable tandem repeat region of surface protein A (*spa* typing), and whole genome sequence typing (WGST).

Pulsed-Field Gel Electrophoresis

PFGE is typically considered the gold standard for typing *S. aureus* isolates, as it is one of the most discriminative methods for studying outbreaks and transmission patterns (Deurenberg, et al., 2007). PFGE typing of *S. aureus* involves the digestion of chromosomal DNA with the restriction enzyme *Sma*1, followed by agarose gel electrophoresis. *Sma*1 cuts genomic DNA at specific, infrequent restriction sites, resulting in ten to twenty very large DNA fragments. These fragments are too large to be separated by traditional gel electrophoresis; however, by constantly changing the direction of the electric field during electrophoresis, PFGE is able to facilitate an efficient separation. PFGE patterns are analyzed using the Dice coefficient and unweighted pair-group matching analysis (UPGMA) settings, allowing for identification of a species and subtype (Deurenberg, et al., 2007). Unfortunately, because small changes in lab protocol can result in different PFGE patterns, inter-lab reproducibility and pattern comparison

are difficult if standardized protocols are not used. Thus, standardized PFGE databases have only been achieved at the national level (Deurenberg, et al., 2007). Additional drawbacks of PFGE typing include high cost and time requirements, and the need for trained lab personnel. ST398 - the strain associated with intensive livestock production, is also "nontypeable" by PFGE using *Sma*1 (Voss, et al., 2005).

Multilocus Sequence Typing

MLST is a relatively new typing method that also demonstrates excellent discriminatory power (Maiden et al., 1998). MLST involves sequence analysis of 0.5-kb internal fragments of seven *S. aureus* housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yquiL*. Differences in these sequences correspond to distinct alleles for each housekeeping gene. Each allele is assigned a unique number; any *S. aureus* isolate can be identified by its allele profile for these seven genes, also known as its "sequence type." A major advantage of MLST over PFGE is that sequence data are truly commutable across laboratories and across countries. Currently, there exists one global database containing all known sequence types (http://mlst.net), to which any researcher can upload new alleles. Global epidemiological investigations of MRSA and MSSA infection are thus possible using MLST. Unfortunately, like PFGE, MLST is also time-consuming and expensive.

MLST does allow some insight into the clonal evolution of *S. aureus*. *S. aureus* strains are grouped into the same "clonal complex" (CC) when 5 out of their 7 housekeeping genes have identical sequences. The founder of a CC is calculated as the ST that differs from the largest number of other STs at only a single locus, rather than the ST that is detected most frequently (as this is subject to sampling bias). This methodology for determining a CC founder takes into account the way clones actually emerge and diversify. Subgroup founders are single-locus or double-locus variants of a CC founder. These variants may become prevalent in the population,

and may diversify independently to produce their own set of single-locus and double-locus variants (Deurenberg, et al., 2007). A constantly updated diagram depicting all known clonal complexes and their relationships to one another is available at http://eburst.mlst.net.

Spa typing

Spa typing is even more discriminatory than MLST, and is much less costly since it involves sequencing of only a single locus. The gene encoding surface protein A is unique to *S. aureus* and contains a polymorphic region X, which is characterized by one to twenty-five 24-bp tandem repeats. Diversity in repeats is attributed to duplications of repeats, deletions, and less commonly, mutations (Deurenberg, et al., 2007). However, the first repeat in region X always begins with the sequence GAG, while subsequent repeats always begin with AAA (M. Sørum, personal communication, November 26, 2011). Sequencing the *spa* gene reveals how many tandem repeats are present in a strain, which repeats they are, and in what order they occur; this corresponds to a certain *spa* type. Researchers can identify *spa* types as well as upload novel spa types using a central *spa* server, available at http://www.spaserver.ridom.de. Because of its simplicity, low cost, and the fact that it can be performed using "in-house" technologies, this typing method is used widely in hospitals and research labs (Deurenberg, et al., 2007; Sørum, 2011).

Usually, once the *spa* type of a specific *S. aureus* strain has been determined, it is possible to infer its sequence type and therefore the clonal complex to which it belongs (Nulens et al., 2008). However, it is important to note that s*pa* typing and MLST result in two different sets of data that are not always comparable. Inferences are usually permissible because there are several known *spa* types that correspond to a single ST type. However, chromosomal recombination has occurred among different *S. aureus* lineages, complicating such assumptions (Nulens, et al., 2008). For example, CC8, has acquired part of its core genome from CC30,

including its *spa* gene. As a result, *spa*-CC012 has a heterogeneous clonal structure that includes both CC8 and CC30, and its ancestry cannot be inferred. To be absolutely sure of a strain's sequence type and clonal complex, MLST must be performed in addition to *spa* typing (M. Sørum, personal communication, November 26, 2011).

Additionally, there exist two competing nomenclature systems for *spa* types which are used worldwide (Harmsen et al., 2003; Koreen et al., 2004). This renders comparison of published *spa* typing results considerably more difficult than comparison of MLST typing results.

Whole genome sequence typing

Whole genome sequence typing (WGST) represents the newest, best available method for tracking *S. aureus* subtypes at both the global and regional scale. Though PFGE is considered the gold standard for typing *S. aureus* isolates and for epidemiological investigations, it is only able to discriminate interregional isolates – i.e., it lacks the power needed to discriminate subtle intraregional variability (Harris et al., 2010). Such discriminatory power is needed to understand the microevolutionary events that lead to clonal differentiation. Unlike full-genome sequencing, which is economically unfeasible and too time-consuming for large population samples, WGST instead maps single nucleotide-polymorphisms (SNPs) in given strains to a reference genome. Next-generation sequencing technologies allow multiple bacterial isolates at be mapped at once, thus making it feasible to quickly generate whole-genome sequence data for a large number of bacteria (Harris, et al., 2010). From this sequence data, the SCC*mec* type, sequence type, and *spa* type for any given bacterial isolate can be determined. Additionally, resistance genes, virulence genes, and other MGEs that may be incorporated in the accessory genome are revealed.

Data produced by WGST is especially useful for generating maximum likelihood phylogenetic trees, which are constructed by comparing the variable sites in the core genomes

of sequenced bacteria. *S. aureus* isolates from specific geographical regions tend to cluster on such trees; clades that are basally located elucidate where an *S. aureus* strain may have originated (Harris, et al., 2010). Phylogenetic trees can also explain whether or not a certain strain was introduced to a country or if it evolved independently. If it was introduced, its origin can typically be determined. Phylogenetic trees constructed from WGST data were used to determine the source of the recent cholera outbreak in Haiti (Hendriksen et al., 2011), as well as to corroborate the assumed source of MRSA ST398 in two adopted Chinese children in Denmark (Stegger et al., 2010). WGST data potentially has sufficient discriminatory power to reveal fine-scale transmission events between or within single hospitals (Harris, et al., 2010). PFGE and *spa* typing findings do not always correspond with evolutionary relationships delineated by WGST (Harris, et al., 2010), suggesting WGST may be a superior tool for epidemiological investigations.

WGST is currently a more expensive *S. aureus* typing method, although prices for next-generation sequencing technology are declining rapidly. Bioinformatics expertise is also required to interpret WGST data and to construct phylogenetic trees. Therefore, WGST may not yet be suitable for routine use outside of highly specialized research laboratories.

Methicillin-resistant *Staphylococcus aureus*

The first case of MRSA was described in the UK in 1961 (Deurenberg, et al., 2007), and the first documented hospital outbreak followed soon after in 1963 (Stewart et al., 1963). MRSA is now endemic globally and is epidemic in many US hospitals, long-term care facilities, and communities (Klein et al., 2007). The US has higher rates of MRSA infection than most other industrialized countries (Bordon, et al., 2010). As of 2005, there were approximately 278,000 hospitalizations related to MRSA in the US (Klein, et al., 2007). Among these hospitalizations, 94,000 were for first-time invasive MRSA infections, 1 in 5 of which resulted in death (Klevens et al., 2006). The most recent national survey of MRSA in US hospitals suggests that prevalence

may be decreasing; from 2005-2008, there was approximately a 28% decrease in hospital-onset MRSA infections and a 17% decrease in community-onset infections (Kallen et al., 2010). It is estimated that MRSA infections cost the healthcare system up to an additional $9.7 billion annually (Klein, et al., 2007). Many MRSA infections tend to be multiply drug-resistant (Bordon, et al., 2010), further compounding the costs of treatment.

As described previously, methicillin resistance is conferred by acquisition of the *mecA* gene, which is carried on the SCC*mec* element. The source of SCC*mec* is unknown, although coagulase-negative staphylococci (CoNS) are thought to have first transferred this MGE to *S. aureus* (Skov, 2011). Evidence suggests that MRSA clones do not have a single ancestor; instead, SCC*mec* was introduced multiple times into different *S. aureus* lineages, resulting in the clonal diversity observed today (Deurenberg, et al., 2007). It should be noted that carriage of the SCC*mec* element is not inherently beneficial to *S. aureus*, as it is associated with a weaker cell wall (Skov, 2011). Therefore, antibiotic pressure is required to facilitate dissemination of SCC*mec* into MSSA.

In 2011, a novel *mecA* gene, homologue $mecA_{LGA251}$ and herein termed "LGA251", was discovered by researchers at the University of Cambridge. LGA251 shares 70% similarity to the conventional *mecA* gene at the DNA sequence level, and 63% similarity at the amino acid level (Fluit, 2011). LGA251 is carried by SCCmec-IX, a novel SCC*mec* element (García-Álvarez, et al., 2011). *S. aureus* isolates carrying this gene were first isolated from bulk milk and English dairy cattle, and then confirmed in archived human isolates from England, Scotland, and Denmark (García-Álvarez, et al., 2011). It is not known when this homologue emerged, although its presence has been confirmed in a Danish human isolate dating as far back as 1975 (Holmes, 2011). It is also unknown whether or not this *mec* gene confers an advantage over the conventional *mecA* gene, although an increasing occurrence in Danish human isolates from

2008-2010 was statistically significant (Holmes, 2011). The LGA251 gene homologue has only been detected in "animal-associated strains" thus far, and has not been detected in any *S. aureus* isolate from the United States (Holmes, 2011). Efforts are now underway to allow for the simultaneous detection of *mecA* and its homologue using clinical diagnostic tools (M. Stegger et al., 2011). The discovery of LGA251 highlights the importance of scrutinizing *S. aureus* isolates that demonstrate methicillin-resistance but are PCR-negative for the *mecA* gene. Other homologues of the *mecA* gene, besides LGA251, may in fact exist.

History of MRSA

Wave 1: 1960s – mid 1970s

The first wave of MRSA to hit Europe was essentially monoclonal, with all archived isolates belonging to a single subgroup of CC8 (Enright et al., 2002). Isolates from this subgroup carried the first characterized SCC*mec* (SCC*mec* I), and most were ST250. This sequence type is now archaic; a defective *ccr* complex prevented SCC*mec* I from transferring effectively to MSSA, causing the first MRSA strains to die out completely by the 1980s (Skov, 2011). While ST250 isolates no longer cause epidemic MRSA disease, a minor variant (ST247–MRSA-I; also known as the "Iberian clone") is one of the major MRSA clones detected in European hospitals and has evolved resistance to most antimicrobial agents (Enright, et al., 2002). MRSA infections from this first wave were largely contained to Europe, with isolated reports of MRSA infection in the US (Chambers, et al., 2009; Skov, 2011).

Wave 2: Late 1970s – present

In the late 1970s, the *mecA* gene was incorporated into two new chromosomal cassettes, SCC*mec* types II and III, both of which were successfully introduced into MSSA strains. Outbreaks were reported globally, and infections became endemic by the 1980s. This MRSA wave was initially dominated by CC5 and CC8, though SCC*mec* types II and III emerged in CC30 in

the 1990s (Skov, 2011). Both of these elements are very large (52 and 66 kb, respectively) due to the accumulation of multiple genes encoding resistance to antibiotics and heavy metals. While selective pressure for these elements exists within hospitals, their large size likely precluded them from ever gaining success in community-associated (CA) MSSA strains (Ma et al., 2002). Both of these SCC*mec* types continue to circulate globally, primarily in hospitals.

Wave 3: Late 1980s – present

The third wave of methicillin-resistance in *S. aureus* was marked by the emergence of the new, relatively small SCC*mec* type IV (20-24 kb). This MGE is uniquely devoid of genes encoding resistance to antibiotics or metals. However, its small size allows strains carrying this element to efficiently compete with natural human flora. SCC*mec* IV first emerged in novel hospital-associated (HA) clones CC22 and CC45, and then began replacing SCC*mec* II and III in CC5 and CC8 (Skov, 2011). By the 1990s, approximately 90% of the HA-MRSA circulating worldwide belonged to only five clonal complexes: CC5, CC8, CC22, CC 30, and CC45 (Skov, 2011). Many of these clonal complexes are still associated with HA infections.

Next, SCC *mec* IV began to transfer to MSSA lineages completely separate from typical HA strains. These new MRSA strains were able to multiply and spread outside of the hospital environment. Reports of CA-MRSA infections date back to the 1980s, but these strains were only able to truly establish themselves in the 1990s. Today, CA-MRSA infections are considered epidemic in many parts of the world, including the US (Kennedy, et al., 2009).

Community-associated MRSA differs from hospital- associated MRSA in many respects. CA-MRSA carries much smaller SCC*mec* elements; in addition to SCC*mec* type IV, many other CA-MRSA-associated SCC*mec* types have emerged, including types V, VII, VIII, and XIII. CA-MRSA typically carries the Panton-Valentine leukocidin (*pvl*) gene, a human-specific virulence factor. CA-MRSA infections present mainly as skin and soft tissue infections, and risk factors include

close contact with an individual colonized with CA-MRSA, contact sports, crowding or living in close quarters, intravenous drug use, and homosexual activity (Skov, 2011). CA-MRSA also tends to be less drug resistant than HA-MRSA, though this is highly strain-dependent. Importantly, CA-MRSA infections can easily occur in healthy individuals. Due to its increased fitness and virulence, CA-MRSA has begun to replace HA-MRSA in the hospital and long-term care facility settings, therefore blurring the traditional distinctions between "HA-MRSA" and "CA-MRSA." Nosocomial outbreaks of CA-MRSA have been reported since 2003 (Otter et al., 2006).

Wave 4: 2004 – present

In 2004, a new strain of MRSA was unexpectedly found in a 6-month old Dutch girl and her parents. The strain was untypeable by PFGE using *Sma*1 and resisted decolonization (Voss, et al., 2005). Neither the girl nor her parents had any traditional risk factors for MRSA colonization; however, they were pig farmers and lived on a farm. Further investigation revealed that some of the family's pigs were carrying the same strain as the girl and her parents, as were several individuals who had recently visited their farm (Voss, et al., 2005). The new strain was designated as ST398, belonging to the new clonal complex 398. By 2005, MRSA ST398 was confirmed in humans and pigs across the Netherlands (Huijsdens, et al., 2006). Livestock-associated (LA) MRSA has since been detected in in pigs and pig farmers in multiple European countries, including France, Denmark, Belgium, and Spain, as well as in South America, China (where LA-MRSA occurs in ST9), Canada, and as of 2009, the United States (Smith et al., 2011). LA-MRSA represents the fourth and most recent wave of methicillin-resistance in *S. aureus*.

Livestock-associated ST398

Origins of MRSA ST398

Though MRSA has been described in livestock since 1972, ST398 represents the first known transmission of MRSA from livestock to humans (Price, et al., 2012). Evidence suggests that MRSA ST398 emerged due to the repeated introduction of SCC*mec* IV and V into precursor MSSA ST398 strains that existed in pigs and in their environment. These precursor strains are thought to be human in origin and were transmitted to pigs an indeterminable length of time ago (Price, et al., 2012). It is not clear whether or not the emergence of MRSA ST398 at varied geographic locations can be attributed to *de novo* emergence on multiple farms from multiple MSSA ST398 reservoirs, or whether transmission among farms and animals is the more probable cause (Smith, et al., 2011).

Characteristics of MRSA and MSSA ST398

Livestock-associated ST398 is highly unique from *S. aureus* strains observed previously. First, almost all livestock-associated ST398 isolates are tetracycline-resistant, and a majority of MRSA ST398, although rarely MSSA ST398, exhibit zinc resistance encoded by the *crcZ* gene (Lina M. Cavaco et al., 2011). A vastly diverse antibiotic resistance pattern is observed among ST398 isolates, indicating that selective pressure for this strain is variable yet sustained (Skov, 2011). Second, the core genome of this strain is markedly different from "human" MSSA strains (Skov, 2011). This corroborates the theory that LA-ST398 evolved in a non-human reservoir. Third, most ST398 isolates carry the SCC*mec* V element, though several novel SCC*mec* types have been discovered in these isolates. Fourth, MSSA and MRSA ST398 isolates typically lack the *pvl* gene, though some exceptions exist (Wulf, & Voss, 2008). Lastly, LA-MRSA has so far proven to be significantly less transmissible among humans than other MRSA strains. Current reports

rate nosocomial transmission of MRSA ST398 as 72% less likely than transmission of non-ST398 MRSA strains (Wassenberg et al., 2011). However, the possibility that transmission of ST398 will increase as the strain becomes better adapted to humans is of great concern. This is particularly alarming since the swine reservoir of ST398 is enormous, and likely cannot be eliminated. Importantly, pigs may not be the only livestock reservoir of ST398; carriage has also been detected in poultry, cattle, and horses (Nemati et al., 2008; Van den Eede et al., 2009; Vanderhaeghen et al., 2010).

ST398 Infection

MRSA and MSSA ST398 have been responsible for a wide diversity of minor and localized infections, including abscesses, urinary tract infections, wound infections, mastitis, and conjunctivitis (Smith, et al., 2011). Much more serious infections include bacteremia, pneumonia, osteomyelitis, otomastoiditis, and post-operative infections (Smith, et al., 2011). Currently, most ST398 infections present as skin and soft tissue infections (Smith, et al., 2011). The vast majority of ST398 infections have occurred in the last five years, although a disease-causing MSSA ST398 isolate was archived in Denmark in 1992 (Smith, et al., 2011).

A rising number of ST398 infections have recently begun to occur in individuals with no documented livestock contact. Isolates causing such infections are often referred to as "human-associated ST398," rather than "livestock-associated ST398." Human-associated MSSA ST398 infections have been documented in China, New York, and New Jersey, and 10% of MRSA ST398 cases in Denmark are now human-associated (Mediavilla, et al., 2012; Skov, 2011; Smith, et al., 2011; Uhlemann, et al., 2012). It is unclear whether these strains are related to the human-associated MSSA precursors that gave rise to LA-MRSA ST398, or whether they have evolved from LA-MRSA ST398. Regardless, analysis suggests that the occurrence of several MGEs, surface adhesins, and the ability to bind to human keratinocytes may make human-associated

ST398 better suited for human infection than traditional livestock-associated isolates (Uhlemann, et al., 2012).

Colonization versus Contamination

It has not clear whether occupational exposure to colonized pigs results in persistent ST398 carriage, or if individuals only become transiently contaminated. Several studies from the Netherlands suggest the latter, while a more recent study from Germany suggests the former. A 2009 study was the first to find that veterinarians who worked with pigs often tested positive for MRSA carriage, but only on the day of their visit (Van Den Broek et al., 2009). A longitudinal study conducted by Graveland et al. later found that MRSA carriage in veal calf farmers was strongly related to duration of animal contact, with odds of carriage significantly reduced during periods of low exposure (Graveland et al., 2011). A third study found veal farmers were 94% less likely to carry MRSA 24 hours after animal exposure, compared to immediately after exposure (van Cleef et al., 2011). However, a 2012 study found that 59% of German pig farmers did not clear MRSA colonization following summer holidays, suggesting that regular contact with swine may lead to persistent carriage (Robin Köck et al., 2012). Overall, these studies present conflicting evidence regarding whether or not MRSA ST398 "carriage" is an artifact of short-term exposure to MRSA-contaminated bioaerosols, or whether carriage truly represents persistent colonization.

ST398 in North America

ST398 and hog farms

ST398 was first reported in North American hogs and workers in 2008. Twenty-five percent of pigs and 20% of workers from 20 farms in southwestern Ontario, Canada were found to carry MRSA, with almost 60% of isolates belonging to *spa* types associated with ST398 (Khanna, et al., 2008). In total, 45% of the sampled farms were contaminated with MRSA,

though not all with ST398. Though a few ST398 infections have been reported, infection by ST398 remains rare in the greater Canadian population (estimated at 0.14%) (Golding, et al., 2010).

The only published report of MRSA ST398 on a US pig farm was from Iowa in 2009. Smith et al. investigated two conventional CAFO systems in Iowa, each comprising over 25,000 animals in total. In the first production system, 209 pigs were swabbed, 70% of which were colonized with ST398. Nine out of 14 workers also carried this same strain. Curiously, in the second production system, no pigs (90 sampled) and no workers (6 sampled) carried ST398 (Smith, et al., 2009). Hypotheses provided for this discrepancy included the confirmed importation of pigs from Canada by the first production system, the presence of nearly twice the number of hogs in the first production system compared to the second, and the older, more established nature of the first production system (Smith, et al., 2009). However, the authors did not research this discrepancy further. Prevalence of MRSA ST398 has not been further investigated in pigs or workers in CAFOs elsewhere in the United States.

ST398 and retail meat

Two recent studies have documented the presence of ST398 on retail meat in the United States. A 2011 study examined the presence of multidrug-resistant *S. aureus*, or *S. aureus* demonstrating intermediate or complete resistance to three or more classes of drugs, on conventionally-raised meat in the US. Researchers discovered MSSA ST398 on turkey, chicken, and pork samples (Waters, et al., 2011). ST398 was the predominant strain recovered from turkey (79%), the second most common strain found on pork (18%), and one of the least common strains found on chicken (4%). ST398 was not recovered from beef (Waters, et al., 2011). Most ST398 isolates recovered from retail meat were multidrug-resistant. A 2012 study isolated MRSA from 6.4% of conventional pork samples and 7.4% of alternative pork samples

(raised without the use of antibiotics) (O'Brien, et al., 2012). Over a quarter of the pork samples that tested positive for MRSA had *spa* types associated with ST398. The authors speculated that the presence of MRSA on alternative pork products might be a result of contamination during processing, as the study's results were incongruous with previous findings in Europe (O'Brien, et al., 2012). Although *S. aureus* is destroyed by cooking, contact with contaminated meat could result in nasal colonization, cross-contamination of surfaces, and/or introduction of ST398 into the home environment.

Prevalence of ST398 in the US population

Carriage of MRSA ST398 in the general US population is unknown due to poor national surveillance, particularly in rural areas. However, no symptomatic infections in the US have been reported (Smith, et al., 2011), and all evidence suggests that prevalence of MRSA ST398 is sharply below that in many other Western, industrialized countries. This may be because the US has a high prevalence of CA- and HA non-ST398 MRSA, and these strains are outcompeting MRSA ST398 in the community European countries like Denmark suffered greatly from the emergence of ST398 because pre-existing MRSA rates were extremely low, due to effective "search and destroy" policies. However, these policies did not target pig farming as a risk factor for MRSA colonization prior to 2006, leaving healthcare systems vulnerable once ST398 emerged. Now, ST398 comprises 30% of Denmark's MRSA cases, and the country is experiencing the highest rates of MRSA since 1985 (Statens Serum Institut et al., 2010).

Prevalence of MSSA ST398 in the general US population appears to be much higher than MRSA ST398, and MSSA ST398 may even be common in some regions. A recent study found that MSSA ST398 was one of the most common noninvasive MSSAs recovered from clinical patients in Northern Manhattan, and invasive MSSA ST398 was recovered, as well (Uhlemann, et al., 2012). The same study found approximately 20% of households in Northern Manhattan to be

contaminated with MSSA ST398, either as a human colonizer or on environmental surfaces. More than one human was colonized with MSSA ST398 in 20% of multi-person households contaminated with this strain; there were fewer concordant dyads for all other *S. aureus* strains examined (Uhlemann, et al., 2012). Another study from the Northeastern US recently described several infections in the Manhattan area due to MSSA ST398 and MSSA ST291, a novel sequence type belonging to CC398 (Mediavilla, et al., 2012). Most isolates were not multidrug-resistant, and all were acquired from individuals with no history of livestock exposure. Both of these studies suggest at least low-level prevalence of human-associated MSSA ST398 in the New York City area, and efficient transmission among households independent of livestock contact.

Unanswered Questions

Livestock-associated MRSA has been actively observed in the human population for less than ten years, and many questions remain regarding its origins, evolution, and changing epidemiology. Of common interest are questions regarding the timing of LA-MRSA emergence in humans. MRSA ST398 has not been detected in any human isolate archived prior to 2004, yet evidence suggests that SCC*mec* IV and V have been introduced into MSSA ST398 multiple times (Graveland, et al., 2011). It is unclear why LA-MRSA never emerged in the years, or even decades, prior to 2004. No changes to husbandry practices were made prior to 2004, nor were any clear changes made in antibiotic use (though it is speculated that Europe's introduction of 3rd generation cephalosporins for use in piglets in the 90s may be related to the emergence of LA-MRSA). Also unclear is why LA-MRSA occurs as ST9 in China and Malaysia (Smith, et al., 2011). Many LA-MRSA ST9 isolates are s*pa* type t899; this same *spa* type is found in ST398 as well, suggesting the two strains are related (Smith, et al., 2011). Most important are uncertainties regarding the future of ST398 in the community. It is unknown whether or not these strains could become better adapted to human hosts, and therefore pose an even greater

risk to global public health. Some evidence shows that accretion of human-specific MGEs, including virulence genes, is already occurring (Skov, 2011). If transmission of ST398 among humans begins to rise, resistance to human-specific antibiotics may also become more prevalent. Serious infections by ST398, including endocarditis, bloodstream infections, and necrotizing pneumonia, may become more common if the fitness, virulence, and/or resistance of ST398 increase.

Multidrug-resistant *S. aureus*

Multidrug-resistant *S. aureus* (MDRSA) is typically defined as *S. aureus* that demonstrates resistance to three of more classes of drugs. MDRSA can include MRSA, but multiply-resistant *S. aureus* that do not exhibit methicillin-resistance are common. Infections with such bacteria can present treatment challenges comparable to MRSA, and multidrug-resistant, *pvl*-positive MSSA strains are thought to be contributing to the ongoing epidemic of CA-MRSA infections in the US (Orscheln et al., 2009). However, prevalence and characteristics of general MDRSA carriage in the US population are unknown. Risks for MDRSA acquisition are also unknown, but are likely similar to risks for MRSA acquisition (See: *Epidemiology of S. aureus carriage)*.

MDRSA Associated with CAFOs

Prior to the emergence of ST398 in the US, the link between industrial livestock production and an environmental reservoir of MDRSA had already been established. A 2006 study found airborne MDRSA downwind from two swine CAFOs in the Midwestern US. *S. aureus* was the most prevalent bacteria sampled from the interiors of these two CAFOs, comprising over 75% of all recovered bacteria. Concentrations of *S. aureus* decreased with increasing distance downwind, although airborne *S. aureus* was still detectable 150 m downwind of both

facilities. Resistance to 4 antibiotic classes was evaluated; a significant difference was detected in *S. aureus* resistance to oxytetracycline (p=.010), tetracycline (p=.014), ampicillin (p=.007), and erythromycin (p=.035) when samples taken upwind and samples taken immediately downwind of the facilities were compared (Gibbs et al., 2006). Several other studies have found *S. aureus* to be the most prevalent microorganism captured by filtration and impaction of bioaerosols inside CAFO barns, although none of these studies have measured antibiotic resistance in *S. aureus* isolates (Butera et al., 1991; Crook et al., 1991; Predicala et al., 2002).

MDRSA has also been isolated from flies collected near poultry CAFOs in Maryland (Graham, et al., 2009). Antibiotic-resistant bacteria can be ingested by flies as they feed on decomposing waste and animal carcasses, or they can stick to their legs, feet, wings, or proboscis. Bacteria can then be transferred to new surfaces through regurgitation or physical contact. A 2009 study found that 10-15% of *S. aureus* isolates collected from flies trapped 100-200 m from poultry CAFOs were MDR (n=27), while approximately 30% of *S. aureus* isolates collected from nearby poultry litter piles were MDR (n=118) (Graham, et al., 2009). While the species of flies collected in this study have a typical traveling radius of 2 miles, individual flies can fly up to 20 miles. Flies carrying MDRSA present an additional route for human exposure.

Research rationale

Staphylococcus aureus is a ubiquitous human and animal commensal carried by approximately one third of the U.S. population, primarily in the nares (Gorwitz, et al., 2008). *S. aureus* is also an adept human pathogen capable of causing a wide range of infections, from surficial to systemic (Loir, et al., 2003; Lowy, 1998). In recent years, rapidly disseminating multidrug-resistant strains of *S. aureus* (MDRSA) have made treatment of *S. aureus* infections more protracted, more burdensome, and less successful (Chambers, et al., 2009). In particular,

strains of *S. aureus* resistant to methicillin (MRSA) have proven increasingly difficult to treat (Chambers, et al., 2009).

Industrial livestock operations may be a source of exposure to antibiotic-resistant *S. aureus*, including MDRSA and MRSA. In addition to confining hundreds of animals in enclosed spaces, industrial livestock operations administer 80% percent of all antibiotics sold in the US to food animals for the purposes of treatment, prophylaxis, and growth promotion (Food and Drug Administration, 2009). These practices have been associated with a growing animal and environmental reservoir of novel antibiotic-resistant pathogens, including antibiotic-resistant *S. aureus* (Frank M. Aarestrup, 1995; Gibbs, et al., 2006; Graham, et al., 2009). Some clones of antibiotic-resistant *S. aureus* can be zoonotically transmitted to individuals in close contact with livestock (Catry et al., 2010), who may then be at an increased risk for infection (Wertheim, et al., 2005). Of particular concern is the recent emergence of a new livestock-associated *S. aureus* clone, clonal complex 398 (CC398), which is responsible for an increasing percentage of MRSA infections in several countries, including Denmark, Germany, and the Netherlands (Smith, et al., 2011; Statens Serum Institut, et al., 2010).

Despite growing attention in Europe, much is unknown about the carriage of antibiotic-resistant *S. aureus* by livestock workers and their household members in the US. Only one published study has examined MRSA carriage in industrial livestock workers (Smith, et al., 2009), and no study has comprehensively examined the antibiotic resistance profiles of *S. aureus* colonizing workers and their household members to determine multidrug-resistance. Furthermore, carriage of antibiotic-resistant *S. aureus* by individuals exposed to antibiotic-free livestock operations has not yet been studied in the US. It is therefore unknown whether or not workers and household members exposed to industrial livestock operations are at a greater risk

of carrying antibiotic-resistant *S. aureus*, including MDRSA and MRSA, than workers and household members exposed to antibiotic-free livestock operations.

North Carolina presents an ideal setting for an investigation of the prevalence of *S. aureus*, MDRSA, and MRSA among livestock workers in the United States. North Carolina is the second largest producer of hogs in the country and the third largest producer of poultry. The vast majority of these animals are housed from birth to slaughter in industry-owned concentrated animal feeding operations (CAFOs). North Carolina, however, is also home to a burgeoning sustainable farming movement (Greene, 2010). These farms raise pork and poultry without the use of antibiotics and with limited reliance on external inputs. Therefore, we conducted a study (1) to compare the prevalence of carriage of MDRSA and MRSA in industrial livestock workers and household members to antibiotic-free livestock workers and household members in North Carolina, (2) to compare antibiotic resistance patterns among *S. aureus*-positive individuals in both groups, and (3) to determine whether or not CC398 is present in North Carolina livestock workers and their household members. Furthermore, based on a lack of consensus in the literature regarding which commercial media is ideal for isolating *S. aureus*, this research aims to compare the sensitivity and specificity of two differential media, Baird Parker and BBL™ CHROMagar™ Staph aureus, in their capacity to detect *S. aureus* from nasal swabs.

CHAPTER 2: METHODS

Study design and timeframe

All participants were recruited for this study by snowball sampling, beginning in early 2011. Industrial workers and household members were recruited with the help of two community organizations based in eastern North Carolina, the Rural Empowerment Association for Community Help (REACH) and the North Carolina Environmental Justice Network (NCEJN). ABF farmers and household members were mainly recruited by a team of researchers at the UNC Gillings School of Global Public Health, though a small number of ABF famers and household members were also recruited by REACH. Enrollment for this study began in May, 2011 and ended in December, 2011.

All workers employed in North Carolina were eligible to participate in this study, providing they reported contact with swine or poultry, were eighteen years or older, and spoke either English or Spanish. Up to two household members, including minors (ages 7-17), were also invited to participate if they reported living in the same household as a livestock worker for the past three months.

This study and all of its protocols were approved by UNC Chapel Hill's Institutional Review Board. Recruitment materials and questionnaires were also available in Spanish.

Administration of Questionnaires and Nasal Swab Sample Collection

Once a participant was enrolled, a follow-up visit was planned at a location of the participant's choosing (usually the home, in the case of IND and PP participants, or the farm, in

the case of ABF participants) to obtain questionnaire data and a nasal swab. The questionnaire assessed risk factors for MRSA colonization, including occupational exposures, household and environmental exposures, personal activities, and medical history, as well as basic demographics, including race, sex, and education level. Each member of a household completed a separate questionnaire.

Next, a swab of each participant's nostrils was taken by the interviewer. A single, sterile BD BBL™ CultureSwab™ was used to swab both nostrils. Most interviewers were trained in proper swabbing protocol by a member of the nursing staff at UNC hospitals prior to the start of the study; all other interviewers were trained by an individual who had attended that training session. Swabs were immediately inserted into Stuart's transport medium and stored at 4°C until arrival at the UNC School of Public Health. Swabs were transported by courier on ice in an insulated storage container within five days of collection.

Isolation of MSSA and MRSA from Nasal Swabs

Upon arrival in the laboratory, swabs were inoculated into 10 ml of Mueller-Hinton broth (MHB) with 6.5% NaCl, then incubated overnight at 37°C (Figure 3). To isolate presumptive MSSA, a loopful of enriched broth was streaked onto Baird-Parker with Egg Yolk Tellurite Enrichment (BP) and CHROMagar™ Staph aureus (CS) plates (Beckton, Dickinson and Company, Franklin Lakes, NJ). Plates were incubated at 37°C for 24 hours. Colonies with 2 out of 3 morphological characteristics for *S. aureus* growth on BP (black, shiny, with clearing) and CS (mauve, matte, with halo) were streaked to isolation on the same media on which they were isolated, then archived in Brain Heart Infusion broth (BHIB) with 15% (by volume) glycerol added for cryopreservation at -80°C. To isolate presumptive MRSA, 1 ml of MHB was transferred to 10 ml of Tryptic Soy broth (TSB) with 2.5% NaCl, 3.5 mg/L cefoxitin, and 10 mg/L aztreonam, and incubated overnight at 37°C. Isolation of presumptive MRSA colonies then followed using

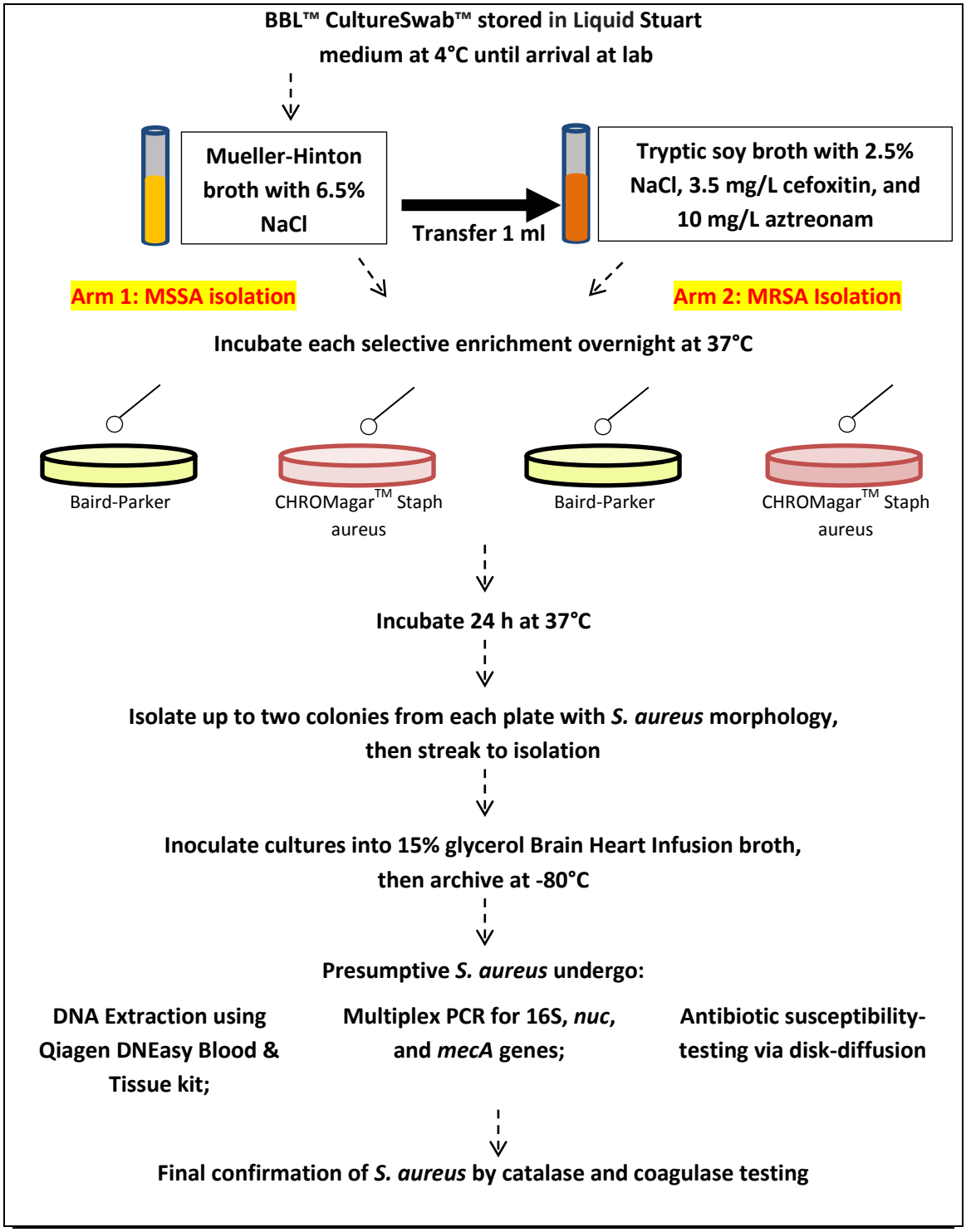

Figure 3. Protocol for isolation and characterization of MSSA and MRSA from nasal swabs. Adapted from: (Davis et al., 2012)

exactly the same procedure as isolation of presumptive MSSA. A more detailed schematic of this process is provided in Figure 3. Approximately 10 swabs were processed each week using this method between May and December, 2011. This protocol was largely adapted from Davis et al. (2012).

Molecular Confirmation of MSSA and MRSA

At least two isolates from each individual with presumptive *S. aureus* (one from each type of media, if available) were regrown from frozen stocks and extracted using Qiagen's DNeasy Blood & Tissue kit, with a modified protocol for gram-positive bacteria recommended by the manufacturer. Primers from a multiplex PCR were used to amplify the 16S, *nuc*, and *mecA* genes in all extracted isolates (Poulsen et al., 2003). Primer sequences are provided in Table 3. PCR conditions were as follows: (25 µl total volume): 2.5 µl 10x buffer (Qiagen, Valencia, CA), 5 nmol each dNTP, 5 pmol each of the 6 primers, 1 unit HotStarTaq DNA polymerase (Qiagen, Valencia, CA), and 1 µl of template. Amplification was carried out on a Bio-Rad Gene Cycler™ thermal cycler with the following program: 15 min at 95°C; then 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C; followed by elongation for 10 min at 72°C. A clinical strain of Mu50 MRSA was used as a positive control (courtesy of Dr. Melissa Miller, UNC School of Medicine). Amplified gene products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Isolates that were positive for the 16S and *nuc* genes but negative for the *mecA* gene were considered MSSA; isolates that were positive for all three genes were considered MRSA. Catalase and tube coagulase testing with rabbit plasma (BD BB™, Franklin Lakes, NJ) were subsequently performed on at least one PCR-confirmed *S. aureus* isolate (MSSA or MRSA) per person to validate molecular findings.

Table 3. Primer sequences used for multiplex PCR

Gene target	Direction	Sequence (5' -- 3')	Product Size (bp)
16S	F	GTG CCA GCA GCC GCG GTA A	886
	R	AGA CCC GGG AAC GTA TTC AC	
nuc	F	TCA GCA AAT GCA TCA CAA ACA G	527
	R	CGT AAA TGC ACT TGC TTC AGG	
mecA	F	GGG ATC ATA GCG TCA TTA TTC	255
	R	AAC GAT TGT GAC ACG ATA GCC	

Source: (Poulsen, et al., 2003)

Antibiotic Susceptibility Testing

At least one isolate per *S. aureus*-positive individual (92 total) was assessed for its susceptibility to 12 classes of antibiotics using the Kirby-Bauer disk diffusion method (Table 4). Standard procedures were used and diameter interpretations were based on Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2010). Inducible clindamycin resistance in erythromycin-resistant isolates was evaluated using the D-zone test (Steward et al., 2005). Additionally, vancomycin resistance was assessed in at least one isolate per *S. aureus*-positive individual (92 total) on Brain Heart Infusion agar supplemented with 5 mg/L vancomycin hydrochloride (Brown et al., 2005).

Isolates that demonstrated full resistance to three or more classes of antibiotics were classified as MDRSA. Because variable resistance profiles are possible among *S. aureus* isolates from the same individual, additional isolates from a subset of individuals determined not to have MDRSA (n=36) were assessed for their susceptibility to the antibiotics listed in Table 4. Complete resistance to oxacillin and intermediate or complete resistance to ceftriaxone were interpreted as methicillin resistance.

Table 4. Antibiotics used in susceptibility testing via disk diffusion

Antibiotic	Class	Concentration	Importance to Human Health[a]
Ampicillin	β-lactam	10 µg	Critical
Ceftriaxone	Cephalosporin	30 µg	Critical
Ciprofloxacin	Floroquinolone	5 µg	Critical
Clindamycin	Lincosamide	2 µg	Important
Erythromycin	Macrolide	15 µg	Critical
Gatifloxacin	Floroquinolone	5 µg	Critical
Gentamicin	Aminoglycoside	10 µg	Critical
Levofloxacin	Floroquinolone	5 µg	Critical
Linezolid	Oxazolidone	30 µg	Critical
Oxacillin	β-lactam	1 µg	Highly Important
Penicillin	β-lactam	10 units	Critical
Quinupristin/Dalfopristin	Streptogramin mix	15 µg	Critical
Rifampin	Rifamycin	5 µg	Critical
Sulfamethoxazole/ Trimethoprim	Sulfonamide mix	23.75/1.25 µg	Highly Important
Tetracycline	Tetracycline	30 µg	Highly Important
Vancomycin	Glycopeptide	5 µg; 5 mg/L	Critical

a. As classified by the World Health Organization (Second World Health Organization Expert Meeting, 2007). Antibiotics were classified from lowest to highest priority: Important, Highly Important, and Critical.

Confirmation of CC398 and additional gene targets

A lineage-specific PCR was used to detect CC398 in one isolate from each *S. aureus*-positive individual, using amplification conditions described elsewhere (Marc Stegger et al., 2011). Two µl of template was used in 2.5x 5 PRIME MasterMix (5 PRIME Inc., Gaithersburg, MD), with final primer concentrations of 2 pM. Amplified gene products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. A ST398 MRSA isolate was used as a positive control (courtesy of Dr. Tara Smith, University of Iowa) and purified PCR-grade water was used as a negative control.

PCR for the *czrC* gene was performed on all MRSA CC398 isolates identified by the lineage-specific PCR described above. Two µl of template was used in 2.5x 5 PRIME MasterMix (5 PRIME Inc., Gaithersburg, MD), with final primer concentrations of 2 pM. Primer sequences are described elsewhere (L. M. Cavaco et al., 2010). Amplification was carried out on a Bio-Rad Gene Cycler™ thermal cycler with the following program: 4 min at 94°C; then 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; followed by elongation for 10 min at 72°C. Amplified gene products were visualized as described above. A ST398 MRSA isolate (courtesy of Dr. Tara Smith, University of Iowa) was used a positive control and purified PCR-grade water was used as a negative control.

PCR was also used to detect the *pvl* gene in one pure isolate from each *S. aureus*-positive individual, using amplification conditions described elsewhere (Lina et al., 1999). Two µl of template was used in 2.5x 5 PRIME MasterMix (5 PRIME Inc., Gaithersburg, MD), with final primer concentrations of 2 pM. Amplified gene products were visualized as described above. *S. aureus* ATCC 25923 was used as a positive control and purified PCR-grade water was used as a negative control.

CHAPTER 3: RESULTS

Prevalence of *S. aureus*, MDRSA, and MRSA

In total, 105 antibiotic-free (ABF) farmers and household members and 99 industrial (IND) workers and household members participated in this study. Additionally, 30 industrial processing plant (PP) workers and household members participated; these individuals were separated from the IND category post-enrollment due to workers' lack of contact with live animals. A detailed summary of study participants is presented in Table 5.

Table 5. Summary of study participants

	Antibiotic-free (ABF)	Industrial (IND)	Processing plant (PP)
Participant			
Worker	92	80	18
Adult household member	4	10	8
Minor household member	9	9	4
TOTAL	105	99	30

S. aureus was isolated from 41 IND participants (n=99), 42 ABF participants (n=105), and 9 PP participants (n=30) (Figure 4). Among these, 37% of *S. aureus*-positive IND participants carried MDRSA, as did 19% of *S. aureus*-positive ABF participants and 33% of *S. aureus*-positive PP participants (data not shown). Additionally, MRSA was detected in 3/41 *S. aureus*-positive IND participants and 3/42 *S. aureus*-positive ABF participants (Figure 4). All six of these individuals were workers reporting direct contact with swine or poultry. Two out of nine *S. aureus*-positive PP participants also tested positive for MRSA (Figure 4), but both of these individuals were household members who did not report contact with swine or poultry, and

who had additional risk factors for MRSA carriage. MRSA prevalence in all three groups was higher than MRSA prevalence in the general US population, 1.5% (Gorwitz, et al., 2008).

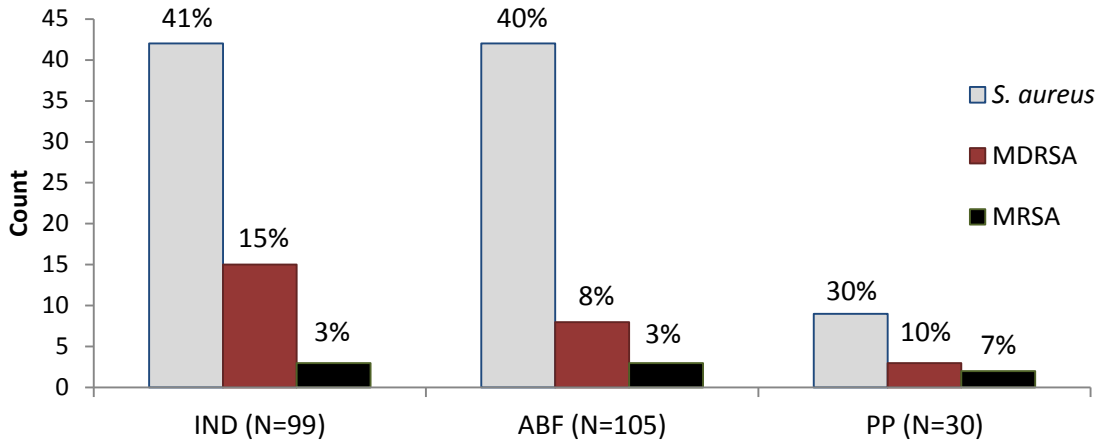

Figure 4. Carriage of *S. aureus*, multidrug-resistant *S. aureus* (MDRSA), and methicillin-resistant *S. aureus* (MRSA) in each study group.
Note: MDRSA and MRSA are sub-categories of *S. aureus* and are not independent of one another.

Not all MRSA detected in this study were multidrug-resistant (Figure 5). Both MRSA-positive individuals from the PP group carried multidrug-resistant MRSA while 2/3 of MRSA-positive individuals from the IND group carried multidrug-resistant MRSA. None of the MRSA isolates collected from the ABF group were multidrug-resistant.

MRSA isolated from one PP participant was *pvl*-positive (data not shown). No other tested *S. aureus* isolate carried the *pvl* gene.

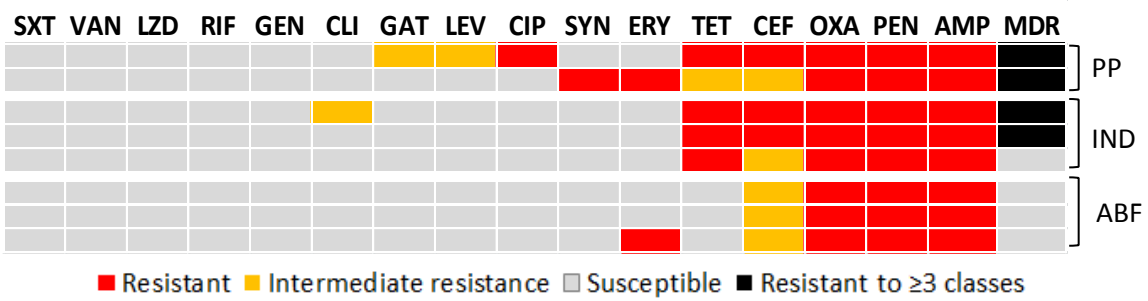

Figure 5. Antibiotic resistance profiles of 8 MRSA isolates collected during study.
SXT: sulfamethoxazole/trimethoprim; VAN: vancomycin; LZD: linezolid; GEN: gentamicin; CLI: clindamycin; GAT: gatifloxacin; LEV: levofloxacin; CIP: ciprofloxacin; SYN: quinupristin/dalfopristin; ERY: erythromycin; TET: tetracycline; CEF: ceftriaxone; OXA: oxacillin; PEN: penicillin; AMP: ampicillin; MDR: multidrug-resistant

It should be noted that the protocol used in this study failed to differentially isolate MRSA and MSSA from nasal swabs, as it was intended to do. Much of the "presumptive MRSA" isolated in Arm 2 of the study protocol (Figure 3) was in fact MSSA, as confirmed by PCR. It is hypothesized that MSSA were not completely eliminated during the overnight incubation in TSB with 2.5% NaCl, 3.5 mg/L cefoxitin, and 10 mg/L aztreonam. Once TSB enrichments were plated on to antibiotic-free BP and CS plates, any MSSA still remaining were able to flourish.

Resistance phenotypes

The antibiotic resistance profiles of *S. aureus* isolated from each study population were also assessed. A broad range of antibiotic resistance was observed in all three groups. Of the twelve classes of drugs tested (Table 5), resistance was observed to all except oxazolidones (linezolid), glycopeptides (vancomycin), and sulfonamides (sulfamethoxazole/trimethoprim). Rifamycin resistance was observed in only one ABF worker, while streptogramin resistance was observed in only one PP household member. Aminoglycoside resistance was unique to the IND group and was observed in two separate workers and an unrelated household member.

In total, ABF workers and household members demonstrated resistance to fewer classes of drugs than either the IND or PP group (5 classes versus 6 and 7, respectively; Figure 6). Furthermore, ABF workers and household members demonstrated the least prevalence of resistance to tetracyclines, macrolides, and β-lactams, the three classes of drugs for which all three groups demonstrated resistance.

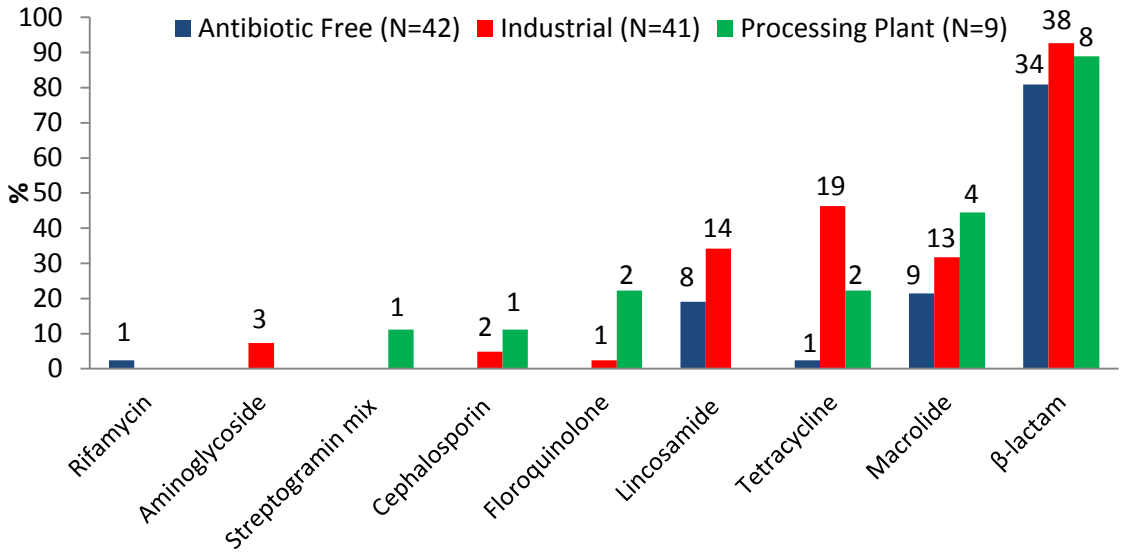

Figure 6. Percentage of *S. aureus*-positive ABF, IND, and PP participants demonstrating full resistance to 9 antibiotic classes.

Tetracycline resistance was unique to the IND group (χ^2=21.92, df=2, p-value <0.0001) with over 40% of all *S. aureus*-positive IND participants demonstrating resistance to this antibiotic, compared with 22% of *S. aureus*-positive PP participants and 3% of *S. aureus*-positive ABF participants (Figure 6). Of the 19 IND participants who carried tetracycline-resistant *S. aureus*, 79% were workers who had direct contact with swine, while the remaining 21% reported direct contact with poultry.

Aminoglycoside resistance was also unique to the IND group, with resistance occurring in 2 IND workers and 1 IND household member, and no other study participant (Figure 6). 67% of individuals carrying gentamicin-resistant *S. aureus* reported direct or indirect contact with poultry.

Prevalence and Distribution of CC398

PCR was used to determine if any *S. aureus* isolated in this study belonged to CC398, the clonal complex commonly associated with zoonotic transmission. CC398 was detected in both workers and household members in this study, providing the first evidence of CC398 in humans

in North Carolina (Figure 7). Seventeen of 41 *S. aureus*-positive IND participants carried CC398, as did 3/42 *S. aureus*-positive ABF participants. Ninety-four percent of CC398-positive participants were workers who reported direct contact with livestock. No CC398 was identified in the PP group.

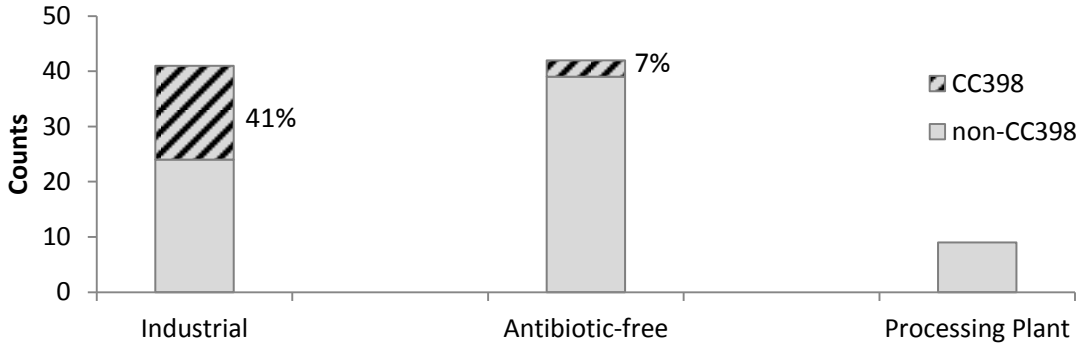

Figure 7. Prevalence of CC398 among *S. aureus*-positive IND, ABF, and PP participants.

No MRSA CC398 was detected among ABF or PP participants, and only 2 cases of MRSA CC398 were identified among IND participants. Neither of these isolates carried the *czrC* gene. However, MSSA CC398 was present in both groups (Figure 8).

In the IND group, over 1/3 of all CC398 was multidrug-resistant (Figure 8). No multidrug-resistant CC398 occurred in the ABF group.

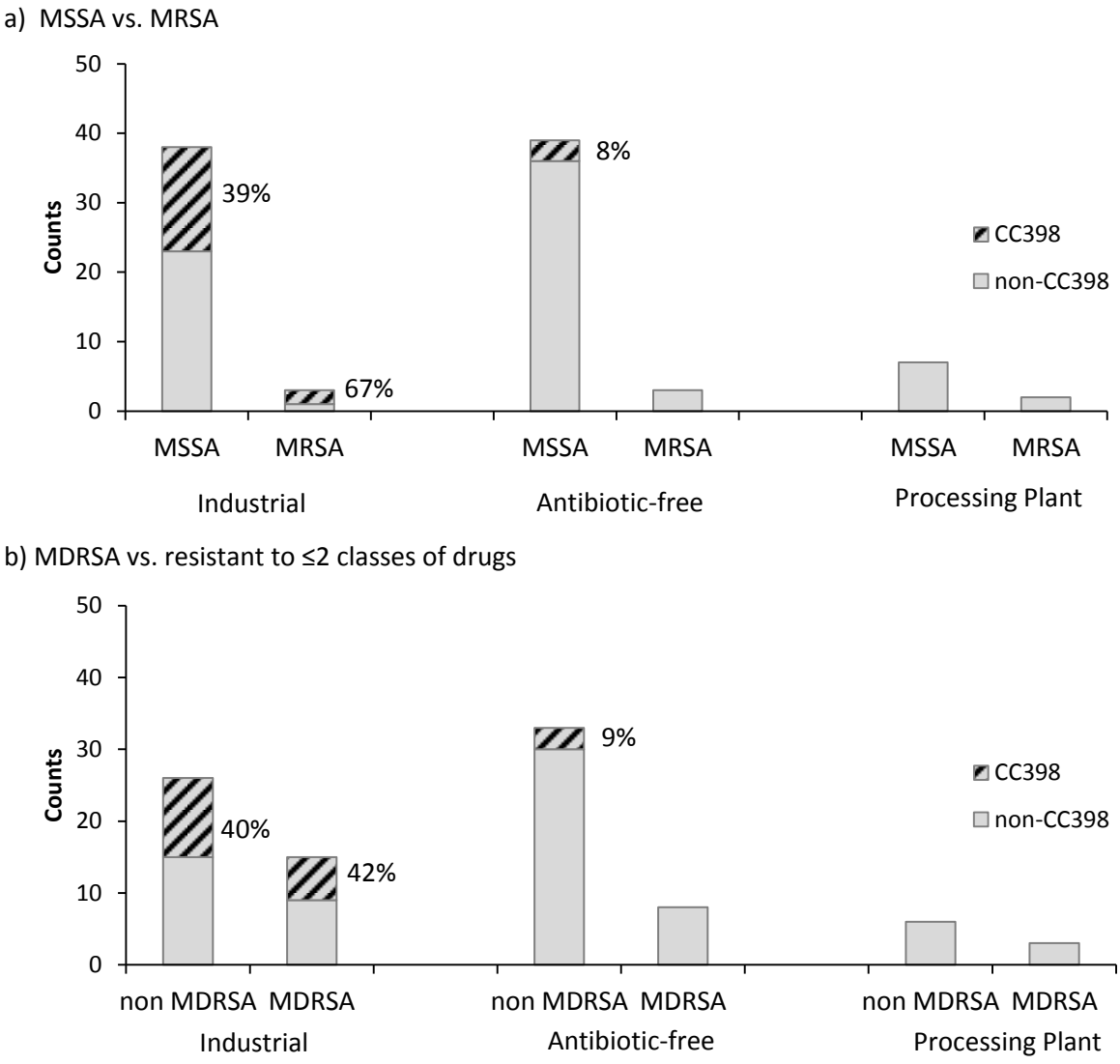

Figure 8. Distribution of CC398 among S. aureus-positive IND, ABF, and PP participants.

Resistance phenotypes of CC398 isolates

Nearly 1/3 of CC398 isolates collected in this study exhibited resistance patterns inconsistent with patterns characteristic of livestock-associated ST398. 1 ABF worker, 1 ABF household member, and 4 IND workers in this study were colonized with tetracycline-susceptible MSSA CC398, despite reporting direct contact with livestock (Figure 9). All six tetracycline-susceptible MSSA CC398 isolates demonstrated resistance to penicillin, and isolates from one IND worker demonstrated additional resistance to clindamycin and erythromycin.

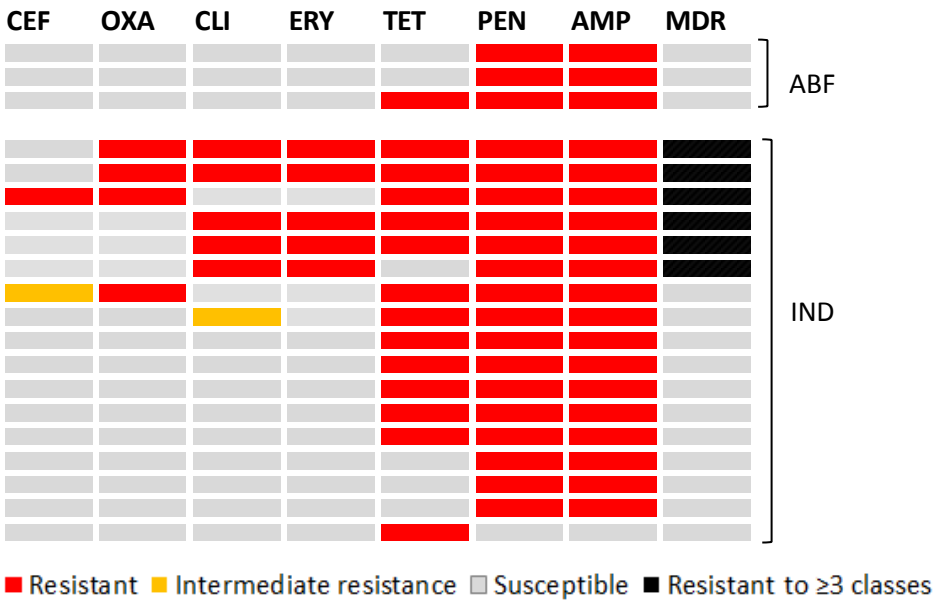

Figure 9. Antibiotic resistance profiles of 20 CC398 isolates collected during study.
CEF: ceftriaxone; OXA: oxacillin; CLI: clindamycin; ERY: erythromycin; TET: tetracycline; PEN: penicillin; AMP: ampicillin; MDR: multidrug-resistant

Sensitivity and specificity comparisons

Results from two different media used to isolate *S. aureus* during this study are shown in Table 6. The sensitivity and specificity for detecting *S. aureus* from human nasal swabs using Baird-Parker media were 80.7% and 95.5%, respectively. Results from 216 individuals were included in these calculations because BP media was unavailable during one week of sample collection. The sensitivity and specificity of CHROMagar™ Staph aureus was 90.1% and 95.4%, respectively. Results from 222 individuals were included in these calculations for the same reason (unavailability of media). Nasal swabs were incubated overnight in selective enrichment broth prior to plating to increase sensitivity on both types of media (Safdar et al., 2003).

Table 6. Sensitivity and specificity of (a) Baird-Parker and (b) CHROMagar Staph aureus media for isolation of *Staphylococcus aureus* from nasal swabs

Baird-Parker

	PCR +	PCR -	Total
Morphology +	67	6	73
Morphology -	16	127	143
Total	83	133	216

CHROMagar™ Staph aureus

	PCR +	PCR -	Total
Morphology +	82	6	88
Morphology -	9	125	134
Total	91	131	222

Identifying colonies as presumptive *S. aureus* based on expression of only 2 out of 3 morphological characteristics was critical in ensuring that no *S. aureus* were missed. Of the 67 individuals correctly identified as *S. aureus*-positive using BP, 17.9% would not have been identified if only those colonies expressing all three characteristics (black, shiny, with clearing) were archived. Of the 82 individuals correctly identified using CS, 26.9% would not have been identified if only those colonies expressing all three characteristics (mauve, matte, with halo) were archived.

Only 69.6% of *S. aureus*-positive participants were identified by both BP and CS; the remaining 30.4% were identified by one type of media or the other. While some of this loss can be attributed to human error and inherent variability in media quality, these results suggest that using at least two types of media to isolate *S. aureus* from nasal swabs improves recovery. However, using two types of media may be cost- and time-prohibitive. Our findings suggest that CHROMagar™ Staph aureus may be a more productive media for *S. aureus* isolation from human nasal swabs than Baird-Parker with Egg Yolk Tellurite Enrichment. CHROMagar™ Staph aureus demonstrated higher sensitivity and comparable specificity to Baird-Parker media in this study.

CHAPTER 4: DISCUSSION

This study provides the first report of CC398 in livestock workers and household members in North Carolina. MSSA CC398 was detected in both IND and ABF participants, although MRSA CC398 only occurred in industrial workers. We observed only 2 cases of MRSA CC398 among 99 sampled industrial participants, a sharply lower incidence than what has been observed in previous studies in North America (Khanna, et al., 2008; Smith, et al., 2009). Most cases of CC398 occurred in individuals with direct exposure to livestock.

Interestingly, 30% of CC398 detected in this study was tetracycline-susceptible. Tetracycline resistance is commonly considered a hallmark of livestock-associated ST398 (MSSA or MRSA) (Frank Møller Aarestrup et al., 2010), though resistance is rare among human-associated ST398 isolates (Uhlemann, et al., 2012). Human-associated ST398 detected in the US thus far has consistently demonstrated resistance to penicillin, and resistance to clindamycin and erythromycin is common as well (Mediavilla, et al., 2012; Uhlemann, et al., 2012). These findings suggest that CC398 in North Carolina may be different from livestock-associated CC398 isolated in previous studies. Whole genome sequence typing will be used to elucidate the evolutionary relationship of all CC398 identified in this study to a diverse collection of ST398 isolates described elsewhere (Price, et al., 2012).

This study provides evidence of a significantly higher prevalence of multidrug-resistant *S. aureus* among individuals exposed to industrial livestock production and processing plants, in comparison to individuals exposed to antibiotic-free operations. However, we cannot calculate adjusted relative risk estimates for MDRSA carriage based on occupational exposure because

the study groups were highly dissimilar – lacking collapsibility. The ABF group was mostly Caucasian, middle class, and college-educated, while individuals in the IND and PP group were largely Hispanic, of lower socioeconomic status, and less educated (data not shown). Additionally, there were wide differences in the distribution of traditional risk factors for MDRSA and MRSA colonization. Members of the ABF group were more likely to have used antibiotics within the past year, to have a pet inside the home, and to have recently visited a gym or played contact sports. However, it is important to that note our findings are in accordance with previous European studies which showed a higher prevalence of antibiotic-resistant *S. aureus* among industrial livestock workers compared with antibiotic-free livestock workers (Blaha et al., 2010; Graveland et al., 2010; Wulf, Nes, et al., 2008). Furthermore, despite having more risk factors for MDRSA and MRSA colonization, ABF participants had a lower prevalence of MDRSA than either the IND or PP group, and had a similar prevalence of MRSA.

Additionally, we observed unique resistance to tetracyclines and gentamicin in individuals from the industrial group. 40% of all *S. aureus*-positive IND participants demonstrated resistance to tetracycline, and 79% of these individuals were workers reporting direct contact with swine. Tetracyclines are one of the most common antimicrobials administered to swine for the purpose of growth promotion. Nearly 50% of the 10 million pounds of antimicrobials fed to swine each year belong to this class (Mellon, et al., 2001), and reliance on tetracyclines in industrial swine production is estimated to be growing (Mellon, et al., 2001). Tetracyclines are also used in poultry production, but less frequently due to high resistance among animals (McEwen et al., 2002). Aminoglycoside resistance was also uniquely observed among industrial participants. Aminoglycosides are used for growth promotion in poultry, and gentamicin is reported to be the most frequently used antibiotic in poultry production (Price et al., 2007). 67% of individuals demonstrating resistance to gentamicin in the

industrial group reported direct or indirect contact with poultry. Aminoglycosides such as gentamicin, neomycin, and streptomycin are administered to swine for therapeutic and prophylactic purposes only.

Lastly, based on our comparison of S. *aureus*-selective media, we conclude that using at least two types of media to isolate *S. aureus* from enriched nasal swabs is helpful to ensure that all *S. aureus*-positive individuals would be identified in a sample population. While CHROMagar™ Staph aureus demonstrated higher sensitivity than Baird-Parker and comparable specificity, 30.4% of *S. aureus*-positive participants would not have been identified if only one type of media had been used in this study. These findings should inform future studies evaluating the presence of *S. aureus* on enriched nasal swabs.

Limitations and Future Research

There were several limitations to our study. First, as described previously, demographic differences in our study populations precluded an exploration of relative risks of MDRSA, MRSA, and CC398 carriage based on occupational exposures, adjusting for potential confounding covariates. This means that there is likely uncontrolled residual confounding in our study. Such wide racial and socioeconomic disparities were not anticipated at the start of our study, but are likely reflective of differences between populations of IND and ABF livestock workers in the Southeastern United States.

Second, we were unable to sample livestock or the production/work-place environment for the presence of antibiotic-resistant *S. aureus*, including CC398. Animal and environmental sampling would be critical in future studies to elucidate sources of transmission of antibiotic-resistant *S. aureus* to livestock workers and their household members, as well as to provide insight into directionality of transmission (i.e., animal to human versus human to animal). Sampling of farms would have thus provided stronger evidence for an association between

carriage of MDRSA, MRSA, and/or CC398 and occupational exposure. However, industrial animal operations in eastern North Carolina were not accessible to our research team.

Third, because our study design was not able to fully target processing plant workers or household members, a smaller number of individuals were sampled. This means that our results cannot be considered characteristic of all PP workers or household members of livestock workers in North Carolina. However, important differences in carriage of MDRSA, MRSA, and CC398 carriage in the PP group compared to the ABF and IND groups were evident even from our small sample size (n=30). High prevalence of multidrug-resistant *S. aureus* among PP participants demonstrates a need for additional research focusing on this population.

Lastly, because we only took a nasal swab from participants at one point in time, we were unable to determine whether carriage of antibiotic-resistant bacteria, including CC398, was due to transient contamination of the nasal passages or true colonization over a longer period of time. While several studies have found carriage of livestock-associated MRSA ST398 to be transient and dependent on animal contact (Graveland, et al., 2011; van Cleef, et al., 2011; Van Den Broek, et al., 2009), a more recent study suggests that carriage may persist regardless of lack of animal contact (Robin Köck, et al., 2012). Future studies should incorporate longitudinal follow-up involving repeated measures of *S. aureus* carriage in IND workers, to help improve understanding of the persistence of antibiotic-resistant *S. aureus* in these populations.

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