# Characterization of *Staphylococcus aureus* at the Human-Swine Interface

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

OF THE UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Dr. Peter R. Davies

November 2016

# Acknowledgements

I would like to express my great appreciation to College of Veterinary Medicine, University of Minnesota for giving me the opportunity to pursue a PhD program.

I would like to thank to my advisor, Dr. Peter Davies who expertly guided me through 4 years of the program. Thank you for spending your valuable time to teach me and make me become an evidence based scientist. There is no doubt that you are the most energetic, enthusiastic and smartest person I know and it was really memorable years under your guidance and mentoring. This journey would not have been possible without your great support.

To my committee, I would like to thank to Dr.Jeff Bender for being a chair of my committee. You were always cheering me whenever I got frustrated from the committee meetings and trying me to think broadly and deeply about my projects. I really appreciate that sometimes you stopped by my cubicles and asked me how everything goes. That encouragement always made me focus and motivate to keep going.

Dr.Srinand Sreevatsan, thank you for being always open to discuss any topics. You always encouraged me to stay focus and keep thinking critically. Also I really appreciate your insightful comments on all the manuscripts I prepared.

Dr. Randall Singer, thank you for constructive feedback on our research and sharing with me your epidemiology and statistic expertise. Your feedback and ideas were absolutely beneficial to the projects I have been working on.

Dr. Marnie Peterson, it was really great opportunity to work on the part of your projects and I would like to thank you for sharing your valuable knowledge of Staphylococcus aureus.

My appreciation also extends to my family and friends. They have always been there for me and supported me. My parents have showed endless love and great support on my decisions. Best friends my sister Sujin, brother in law Kwanhoon and lovely niece Nayeon visited me every year so I never felt I was alone in Minnesota. Special thanks to Minjung Park, thanks for sharing with me the great memories since we met each other first time in the flight on way to Minnesota in Aug 8th, 2012. We always laugh together and cry together I can't imagine my graduate life without you. Most important person, my husband Dustin Haan, thanks for great support through this journey and you have been very patient and helped make this work possible. I can't wait to see our next chapter of life we can go through together. Also I would like to thank my law family who has supported me a lot and treated me and love me as their own daughter or sister.

I met so many great people and sharing friendship through this journey and can't even list here all but I always thank you all for sharing great moment together and supporting me.

## Research Abstracts

### Chapter 2: Prevalence and Characterization of *Staphylococcus aureus* in Growing Pigs in the USA

A decade of research of methicillin-resistant *S. aureus* (MRSA) in pigs shows that the prevalence and predominant genotypes (i.e., ST398, ST9, ST5) of MRSA vary widely geographically, yet knowledge of the epidemiology of *S. aureus* generally in swine remains rudimentary. To characterize *S. aureus*, including MRSA, in the US swine industry, we sampled 38 swine herds in 11 states in major swine producing regions. The herds sampled included pigs sourced from 9 different breeding stock companies, and the sample was likely biased towards larger herds that use regular veterinary services. Twenty nasal swabs were collected from 36 groups of growing pigs by 36 swine veterinarians, 2 more herds were sampled opportunistically, and a historically MRSA-positive herd was included as a positive control. *S. aureus* was detected on 37 of the 38 herds, and in 77% of pigs sampled. Other than the positive control herd, no MRSA were detected in the study sample, yielding a 95% upper confidence limit of 9.3% for MRSA herd prevalence. All but two (ST1-t127; ST2007- t8314) of 1200 isolates belonged to three MLST lineages (ST9, ST398, and ST5) that have been prominent in studies of MRSA in pigs globally. A total of 35 spa types were detected, with the most prevalent being t337 (ST9), t034 (ST398), and t002 (ST5). A purposively diverse subset of 128 isolates was uniformly negative on PCR testing for major enterotoxin genes (A-E). The findings support previous studies suggesting a relatively low herd prevalence of MRSA in the US swine industry, but confirm that methicillin susceptible variants of the most

common MRSA genotypes found in swine globally are endemic in the US. The absence of enterotoxin genes suggests that the source of toxigenic *S. aureus* capable of causing food borne enterotoxicosis from pork products is most likely post-harvest contamination.

**Chapter 3: Longitudinal Study of *Staphylococcus aureus* Colonization and Infection in a Cohort of Swine Veterinarians in the United States**

People working with live pigs are at elevated risk of harboring methicillin resistant *S. aureus* (MRSA) in their upper respiratory tract, and this is attributable to occupational exposure to animals harboring livestock adapted variants of *S. aureus*. To obtain insight into the biological nature of occupationally related nasal culture positivity we conducted a longitudinal study of 66 swine veterinarians in the USA. The study cohort was resident veterinarians in 15 US states, who worked predominantly with swine. Participants submitted self-collected nasal swabs monthly over 18 months, and concurrently completed a survey reporting recent exposure to pigs and other animals; the occurrence of work related injuries during the preceding month; and any relevant health events such as skin and soft tissue infections or confirmed staphylococcal infections. Nasal swabs were cultured using selective methods to determine the presence of MRSA and methicillin susceptible *S. aureus* (MSSA), and isolates were characterized by *spa* typing and MLST. The prevalences of *S. aureus* (65% of all samples; monthly range from 58 to 82%) and MRSA (9% overall; monthly range from 6 to15%) were higher compared to prevalence reported for the US population (30% and 1.5% respectively). Predominant *spa* types were t034 (ST398, 37%), t002 (ST5, 17%) and t337 (ST9/ST398 13%), a distribution similar to that found in a concurrent study in pigs in the USA. Based on

detection patterns, veterinarians were classified into three groups: Persistent carrier (PC, 52%), Intermittent carrier (IC, 47%) and Non-carrier (NC, 1%). True persistent carriage of a single *spa* type was observed in 14 (21%) participants. Based on a single quantitative assessment of a subset of 41 subjects PC veterinarians carried significantly higher numbers of *S. aureus* CFU (colony forming unit) than IC. Among IC veterinarians, culture positivity was significantly associated with recent contact with pigs. Exposure did not lead to prolonged colonization in most subjects, and the higher numbers of SA in PC subjects suggests that unknown host factors may determine the likelihood of prolonged colonization by SA of livestock origin. Although the period of follow up was limited, MRSA carriage and persistence among swine veterinarians was common but rarely associated with any *S. aureus* disease.

**Chapter 4: Antimicrobial Resistance of *Staphylococcus aureus* Isolated from Swine and Swine Veterinarians in the USA**

The recognition that livestock populations can be reservoirs of methicillin resistant *S. aureus* (MRSA) has raised concern about the implications for human health. To obtain broader insight into antibiotic resistance in *S. aureus* in the US swine industry, 128 isolates (including 2 MRSA) from pigs on 36 farms, and 113 isolates (including 21 MRSA) from 66 swine veterinarians were tested for susceptibility to 17 antibiotics and zinc, and for the immune-evasion cluster (IEC) genes that have been linked to human adaptation. Data on current and recent exposure to antibiotics was obtained for 35 swine herds sampled. In both the pig and veterinary populations, >95% of isolates belonged to sequence types ST398, ST9 and ST5 and a majority were resistant to spectinomycin,

tetracycline, macrolides, clindamycin and ampicillin. Resistance was least common to sulfonamides, enrofloxacin and ceftiofur. In general, patterns of resistance were similar for the swine and veterinary isolates, although some differences were observed. Resistance to several antibiotics was associated with MLST sequence type. Significant associations were found for phenotypic resistance between 5 pairs of antibiotics in isolates from both groups. Phenotypic zinc resistance was relatively common (22%) and observed in all MLST types, but the resistance was likely associated with carriage of *czrC* exclusively in the ST398 linage of MRSA. Resistance patterns were extremely diverse with the most common resistotype found in only 6% of isolates. Significant associations between antibiotic exposure and the presence of resistance in swine isolates were observed for tetracyclines and sulfonamides.

**Chapter 5: Genomic Characterization of *Staphylococcus aureus* at the Swine-Human interface**

It is evident that swine and people working with pigs can be reservoirs of livestock associated (LA) *Staphylococcus aureus* or methicillin resistant *S. aureus* (MRSA). Relatively established characterizations of ST398 LA-MRSA lineage in Europe have been shown less virulence and less transmissibility than other endemic MRSA variants causing human infections. However, there is sparse information about genomic characteristics of livestock associated *S. aureus* in the USA, particularly ST9 or ST5 which are the most predominant types besides ST398. To obtain perspectives of virulence factors and antibiotic resistance genes profiles across presumably animal origin *S. aureus*, whole genome sequencing (WGS) analysis was conducted with 76 isolates

from pigs (n=30) and swine veterinarians (n=46). MLST types of isolates were ST9 (n=47), ST398 (n= 19) and ST5 (n = 9) and ST72 (n = 1). Each MLST lineage carried distinct profiles of virulence genes and antibiotic resistance genes suggesting that they have evolved independently by lineage in swine population. ST398 isolates harbored the fewest number of putative virulence genes and absence of human immune response genes (*chp, sak, scn*) corresponding with previous studies. Interestingly, we observed the novel variants (NV), which were putatively believed to be ST9 based on the *spa* typing, were assigned to ST398 using the MLST database and WGS data. These isolates (ST398/t337 and ST398/t3446) were clustered together with other ST398 isolates instead of ST9/t337 and ST9/t3446 on the phylogenetic analysis. Further characterizations are required to understand the genetic variations between these isolates. Overall, we confirmed genetic diversity between sequence types but no significant difference between pigs and veterinarians' isolates. *S. aureus* isolates at the swine-swine veterinarians interface are more likely to be animal origin carrying limited number of virulence genes and multiple antibiotic resistance genes to adapt to the antibiotic pressures in livestock environments.

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# Chapter 1. Literature Review

**General introduction of literature review**

The work presented in this thesis seeks to advance understanding of the epidemiology of *Staphylococcus aureus* in the US swine industry. The work was motivated by ongoing uncertainties about the potential human health risks associated with interspecies transmission of *S. aureus*, particularly with respect to people who are occupationally exposed to pigs. *S. aureus* is a common commensal of both humans and swine, and an opportunistic pathogen of premier significance in people, but has minimal clinical significance in pigs. Consequently, *S. aureus* has been among the most researched of human pathogens, while only recently attracting the attention of researchers in food animals. This recent shift in emphasis was driven by the discovery that livestock populations in many countries may be reservoirs of methicillin resistant *S. aureus* (MRSA), one of the organisms of greatest concern in the current global crisis surrounding antibiotic resistance in human medicine. The research findings are preceded by a general and selective review of literature on *S. aureus* that is most pertinent to the work undertaken, and more detailed review of specific aspects of the literature is presented in the respective research chapters.

## 1. Characterization of *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus*) has had a long history of co-evolution with humans before and since being discovered by Alexander Ogston in 1880 (Ogston. 1882). Normally *S. aureus* dwells on skin, nasal epithelium or mucosal surfaces and 30-50% of healthy adults harbor *S. aureus* intermittently, while 10-20% are persistently colonized

(Eriksen et al. 1995). However, *S. aureus* is also an important opportunistic pathogen that can cause life-threatening invasive infections in people worldwide (Dayan et al. 2016). Various virulence factors in *S. aureus* play significant roles in interactions with hosts, leading to diverse clinical manifestations including soft and skin tissue infection, toxic shock syndrome, pneumonia, septicemia or even death. The majority of virulence genes are associated with nasal colonization and toxin production (Lin and Peterson, 2010). While some virulence genes are located on the core genome, other virulence genes are encoded on mobile genetic elements (MGE), which are exchangeable components such as plasmids, transposons or bacteriophages (Chambers and Deleo, 2009).

*S. aureus* has multiple surface proteins called 'microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)' which play an important role in adhesion to host proteins. For example, *S. aureus* wall teichoic acid (WTA) encoded by the *tagO* and *tarK* genes plays a critical role in cell surface dynamics, and is thought to impact the initial stage of colonization (Baur et al. 2014). On the other hand, fibronectin binding protein, clumping factor B, elastin binding protein, and fibrinogen binding protein appear to become involved in the later stages of colonization. In addition, adhesion molecules (*isdA, fnbA* and *eap*) and immune-modulatory factors *(sak, chp* and *scn*) are increasingly expressed during colonization in human nares (Johannessen et al. 2012). However, *S. aureus* appear to have diverse combinations of surface proteins that influence the survival or *S. aureus* as a commensal and its success as a pathogen (Foster et al. 2014).

Once *S. aureus* has colonized successfully, biofilm formation or toxin production may ensue depending on host immunity or other factors. *S. aureus* is well known as a biofilm

forming species, like other bacteria such as *Listeria monocytogenes, E.coli*, *Salmonella* and *Pseudomonas aeruginosa* (Wang et al. 2013; Giaouris et al. 2015). The formation of biofilms on the surface of medical devices, tissue engineering constructs (i.e, artificial heart valves) or damaged host tissue is associated with increased mortality in patients and difficulty of antibiotic treatment. The last stage of biofilm, 'dispersion' is controlled by the mechanism of quorum sensing regulated by *agr* genes (accessory gene regulator), is able to spread highly populated *S. aureus* inside of biofilm and cause bacteremia such as endocarditis, arthritis or osteomyelitis (Boles and Horswill, 2011). Interestingly, MSSA and MRSA have different mechanisms to form biofilms, and different lineages of *S. aureus* are capable of different levels of biofilm formation indicating genetic components should be considered as tools for control and prevention against biofilm formation (Nicholson et al. 2013; McCarthy et al. 2015; Jotic et al. 2016).

Regarding the numerous toxins produced by *S. aureus*, pyogenic superantigens bind to major histocompatibility complex (MHC) class proteins, release cytokines and stimulate T cell activation (Bernal et al. 1999). For example, cytotoxins such as alpha toxin mediate proinflammatory responses and pore formation. The exfoliative toxin (*ext*) and the Panton-Valentine leukocidin (PVL) are associated with staphylococcal scalded skin syndrome (SSSS) and severe skin disease in community-acquired infections, respectively. Toxic shock syndrome toxin (TSST) was originally identified in menstrual TSS linked to tampon usage in women while non-menstrual TSS occurs mostly as a post-surgical problem (McCormick et al. 2001). The production of heat stable enterotoxins is the central mechanism of foodborne enterotoxicosis (staphylococcal food poisoning), and only a small amount of toxin can cause illness, particularly in immunodeficient patients

or infants (Murray. 2005). Phenol-soluble modulins (PSMs) causing cytolytic peptide mediated neutrophil lysis, are secreted proteins identified in the community associated MRSA (CA-MRSA) USA300 lineage (Wang et al. 2007). Further studies showed that the expression of PSM contributes to biofilm formation controlled by accessory gene regulator (*agr*) and PSMs may be useful virulence factors to track the evolution of CA-MRSA (Otto. 2013).

**Detection of *S. aureus***

*S. aureus* are gram-positive bacteria that grow on blood culture plate to form golden round colonies surrounded by beta hemolysis zone. Methods for *S. aureus* isolation are well established and relatively simple, and identification can be confirmed by several methods using PCR, or biochemical methods such as hemagglutination and coagulase tests (Ji 2014). Columbia agar formulated by Ellner in 1966 has traditionally been used to culture gram-positive bacteria isolation (Ellner et al. 1966). Later, Colombia agar was optimized for *S. aureus* by adding colistin and nalidixic acid with 5% blood to selectively inhibit the growth of other bacteria and to observe clear hemolysis zones (Murray et al. 2003). Phenotypically, *S. aureus* can be differentiated from coagulase negative staphylococci (CoNS) with the coagulase test. *S. aureus* produce staphylocoagulase which binds with coagulate-reacting factor (CRF) (Murray et al. 2003). More specifically for MRSA, a commercially available penicillin binding protein 2a (PBP 2a) latex agglutination test has been used to identify MRSA, showing 100% correlation between detection of PBP 2a and presence of the *mecA* gene (Miller et al. 2005). Because conventional culture methods usually require more than a day to culture and identify

(Martinez et al. 2007), real time PCR (RT-PCR) approaches to have been introduced reduce turnaround times. Using 16S rRNA as an internal control, *nuc* encoding staphylococcal nuclease, *femA* encoding aminoacyltransferase for *S. aureus* and *mecA* for MRSA, PCR enables rapid identification directly from clinical specimens such as blood tubes or nasal swabs (McDonald et al. 2005; Paule et al. 2005; Pasanen et al. 2010).

**Subtyping methods**

Subtyping tools are useful in terms of tracking the origin of outbreaks or understanding the evolutionary history of pathogens. A broad range of phenotypic methods such as phage typing and antibiograms, and genotypic methods have been applied to typing of *S. aureus* and MRSA (Weller. 2000). Increasingly genotypic methods have become predominant, with three commonly used subtyping methods for *S. aureus* being Pulsed-field Gel Electrophoresis (PFGE), Multi-locus sequence typing (MLST), and *spa* typing. PFGE applies high voltages from 6 different angles to create pulse electrical fields to separate and migrant DNA fragments resulting from digestion of DNA by restriction enzymes such as *SmaI* (Bannerman et al. 1995). PulseNet, operated by the Centers for Disease Control and Prevention (CDC) has widely employed PFGE methods to investigate outbreaks of food borne or zoonotic disease (http://www.cdc.gov/pulsenet/index.html). Using PFGE has benefits of high reproducibility allowing data accumulation and comparisons between isolates from different locations. However, it is expensive to set up, time-consuming, and demands considerable expertise to generate reliable results. In 2000, Enright proposed the use of

multi-locus sequence typing (MLST) for subtyping *S. aureus* (Enright et al. 2000). Seven housekeeping genes - *arcC, aroE, gmk, glpf, pta, tpi* and *yquil*, which are considered well conserved regions that reflect evolutionary footsteps of *S. aureus*, are sequenced to obtain allelic numbers for each gene from the database, and combinations of allelic numbers define sequence types, ST (http://saureus.mlst.net). MLST methods have similar discriminatory power to PFGE but are more useful for isolates that are nontypable with PFGE method (Grundmann et al. 2002). However MLST currently costs approximately $50 per isolate and is labor intensive for assembling sequences from all seven genes. Introduction of *spa* (*staphylococcus aureus* protein A) typing has changed the trend of molecular diagnostics of *S. aureus* due to its relative simplicity and discriminatory power (Harmsen et al. 2003). The *spa* gene is comprised of immunoglobulin G-binding regions and the COOH terminus, which include the short sequence repeat (SSR) (Shopsin et al. 1999). After obtaining SSR sequences in polymorphic regions X, repeats are assigned a "repeat succession numbers" by the online Ridom database (http://www.spaserver.ridom.de) and combined repeat numbers define the *spa* type of isolates designed with the letter "t" and a number, for examples t034, t011 or t002. There is relatively high level of correlation between the results of MLST and *spa* typing (O'Hara et al. 2016).

Staphylococcal cassette chromosome (SCC) elements harboring *mecA* genes encoding methicillin resistance were first found in 1961 in the UK (ERIKSEN. 1961) and characterized by several features (Ito et al. 2001). SCC*mec* elements are comprised of *mec* gene and chromosome recombinase (*ccr*) complex, and frequently carry insertion sequences (ISs), transposons or plasmids together harboring antibiotic resistances genes

or virulence genes. Due to the diversity of SCC*mec* structure, SCC*mec* typing is useful method to differentiate the origin of MRSA isolates (Turlej et al. 2011). Commonly USA100 (ST5) and USA300 (ST8) MRSA are harboring SCC*mec* type II and SCC*mec* type IV, respectively (Tenover and Goering, 2009). The majority of LA-MRSA ST398 from Europe were identified SCC*mec* type V but livestock associated LA-MRSA ST5 in the USA harbored SCC*mec* type III and IV (Hau et al. 2016). The components of SCC*mec* are labile, and novel structures have been discovered over time. Thus, SCC*mec* typing provides useful evolutionary insight for epidemiological investigations (Shore and Coleman, 2013)and to date, 18 different SCC*mec* types have been identified (http://www.sccmec.org).

Rapidly advancing sequencing technology has allowed obtaining the resolution of whole genome sequences (WGS) in *S. aureus* (Kuroda et al. 2001). WGS data for *S. aureus* provide the fine details throughout the genome of approximately 2.9 Mbps enabling comprehensive analysis of genetic information and differentiation between closely related bacterial strains. More detailed reviews of the WGS are presented in the final section of this review.

**Methicillin resistant *Staphylococcus aureus***

The public health burden of *Staphylococcus aureus* infection was significantly reduced after the discovery of penicillin that saved tens of million patients from fatal infections (Huttner et al. 2013). However, *S. aureus* conferring resistant to penicillin mediated by *blaZ* encoding β-lactamase emerged rapidly (Lowy. 2003). Subsequently methicillin, a penicillinase resistant analogue of penicillin inhibiting the synthesis of *S. aureus* cell

walls, was developed. In the early 1960s, the first methicillin resistant *S. aureus* was reported soon after the introduction of methicillin in 1959 (ERIKSEN. 1961). The *mecA* gene inserted on the staphylococcal cassette chromosome (SCC) encodes the low-affinity PBP2a (penicillin binding protein 2a) conferring resistance to high concentrations of methicillin (Chambers and Deleo, 2009).

Depending on the epidemiological setting, MRSA infections are classified into hospital associated (or healthcare associated, HA-MRSA) and community associated MRSA (CA-MRSA). MRSA infection within 48 hours of hospitalization is considered as the CA-MRSA by CDC definition (Buck et al. 2005). The infections by community onset are more likely to cause mainly skin infections or severe SSTIs (skin soft tissue infections). HA-MRSA, MRSA in healthcare settings, is a common complication of dialysis, SSI (surgical site infections) or respiratory tract infections in the patients. Immune compromised patients may be more likely to suffer severe blood stream infection (BSI), pneumonia, sepsis or death (Wang et al. 2015).

In 2011, the CDC reported the incidence rates of HA-MRSA infection had decreased from 21 per 100,000 population per year in 2005 to 15 per 100,000 population per year in 2011 due to effective interventions such as hand hygiene, thorough sterilization of surgical tools or precautions to prevent surgical sites from contamination (Dantes et al. 2013).

For several decades, MRSA infections outside of health care settings remained uncommon. In the 1990s, CA-MRSA cases were identified in healthy people in the community such as prisoners, sport players or students involved in crowd activities without any history of contact with health care settings. Also HIV patients or intravenous

drug users were also deemed as risk groups. Incident rates of CA-MRSA between 2005 and 2011 decreased by only 5% decline from 5.9 per 100,000 population per year to 5.2 per 100,000 population per year reflecting the greater difficulty of making interventions across the broad range of communities settings (Dantes et al. 2013; Wang et al. 2015). Typically, the most prevalent genotypes by PFGE in HA-MRSA and CA-MRSA in the USA were US100 (CC5) and US300 (CC8), respectively (McDougal et al. 2003). Due to increased importation and transmission of CA-MRSA into health care settings, however, the straightforward standards by genotyping methods to differentiate HA-MRSA with CA-MRSA isolates are not useful anymore (David and Daum, 2010).

**2. Epidemiology of livestock associated MRSA (LA-MRSA)**

Although humans are considered the primary host of *S. aureus*, the organism also is a common commensal organism in many other homeothermic species, and notably livestock (e.g., swine, bovine, avian, equine) species. The most important staphylococcal infections in livestock are exudative epidermitis in pigs cause by *S. hyicus* and mastitis in cattle caused by *S. aureus* (Barkema et al. 2006; Foster. 2012). There are many reports of isolation of *S. aureus* from various anatomical sites in pigs and it is generally assumed to constitute part of the normal bacterial flora of swine (Linhares et al. 2015). However, prior to 2004, *S. aureus* was considered an unimportant opportunistic pathogen of pigs, and had attracted little research interest.

The first detection of methicillin resistant *S. aureus* in pigs in France (Armand-Lefevre et al. 2005) then from people with occupational exposure to pigs in the Netherlands led to the recognition that pigs may constitute an significant reservoir of MRSA of potential

public health importance (Voss et al. 2005). Using MLST, swine associated isolates in the Netherlands were determined to belong uniformly to a novel sequence type, ST398. Similar isolates were found in other livestock species (van Duijkeren et al. 2010; Graveland et al. 2012) and the ST398 MRSA lineage was described as 'livestock associated' MRSA (LA-MRSA). Subsequently, many investigations were undertaken to assess the prevalence and genotypes of MRSA in swine populations in several countries. Although ST398 were predominant in European countries (European Food Safety Authority. 2009), studies in several Asian countries reported a predominance of another lineage, ST9 (Chuang and Huang, 2015). In North America, MRSA isolates (ST398 and ST5) from pigs were first reported from Ontario, Canada (Khanna et al. 2008), and ST398 MRSA were found in pigs and people in one swine production system in Iowa (Smith et al. 2009). Other US studies reporting MRSA prevalence in the US swine have reported ST398, ST5, and ST9 in pigs (Gordoncillo et al. 2012; Osadebe et al. 2013; Buyukcangaz et al. 2013; Smith et al. 2013). However, all were convenience samples of herds in geographically limited regions, and reported only MRSA isolates without any information on methicillin susceptible *S. aureus*. A pilot study of pigs on 2 farms in Minnesota, USA found 3 lineages (ST398, ST9, ST5) of methicillin susceptible *S. aureus*, and multiple lineages occurred on both farms (Linhares et al. 2015).

**Risk factors for LA-MRSA in pigs**

**1) Age**

The prevalences of LA-MRSA in different age groups of pigs have varied among different cross sectional studies (Broens et al. 2011a; Crombe et al. 2012; Dressler et al.

2012; Espinosa-Gongora et al. 2012; Osadebe et al. 2013; Smith et al. 2013; Fang et al. 2014; Yan et al. 2014). However, two longitudinal studies following the same groups of pigs for 70 days or until the market age, respectively, found that weaned pigs were more likely to be positive for MRSA than fattening or market age pigs (Weese et al. 2011a; Hawken et al. 2013). In contrast, a longitudinal study of MSSA colonization of pigs on 2 farms in Minnesota found prevalence increased with age (Linhares et al. 2015). Bangerter (2016) suggested that nasal colonization of piglets was dependent on colonization status of their sows or a contaminated environment due to the possibilities of vertical or horizontal transmission (Bangerter et al. 2016). The longitudinal studies from Burns and Verhegghe elucidated that pigs were colonized with either different MRSA variants over time or frequently changed between positive and negative status (Verhegghe et al. 2013; Burns et al. 2014). The conflicting results among different studies may reflect methodological issues (i.e., limited sensitivity of culture methods), or could imply that nasal colonization of pigs with MRSA is dynamic and influenced by animal and environmental factors.

**2) Herd size and system**

Contact rate between animals is a critical determinant of transmission rates of infectious pathogens (Correia-Gomes et al. 2014). Thus, herd size, animal flow and density are likely key factors in terms of biosecurity and risk of MRSA introduction to herds. According the European Food Safety Authority (EFSA) report in 2009 (EFSA,. 2009), the pigs from the herds of 400-1000 sows were twice as likely to be positive for LA-MRSA compared to pigs from the herds of less than 100 sows. In addition, several

studies of risk factors from Germany, the Netherlands and Italy (Battisti et al. 2010; Alt et al. 2011; Broens et al. 2011a) indicated similar results where herds with more than 500 finishers or 500 sows were more likely to harbor MRSA compared to smaller herds (Battisti et al. 2010; Alt et al. 2011; Broens et al. 2011). Therefore the larger herd size is considered as the strongest associated factor for herd infection, and transportation of piglets or introduction of gilts replacements is a potentially important source for spreading MRSA to other susceptible herds (Broens et al. 2011b).

**3) Use of antibiotics, heavy metals and disinfectants**

The use of antibiotics, particularly tetracyclines, has been implicated as a contributing factor of the emergence of ST398 LA-MRSA since the comparative genomic study from Price found the *tetM* gene encoding tetracycline resistance was located together with the *mecA* gene encoding methicillin resistance on the SCC*mec* element in these organisms (Price et al. 2012). However, tetracycline resistance is also prevalent in swine MSSA isolates (Aarestrup et al. 2010), and there is no concrete evidence linking specific uses of antibiotics to the emergence of LA-MRSA. Cephalosporin use has also been proposed as a selection pressure associated with the emergence of LA-MRSA in pigs based on an ecological study (Dorado-Garcia et al. 2015) but with the roles of antibiotic use and other environmental factors remain unclear. Recently, several antibiotic resistant genes such as *vgaC, vgaE* or *aadD* encoding pleuromutilin or aminoglycoside resistance, respectively, were found on the plasmid in ST398 and therefore constitute potential mechanisms for co-selection of MRSA (Kadlec and Schwarz, 2009; Li et al. 2014). Furthermore, heavy metals such as zinc or copper which are used as dietary supplements

in pigs to prevent enteric disease and promote rapid growth rate, also may be implicated for emergence of LA-MRSA. Zinc administration in feed could co-select for MRSA variants due to co-location of the *czrC* (cadmium-zinc resistance) and *mecA* genes on SCC in ST398 (Aarestrup et al. 2010; Cavaco et al. 2011). Also disinfectants used to clean the facilities, particularly quaternary ammonium compounds (QAC) were also strongly associated with MRSA prevalence. All MRSA were harboring at least one QAC resistance gene (Slifierz et al. 2015).

**4) Miscellaneous**

Putative risk factors for MRSA prevalence suggested from other studies include were pig-density area, type of floor (slatted floor), use of disinfectants, lower hygiene scores or animal trading (Fromm et al. 2014).

Production type and pig flow may also influence LA-MRSA prevalence. For example, wean-to-finish farms were more likely to be positive for MRSA than other farm types such as farrow-to-finish or grow-finish (Alt et al. 2011). The study from Friese also found fattening farms seemed to have higher positive rates for LA-MRSA than breeding farms (Friese et al. 2012). However, these hypothesis generating studies were conducted in different countries and employed different methods. Therefore, more extensive research is needed to verify the importance of these apparent indicators of MRSA risk.

**Occupational and public health risk of LA-MRSA**

The index case of human ST398 LA-MRSA colonization occurred in a pig farmer's 6-month old daughter and was detected in pre-operative screening (Voss et al. 2005). The

potential health concerns about living or working on farms led to numerous investigations of LA-MRSA in farmers, swine veterinarians, abattoir workers and residents of pig dense regions.

**1) Farmers**- Studies of exposure risk for LA MRSA have primarily focused on farmers who commonly have regular and prolonged contact with pigs, particularly in modern intensive farms. Overall, relatively high prevalences (30-86%) have been reported in swine farmers in Northern European countries (The Netherlands, Germany, Denmark and Belgium etc.,) where LA-MRSA is prevalent in pigs, and where most of the research has been conducted (van Cleef et al. 2011; Graveland et al. 2011; Bisdorff et al. 2012; Vandendriessche et al. 2013). Consistently, nasal colonization with LA-MRSA was associated with contact with livestock, in particular pigs, regardless of age, gender or antibiotic use (Fang et al. 2014). Interestingly, wearing personal protective equipment (PPE) such as gloves, a mask has generally not been shown to play a protective role against MRSA nasal colonization (Wulf et al. 2008).

To date the majority of MRSA isolated from swine farmers have been considered to be of livestock origin based on characteristics such as absence of genes linked to human immune evasion cluster (IEC), and the presence of tetracycline resistance or resistance to multiple antibiotics (Ye et al. 2016). A key question regarding occupational exposure to *S. aureus* is whether positive cultures (typically from nasal swabs) represent transient contamination of mucosal or skin surfaces, versus populations that are established components of the bacterial flora.

**2) Veterinarians**

Veterinarians have unique relationships with livestock populations as they typically contact diverse animal populations on many farms and may work with multiple livestock species. Although there is evidence of lower prevalence of LA-MRSA colonization in veterinarians than farmers, there are also concerns about the possibility that veterinarians may act as vehicles of transmission between farms (Moodley et al. 2008; Walter et al. 2016).

Initial studies of LA-MRSA in veterinarians who attended conferences reported 3-12% prevalence, higher than expected in the general population but substantially lower than seen with farmers. However, due to lack of knowledge about the duration of colonization of people with LA-MRSA, this may have been influenced by the time since animal contact due to traveling to conferences, among other factors (Wulf et al. 2008; Moodley et al. 2008). Frana et al (2013) found that the majority of veterinary students visiting LA-MRSA positive farms became culture positive (nasal swabs) for MRSA after animal contact, but was all were negative when retested 24 hours after visiting farms (Frana et al. 2013). However, a pilot study from Germany compared the prevalence of MRSA in farmers during workdays and after vacation periods of 1-2 weeks. The majority of 59 farmers who were colonized before the vacation were still culture positive after up to 2 weeks without working (Kock et al. 2012).

Verkade (2014) conducted the only longitudinal study of 137 veterinarians (swine and veal calves) to investigate the patterns of nasal colonization with MRSA. Sampling involved five events of nasal swab collection over two years (Verkade et al. 2013). Overall, 44% of swabs were ST398 MRSA positive, and 72% were positive for *S. aureus*,

but the genotypes of MSSA were not detailed. Based on consistent culture positivity at all 5 sampling events, 23 % of the subjects were deemed to be persistent carriers and the majority of them were considered to have been transiently contaminated through contact with pigs or calves on farms.

Regarding the transmission risk to house members of farmers or veterinarians, the evidence suggests that family members of farmers and veterinarians are at lower risk compared with swine workers but at higher risk than the general community (Cuny et al. 2009; Verkade et al. 2014). For example, in a region of Germany known to have a high prevalence of ST398 MRSA in pigs, 86% (97/113) of farmers were positive for MRSA but only 4.3% (5/116) of their family members were positive for ST398. Similarly, 45% (22/49) of swine veterinarians and 9% (4/44) of their family members were positive for ST398 MRSA. Although there is evidence that human-to-human transmission occurs less often with LA-MRSA than common human MRSA variants (Wassenberg et al. 2011), there is evidence of some risk of person to person spread of LA-MRSA.

**3) Residents of regions of high pig density**

Although risk of exposure to LA-MRSA is obviously of concern for people with direct animal contact, *S. aureus* is readily isolated from the air inside and outside of swine barns, and it is known that airborne contamination with LA-MRSA can occur up to 300m by downwind from infected animal populations (Gibbs et al. 2006; Schulz et al. 2012; Ferguson et al. 2016).

In the Netherlands, 21% of individuals that were positive for ST398 LA-MRSA in pig dense regions had no known contact with livestock (van Rijen et al. 2014), further

raising concerns about potential community exposure for people without livestock contact. Prevalence studies of residents of swine dense regions to assess the relative risk of exposure of people who are, or are not, occupationally exposed to pigs have indicated minimal risk (based on nasal swabs) for individuals without occupational exposure (Cuny et al. 2009; van Cleef et al. 2010; Bisdorff et al. 2012). Across these studies, some positive cultures (0.24% collectively) occurred in the non-exposed group, but the prevalence was 44% (prevalence ratio of 180) for occupationally exposed people and their family members were intermediate at 5.4% prevalence (Davies et al. 2013). On the other hand, some studies have inferred elevated risk of LA-MRSA, or MRSA, infection of residents in swine dense regions using case-control (Schinasi et al. 2014; Carrel et al. 2014) or ecological study designs (Casey et al. 2013). However, limitations in the design or inference in some of these studies have been identified (Davies et al. 2013; Perencevich et al. 2014). In some cases, MRSA risk has been associated with livestock exposure, without evidence of the presence of LA-MRSA variants, or even when LA-MRSA have been absent among characterized isolates (Casey et al. 2014).

Overall, the evidence suggests the community exposure risk for residents without livestock contact is non-zero, but substantially lower than for livestock workers, and the routes of exposure remain to be determined.

**The molecular characterization and the pathogenicity of ST398 livestock associated *Staphylococcus aureus* lineage**

The majority studies related to livestock associated MRSA have focused on ST398 in European countries, and relatively little information is available about other MRSA

lineages (e.g., ST5, ST9) detected in pigs in other regions. Distinct features of ST398 MRSA isolates from livestock compared to human isolates have been documented. Comparison of *S. aureus* isolates using microarray assays indicated that immune evasion cluster (IEC) genes, particularly *chp*, *sak* and *scn* genes carried on bacteriophage φSa3, were predominantly found in human isolates (Sung et al. 2008). More specifically focused on ST398, Price et al (2012) reported the absence of the Panton-Valentine leukocidin (PVL) gene in isolates from animals but the presence of genes encoding resistance to methicillin (*mecA*) and tetracycline (*tetM*) on SCC (type V), which also contained the *czrC* gene encoding cadmium-zinc resistance (Price et al. 2012). PVL toxin encoded by luks/f-pv gene has been mostly absent in isolates from animals. McCarthy et al (2012) used microarray analysis to study virulence genes and antimicrobial resistance genes in 100 CC398 isolates from humans and animals (McCarthy et al. 2012b). Interestingly, 77 isolates from pigs and humans who had contact with pigs were clustered together as one clade (P) while 33 isolates from humans with no contact with pigs formed a genetically distinct clade. However, some isolates from humans without any pig contact were in the pig clade (P). This study indicated that attribution of human MRSA infections as being of livestock origin based on MLST type alone may be unreliable, and more discriminating methods are required (Larsen et al. 2015).

*Staphylococcus aureus* enterotoxins cause acute gastrointestinal illness from undercooked meats or careless meat handling (Cheng et al. 2016). Another type of pyrogenic toxin superantigens (PTSAgs) is the toxic shock syndrome toxin-1 (TSST-1) that causes the menstrual and non-menstrual TSS (Chesney et al. 1984). To date enterotoxin and *tsst*

genes have been rarely found in CC398 MRSA isolates from chickens (Wendlandt et al. 2013), cattle and pigs (Fessler et al. 2010; Argudin et al. 2011). Although staphylococcal enterotoxicosis is not uncommonly associated with pork products (Wallin-Carlquist et al. 2010), the prevalence of enterotoxin genes in *S. aureus* lineages other than 398 is not well documented.

One of the important steps in pathogenesis for *S. aureus* is adhesion to host cells for colonization (Wertheim et al. 2005; Weidenmaier et al. 2012; Baur et al. 2014). Interestingly, a phenotypic adhesion assay used by Uhlemann in 2012 showed significantly different affinity of attachment to human keratinocytes between human ST398 MSSA and animal ST398 MRSA isolates suggesting possible differences in host tropism but the mechanism of this difference is still undetermined (Uhlemann et al. 2012).

ST398 isolates have been reported to harbor various antibiotic resistance genes such as *dfrK* encoding trimethoprim resistance, *vgaC* for streptogramins and lincosamides and the multidrug resistance gene *cfr* (Kadlec and Schwarz, 2009; Kadlec and Schwarz, 2010). The existence of multiple resistance genes in livestock associated lineages of MRSA has raised concerns about potential transmission to humans. However, to date there has been no systematic effort to describe antibiotic resistance in *S. aureus* (both MSSA and MRSA) in the US swine industry.

**Human infection with livestock associated *S. aureus* lineages**

Although current evidence suggests that LA-MRSA are less virulent and less likely to transmit among humans than MRSA of human origin (Bootsma et al. 2011), numerous cases of clinical infection linked to CC398 have been reported, particularly in European countries (Kock et al. 2013; Kock et al. 2014; Larsen et al. 2015). The overall impact of LA-MRSA on public health remains uncertain, and is complicated by the fact that some reports do not discriminate cases of MRSA detection by screening from cases with overt clinical infections (Huijsdens et al. 2009). An additional difficulty relates to the possibility that some ST398 infections may be unrelated to animal reservoirs (Uhlemann et al. 2013). Smith and Wardyn (2015) summarized 74 cases of CC398 *S. aureus* infections (including MRSA infections) in 19 different countries between 2005-2014 (Smith and Wardyn, 2015). Most clinical infections occurred in immune-compromised patients or elderly people with no history of animal contact. Furthermore, isolates from some patients had genetic profiles that were atypical for livestock associated isolates as the presence of PVL toxin gene and IEC genes, and sensitivity to tetracyclines (Cuny et al. 2015). The isolate from a fatal case in Japan was closely related with recent Chinese ST398 isolates harboring PVL genes but not associated with any animal contacts (Koyama et al. 2015). Therefore, it is evident some reported ST398 infections are unrelated to livestock reservoirs. Also, a low incidence of clinical LA-MRSA infection has been reported in people without animal contact (van Rijen et al. 2014; Lekkerkerk et al. 2015). Although the routes of these community infections are unknown, foodborne transmission of LA-MRSA appears to be of minimal importance despite the fact that LA-MRSA can be detected in meat (de Boer et al. 2009; Bortolaia et al. 2016; Larsen et al. 2016b).

In Denmark, MRSA surveillance during 1999-2011 described that 151 clinical cases among 7,429 cases were identified as ST398 LA-MRSA and 36% (54/151) of them were from patients who had no history of livestock contact (Larsen et al. 2015). Also some fatal cases occurred without any correlation with livestock (Nielsen et al. 2016).

Within the USA, *S. aureus* associated infections were recently analyzed retrospectively in Iowa, the most pig dense state in the USA. Among 2,226 clinical cases of *S. aureus*, only 1% (25 cases) of isolates were confirmed as ST398 or ST9 livestock associated lineages suggesting a minimal contribution of livestock associated infections to the overall burden *S. aureus* disease in that state (Nair et al. 2016).

Although there is ample evidence that ST398 MRSA of livestock origin can cause a similar range of clinical infections as MRSA variants of human origin, the implications at a population level are still not clear.

**3. Antibiotic resistance in *S. aureus* in swine**

Antibiotics have played integral roles in the treatment and control of infectious diseases of pigs since the 1950s. In addition to therapeutic uses (treatment, control and prevention), for more than 50 years antibiotics have been used to enhance performance of pigs by promoting growth and improving the efficiency of food conversion (Cromwell. 2002). The animal health benefits of antibiotic use in food animals are generally accepted, as is the inevitability that antibiotic use will lead to selection of antibiotic resistant organisms. For example, a systematic review concluded that the oral use of antibiotics in pigs increased the prevalence of antibiotic resistance in *E. coli*, although

there was inadequate information about the effects of dosage and duration of exposure (Burow et al. 2014).

The magnitude of any impact on public health of antibiotic resistant organisms that may result from antibiotic use in food animals is an unresolved debate that is beyond the scope of this review. Historically, debate has focused on major foodborne pathogens (i.e., *Salmonella, Campylobacter*), or particular commensal organisms such as vancomycin resistant enterococci in which resistant organisms in people have been linked to antibiotic use (avoparcin) in food animals (Bager et al. 1997). A detailed review of this question stated that 'several lines of reasoning support the conclusion that agricultural antibiotics are associated with resistance, yet most public policy is based on expert opinion and consensus' (Landers et al. 2012). Use of medically important antibiotics for growth promotion was banned in the European Union in 2006, and similarly will become illegal in the USA from January 1, 2017. (http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM299624.pdf). More extensive efforts to curtail antibiotic use in livestock were implemented in the Netherlands and resulted in a measurable reduction in antibiotic use in food animals including swine. Importantly, the implementation of these measures was motivated by concerns related to the emergence of LA-MRSA in that country (Speksnijder et al. 2015). Antibiotic resistance in *S. aureus* in pigs is relevant to both public health and for understanding the apparently recent emergence of these organisms.

Use of antibiotics in livestock is an obvious mechanism that could have played a part in the emergence of LA-MRSA. However, the mechanisms for emergence are still

unknown (Argudin et al. 2016). Price et al. (2012) concluded that ST398 LA-MRSA originated from human MSSA CC398 after the acquisition of *mecA* and tetracycline resistance genes (Price et al. 2012). The potential coselection was confirmed through genomic analysis indication co-located of *tetM* and *mecA* gene on SCC*mec* elements of ST398 MRSA, unlike other MRSA lineages. The association between use of tetracycline and tetracycline resistance has been reported for decades from *Salmonella* or *E.coli* in pig studies (Haley et al. 2012; Jurado-Rabadan et al. 2014; Johnson et al. 2015). Sequencing of plasmids showed livestock associated ST398 and ST9 *S. aureus* harbored unique combinations of resistance genes. For instance, in swine associated ST398 MRSA the *vgaC, tetL, dfrK* and *aadD* genes were identified on plasmid pKKS825 encoding resistance to pleuromutilins, lincosamides, streptogramin A, tetracycline and aminoglycosides (Kadlec and Schwarz, 2009; Kadlec et al. 2012). Similarly, ST9 MRSA isolates from pigs harbored the pleuromutilin–lincosamide–streptogramin A resistance gene *lsaE* (Li et al. 2013). The occurrence of multiple resistance genes that are inherited in clusters complicates interpretation of relationships between antibiotic use and resistance in animal populations, as resistance genes are likely to persist in the absence of exposure to their corresponding antibiotics (Johnson et al. 2016). Some inferences have been drawn with respect to the use of antibiotics, particularly tetracycline, and the emergence of LA-MRSA. However, limited studies have indicated that MSSA in swine are also commonly resistant to tetracycline, and other factors such as high zinc administration may be linked to LA-MRSA emergence (Cavaco et al. 2011). In order to have better insight into the possible roles of antibiotics in the emergence of LA-MRSA, it

is necessary to understand profiles of resistance in *S. aureus* isolates in the context of both host species and geographic setting.

**4. Whole genome sequencing (WGS) of *S. aureus***

The description of the completed whole genome sequence of MRSA was published in 2001 (Kuroda et al. 2001). The authors used the shot-gun random sequencing method that creates longer reads from chopped DNA than the Sanger sequencing method, and assembled from overlapping reads into a reconstructed sequence. Later, more advanced technologies for genome sequencing have been developed to enable much longer reads and higher coverage. The advent of affordable WGS methods has provided unprecedented opportunities to characterize and compare microbial populations. This has obvious advantages in the context of interspecies transmission of organisms such as *S. aureus*.

Unlike other conventional methods such as *spa* typing or MLST for *S. aureus*, which interrogate a few genetic loci by PCR, WGS enables analysis of the entire genome including the core genome, core variation (CV) parts, and mobile genetic elements. This provides more precise and robust assessment of population structures of isolates. For example, recent WGS studies analyzed LA-MRSA outbreaks over 12 years in humans in the Netherlands (Bosch et al. 2016b), and CC398 isolates from different host species in the USA (Uhlemann et al. 2012). Uhlemann et al (2012) reported that the genomic size of animal isolates was smaller than that of human isolates due to absence of some mobile genetic elements that may be linked to adapting to the animal host.

In a 12-year (2003-2012) Dutch study, 118 isolates of three predominant *spa* types (t011, t034 and t108) were sequenced (Bosch et al. 2016a) These 3 ST398 *spa* types showed relatively distinct genetic distances from each other. The t034 *spa* type was the most diverse and had rapidly increased in frequency of colonization in humans without swine contact, suggesting it may be the most successful genotype for colonizing the human population.

Whole genome sequencing also allows screening and predicting antibiotic resistance genes in *S. aureus* which has a crucial role in clinical bacteriology to provide appropriate treatment with patients (Koser et al. 2014). Practically, antimicrobial susceptibility testing is typically cheaper, faster and more convenient compared to WGS. However, in case of slowly growing bacteria such as *Mycobacterium* species, WGS can also have practical advantages (Price et al. 2013). Also WGS can detect mutation sites in antibiotic resistance genes which can give insight into their evolutionary mechanisms and lead to new strategies for a novel antibiotics development (Koser et al. 2012).

Although there often is concordance between phenotypic and genotypic methods for assessing antibiotic resistance, WGS enables detection of all known resistance genes in an organism. Gordon compared WGS data with phenotypic testing found 97% sensitivity and 99% specificity (Gordon et al. 2014).

Furthermore, WGS has contributed to discovery of novel virulence genes. The first study of entire genome sequences from two MRSA isolates N315 and Mu50 published by Kuroda elucidated more than 70 novel candidate virulence factors such as the *S. aureus* pathogenicity islands (SaPIs) harboring multiple virulence and antibiotic resistance genes (Kuroda et al. 2001).

Lastly, evolutionary history including host adaption or spill-over can be assessed by WGS data. Spoor et al (2013) showed evidence of microevolution of CC97 *S. aureus* isolates from bovine, humans and pigs. The genetic distances indicated livestock to human transmission based on the calculation of molecular clocks using Bayesian analysis (Spoor et al. 2013). Likewise, another population genetic study from Lowder in 2009 indicated a host shift of ST5 *S. aureus* lineage from humans to chickens (Lowder et al. 2009). Phylogenetic analysis indicated multiple genetic variations and permitted discrimination between ST5 isolates from humans and chickens, with evidence of direction of transmission and identification of some host specific factors related to the adaptation.

Therefore, phylogenetic analysis based on whole-genome sequencing (WGS) data can be useful for not only outbreak investigation to infer the origin of isolates, but also understanding short or long term transmission events through accumulated genomic information on the core genome or virulence genes and antibiotic resistance genes on MGEs.

To date (07/08/16), there are WGS genome data from 9,038 *S. aureus* isolates and completed sequences data from 172 isolates on the database (www.patricbrc.org). The majority of the sequence set (61%; 5,658/9,210) were from humans, but only 0.5% (49/9,210) were from pigs. The genetic reference base for *S. aureus* from swine is therefore very limited, and appears heavily biased to European isolates where LA-MRSA has been intensively researched. In particular, there has been little effort to document genotypes of MSSA in swine generally, including in the USA swine population.

Unlike the scenario of LA-MRSA in Europe where ST398 MRSA isolates predominate in swine, the LA-MRSA in North America appear to be relatively diverse (Khanna et al. 2008; Osadebe et al. 2013; Smith et al. 2013; Frana et al. 2013). However, to date individual studies have had relatively little geographic coverage, and/or have generally focused on MRSA with minimal investigation of MSSA. In addition to traditional methods of characterization using MLST and *spa* typing, there is a need to document both the antibiotic resistance genes and putative virulence genes in the US swine population, as these are likely relevant to both the population dynamics of *S. aureus* in pigs and their potential impact on public health.

# Chapter 2. Prevalence and Characterization of *Staphylococcus aureus* in Growing Pigs in the USA

This work has been published in :

Sun, J., Yang, M., Sreevatsan, S., Davies, P.R., 2015. Prevalence and Characterization of Staphylococcus aureus in Growing Pigs in the USA. PLoS One 10, e0143670.

## Introduction

Prior to the recognition that pigs and other livestock species can be reservoirs of methicillin resistant *Staphylococcus aureus* (MRSA) (Voss et al. 2005), *S. aureus* was considered a relatively unimportant organism in swine. Mounting concerns regarding the occupational and public health implications of MRSA in livestock populations have stimulated research of MRSA in animals, and particularly pigs, in many countries (Cuny et al. 2009; Alt et al. 2011; Broens et al. 2011a; Crombe et al. 2012; Boost et al. 2012). Although the ST398 lineage of *S. aureus* was the first designated to be 'livestock associated' in Europe, broader investigations have confirmed that MRSA of other MLST types (e.g., ST9, ST5) also occur in swine populations (Guardabassi et al. 2009; Cui et al. 2009; Frana et al. 2013). Furthermore, the relative prevalence of these lineages, and subtypes within lineages, appears to vary geographically (European Food Safety Authority. 2009; Battisti et al. 2010). ST398 variants have been predominant in studies of pigs in Europe, ST9 in studies from most Asian countries, while both ST398 and ST5 have been relatively common in North American studies (Khanna et al. 2008; Smith et al. 2009; Molla et al. 2012; Frana et al. 2013; Smith et al. 2013). Within the ST398 lineage in Europe, spa types t108 and t011 have been predominant in the Netherlands, while spa type t034 is predominant in Denmark (de Neeling et al. 2007; EFSA,. 2009; Agerso et al. 2012). Similarly, the predominant spa types of ST9 MRSA isolated from pigs vary among Asian countries (Chuang and Huang, 2015).

Until recently, research of *S. aureus* in pigs has been heavily focused on MRSA, with relatively little attention given to the ancestral organism (Armand-Lefevre et al. 2005; Osadebe et al. 2013). The need for a more holistic approach to *S. aureus* epidemiology is

illustrated by a recent study in China in which all MRSA isolates from pigs were ST9, but 61% of methicillin sensitive *S. aureus* (MSSA) isolates from the same population were ST398 (Yan et al. 2014). There has been no comprehensive study to determine the prevalence of MRSA in pigs in the USA or Canada, but several studies have reported observations on MRSA and/or MSSA in geographically limited studies using convenience sampling (Table 2.1).

MRSA in pigs in North America was first reported in 9 of 20 swine herds in Ontario, Canada (Khanna et al. 2008), where ST398 related spa types comprised 75% of isolates, but 3 closely related spa types (t002, t067, t653) likely to belong to the ST5 lineage were also found in 3 herds. Subsequently, a more representative study across Canada found MRSA in fewer herds (5 of 46; 11%), again with ST398 variants predominating and ST5 variants accounting for most of the remaining isolates (Weese et al. 2011b). In the USA, MRSA in pigs (ST398) was first reported in one of 2 production systems in the US Midwest (Smith et al. 2009). A broader study of 45 herds (including 21 herds classified as 'antibiotic free') in 4 US states identified 4 (9%) MRSA positive herds (ST398, ST5 and ST9), (Smith et al. 2013) although 2 herds were affiliated with the one system previously known to be positive from an earlier study (Smith et al. 2009). Frana et al (2013) detected MRSA in 12 (30%) of 40 herds in Iowa, with ST5 isolates (t002, t548) comprising the majority (82%) detected in pigs, and ST398 the remainder (Frana et al. 2013). Similarly, a study of 10 herds in Ohio found that 3 (30%) herds were MRSA positive, and detected the ST5, ST398, and ST9 lineages (Molla et al. 2012). Notably, MRSA prevalence in pigs was low overall (2.9%) in that study, even among pigs from positive herds (7 of 72 pigs; 10%), in contrast with most reports in which pig prevalence

typically exceeds 50% on positive herds (Khanna et al. 2008; Frana et al. 2013; Smith et al. 2013). Thus, observed prevalence and diversity of MRSA in US pig herds have varied among studies, and methicillin susceptible *Staphylococcus aureus* (MSSA) isolates have not been well characterized other than one study of small scale producers in Connecticut (Osadebe et al. 2013).

To guide the design of the current study, we conducted a pilot investigation of *S. aureus* ecology in two multiple-site production systems in Minnesota (Linhares et al. 2015). Neither herd yielded MRSA isolates, but *S. aureus* was detected in 91% of pigs sampled, with all MSSA isolates belonging to the ST398, ST9, or ST5 subtypes. Within systems, multiple spa types (>5 types) and MLST types (2 or 3 sequence types) occurred, and individual pigs frequently carried multiple *S. aureus* variants. The goal of the current study was to estimate the prevalence and diversity of *S. aureus* in growing pigs in a geographically diverse sample of commercial herds in the USA. Given that pork products, particularly ham, are often implicated in cases of staphylococcal enterotoxicosis in people (Bennett et al. 2013), we also tested for major enterotoxin genes A to E to assess the potential importance of the swine reservoir as a source of enterotoxigenic *S. aureus*.

## Materials and Methods

### Selection of Herds and Animals

A cross sectional study was conducted on 36 swine herds located in 11 states of the USA (Mid- west: IA, IL, IN, MI, MN, NE, SD and Non-Midwest: AL, NC, PA, TX). All procedures were approved by Institutional Animal Care and Use Committee (IACUC) at University of Minnesota (1303-30452A), and sampling was conducted from June 2013 to

November 2014. Herds were selected by 36 swine veterinarians, who were purposively chosen from a cohort of swine veterinarians participating in a separate longitudinal study of MRSA colonization and infection in people (Sun et al. 2015a). To maximize diversity in the herds included, each veterinarian selected by convenience one client herd for sampling, and no more than 2 herds were serviced by the same veterinary clinic. The veterinarians were mailed sampling instructions for obtaining nasal swabs from 20 growing pigs aged 4 weeks or older in one client herd. Nasal swabs were mailed to University of Minnesota for processing. The same process was used to collect samples from one positive control herd known to be MRSA positive from previous studies (Smith et al. 2009; Smith et al. 2013). Nasal swab sampling was also conducted opportunistically on pigs at another 2 herds in Minnesota visited for educational purposes by our group. The sample size of 20 pigs per herd was calculated based on the pilot study that found the apparent prevalence of *S. aureus* in nasal swabs of pigs to be greater than 60% (Linhares et al. 2015). Based on an expected prevalence of 60%, it was anticipated there would be an average of 12 positive isolates per herd sampled, and that at least 8 isolates would be obtained in 97% of herds sampled. A total of 739 pig nasal swabs were collected on the 36 veterinary selected herds (20 pigs on 29 of the herds; 21 on 1 herd; 22 on 4 herds; and 25 on 2 herds). Twenty pigs were also sampled on the positive control herd, and on the two educational visits swabs were collected from 37 and 30 pigs respectively. For detection of MRSA at the herd level, a conservative estimate of within-herd prevalence of 10% of MRSA positive pigs would yield and herd sensitivity of 87.8% if 20 pigs were tested. In turn, sampling of 36 herds yields 97% probability of detecting at least one infected herd if 10% of herds were positive.

Bacterial Isolation and Characterization

Isolation of *S. aureus* was performed using the methods described previously (Linhares et al. 2015). Nasal swabs were double enriched in Mueller-Hinton broth (BBLTM, MD, USA) supplemented with NaCl (6.5%) and in Phenol-Red Mannitol broth (BBLTM, MD, USA) supplemented with 4ug/ml Oxacillin (Sigma-Aldrich, MO, USA). Broths showing a color change to yellow were selected for inoculation on chromogenic agar plate (BBL CHROM agar MRSA, MD, USA) and Factor plate (Veterinary Diagnostic Laboratory, University of Minnesota, MN, USA) to culture MRSA and *S. aureus*, respectively. Two colonies per sample were collected for further characterization. DNA was extracted from one colony on the plate with 19.5µl 10mM Tris-HCl and 0.5µl Lysostaphin (both Sigma-Aldrich, MO, USA) at 37°C for 30 min. PCR were used to detect the mecA gene and perform spa typing. The primers for the mecA gene were [F: 5' GTA GAA ATG ACT GAA CGT CCG ATA A 3', R: 5' CCA ATT CCA CAT TGT TCG GTC TAA 3'], and the spa gene [F: 5' AGA CGA TCC TTC GGT GAG C 3', R: 5' GCT TTT GCA ATG TCA TTT ACT G 3']. PCR master mix (USB HotStart-IT FideliTaq, affymetrix, CA, USA) was used to amplify DNA under the following condition: 95°C for 2min, 94°C for 30s, 55°C for 30s, 72°C for 1min with 30 cycles and 72°C for 10 min (Harmsen et al. 2003). All PCR products were visualized on 1% agarose gel with SYBR Safe dye in 1X TAE buffer (Tris-Acetate-EDTA, Thermo Fisher Scientific Inc., MA USA) for 40min at 200 V.

Molecular Typing and Analysis

All selected *S. aureus* isolated were subtyped using spa typing (Harmsen et al. 2003). After amplification of the spa gene, PCR products were cleaned up with Illustra

Exoprostar, (GE Healthcare Bio-sciences, PA, USA) then submitted to the Biomedical Genomic Center (BMGC, University of Minnesota, MN, USA) to obtain gene sequences. After aligning sequences using Sequencher 5.1 software (Gene Codes Corporation, MI, USA), each sequence was submitted to Ridom spa typing database (http://spa.ridom.de/index.shtml).

Multi-locus sequence typing (MLST) of *S. aureus* was performed following the methods previously reported (Enright et al. 2000). Briefly, seven housekeeping genes (carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferrase (*pta*), triose-phosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*)) were amplified and sequenced. Specific allelic numbers of each isolate and sequence type were obtained via the MLST database of *S. aureus* (http://saureus.mlst.net).

Detection of Enterotoxin Genes

The presence of enterotoxin genes A to E was tested by PCR in a subset of 128 isolates purposively selected to include at least one isolate of each spa type detected on each herd (educational herd samples excluded). A multiplex PCR was used to detect genes for *S. aureus* enterotoxins A (*sea*), B (*seb*), C (*sec*), D (*sed*), and E (*see*). Primer sequences for the five enterotoxin genes were selected using published research (Mehrotra et al. 2000). The primer mix to run a single PCR reaction contained 5.5µL nuclease-free water, 12.5µL HotStart-It Fideli Taq Master Mix, (Affymetrix), 0.5µL of each 10µM forward and reverse enterotoxin primers (for *sea, seb, sec,* and *see*), 1.0µL of 10µM *sed* primers, and 1.0µL of extracted *S. aureus* DNA. DNA amplification was conducted with the following thermal cycling profile: an initial denaturation at 94°C for 2 min was followed

by 30 cycles of amplification (denaturation at 94°C for 2 min, annealing at 56°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 5 min. PCR products were visualized under the same conditions used for mecA PCR. *S. aureus* isolates ATCC 13565, ATCC 14458, ATCC 19095, ATCC 23235 and ATCC 27664 were used as positive controls for enterotoxin genes A to E, respectively.

Survey Questions and Statistical Analysis

Each swine veterinarian was requested to complete an online questionnaire to obtain information about the herd from which samples were collected. The questionnaire was administered via Survey Monkey (http://www.surveymonkey.com) and questions included herd size, age of pigs sampled, type of herd, genetic origin, and number of sources providing pigs to the group sampled (surveys were not conducted on the 2 educational visits). Descriptive statistics of the detection of *S. aureus* by herd characteristic were calculated as prevalence at the pig level, using prevalence ratios to compare subgroups. Mixed models to account for clustering at herd level could not be conducted due to sparsity of data in some categories, resulting in quasi complete separation. Within herd prevalence was highly skewed, therefore univariable analyses of associations between within-herd prevalence of *S. aureus* and herd characteristics were conducted by Kruskal-Wallis one-way analysis of variance using Statistix 10.0 (Analytical Software, Tallahassee, FL, USA).

## Results

Thirty-six herds from 11 states in the USA were sampled by veterinarians, mostly (29 of 36, 81%) in the Midwest region where pig production is concentrated, plus 2 additional herds in Minnesota. Completed surveys were obtained for 35 of the 36 herds (97%), and for the MRSA positive control herd. Excluding 7 herds where the genetic sources of pigs were unknown, pigs on the herds sampled by veterinarians originated from the following breeding stock suppliers: Pig Improvement Company (10 herds); Choice Genetics (5 herds); Danbred (4 herds); Genetiporc (3 herds), Fast Genetics, Genesus Genetics, and Topigs/Norsvin (2 herds each); Smithfield Premium Genetics and Hypor (1 herd each). Herd sizes ranged from 40 to 12,000 head and most (22 of 36, 61%) were nursery herds. Mixing of pigs from multiple sources was not widely practiced, with 29 herds (81%) receiving pigs from a single source. The age of pigs sampled ranged from 4 to 20 weeks. Overall among the veterinary sampled herds, 739 nasal swabs were collected, of which 558 (76%) were culture positive for *S. aureus*, and positive pigs for *S. aureus* were detected on 35 of the 36 herds (97%). However, no MRSA were detected in any of the herds sampled, apart from the positive control herd on which all 20 pigs were positive for MRSA. The prevalence of *S. aureus* at herd level varied from 0 to 100% (Fig 1). For the majority of herds (60%, 21/35) prevalence exceeded 80%, including 12 (34%) herds with all pigs positive, while prevalence was less than 50% on only 7 herds. The two additional herds visited were also negative for MRSA, and had high prevalences (90%, 100%) of *S. aureus*.

Molecular Characterization of *S. aureus*

Among the 35 *S. aureus* positive herds sampled by veterinarians, and the 2 opportunistically sampled herds, there was considerable diversity found with 35 spa types detected across 5 MLST sequence types (Table 2.2; Supplement table 2.1). The most predominant spa types (sequence type) were t337 (ST9), t034 (ST398) and t002 (ST5) which together accounted for 54% (653 of 1200) of all isolates. Seven spa types (t3232, t2582, t5883, t1793, t5462, and 2 unknown types) that were detected only once were closely related to the predominant spa type in their respective herd. For example, t2582 (ST398) was found on a herd where t034 was the predominant type. The repeat succession in the spa genes of t034 and t2582 comprised X1-K1-A1-O1-A1-O1-B1-Q1-O1 and X1-K1-A1-O1-A1-O1-A1-O1-B1-Q1-O1 respectively, suggesting that an insertion-deletion event of an A1-O1 repeat likely occurred in the population. All MRSA isolates in the positive control herd were t034 (ST398), as reported previously (Smith et al. 2009; Smith et al. 2013).

Associations between Herd Attributes and *S. aureus* Nasal Colonization in Pigs

Due to the absence of MRSA in all pigs tested, no analysis of MRSA occurrence was possible and analyses were limited to *S. aureus* prevalence. Also, as only one herd was negative for *S. aureus*, no analysis was possible for herd status (positive/negative). Based on prevalence ratios at pig level, unadjusted for clustering by herd, there was no indication of a difference in *S. aureus* prevalence by geographic region or herd size (Table 2.3). However, the data indicated that prevalence was lowest in nursery pigs compared to other farm types and in the youngest pigs 4–6 weeks old (noting that pig age is confounded with farm type, as nursery facilities only house young pigs), and there was some suggestion that prevalence was higher in commingled groups. Based on non-

parametric analysis of variance of prevalence at herd level, prevalence was significantly lower in nursery facilities (P = 0.02), and tended to be positively associated with age (P = 0.07), but the effect of commingling was not significant (P = 0.82).

Genotypic Diversity of *S. aureus* within Herds and Presence of Enterotoxin Genes

In 9 herds, all *S. aureus* isolates were of a single spa type, including six herds with ST9 variants [spa type t337 (5 herds) or t2498 (1 herd)], and 3 with ST398 variants [spa types t034 (2 herds); t5838 (1 herd)]. The most frequent scenario (15/37, 41%) was detection of two spa types in a herd, but 8 spa types were isolated from one herd. Genotypic diversity within herds did not appear to be related to genetic source, geographic location, herd size or age at sampling. All 128 isolates tested negative on PCR for enterotoxin genes A-E.

**Discussion**

Despite over a decade of angst about MRSA associated with swine, the epidemiology of *S. aureus* generally in pigs has been remarkably neglected (Linhares et al. 2015). Furthermore, considerable uncertainty remains regarding MRSA prevalence in pigs in the USA where previous studies have had limited geographic scope and delivered mixed results (Table 2.1). The current study also cannot claim to be representative of the US swine population as herd selection was by convenience rather than formal random sampling from an identified national register of herds. However, the study has some features making it more likely to be representative of mainstream commercial swine production than any preceding studies, some of which purposively targeted small herds or niche populations more than conventional commercial herds (Dressler et al. 2012; Gordoncillo et al. 2012; Smith et al. 2013; Osadebe et al. 2013). Firstly, the current study

was more geographically diverse, encompassing herds in 11 states (including the 6 leading pig producing states), mostly located in the major swine producing regions of the Midwest and South East. Secondly, by restricting sampling to one herd per veterinarian (and 2 herds per veterinary practice), we should have minimized local clustering of sampling within states which occurred in some earlier studies (Molla et al. 2012; Frana et al. 2013). Using veterinarians to select herds would be expected to bias sampling towards larger herds which supply the bulk of the US pork supply, as larger herds are more likely to use veterinary services regularly. This is likely reflected in the fact that 9 of the study herds had more than 5000 animals while only 8 herds had less than 1000 animals. However, theoretical selection biases that are inherent in convenience sampling, such as the processes influencing the veterinarians to select a herd or the farmers being willing to participate, cannot be ruled out.

Perhaps more importantly, the current study included animals of diverse genetic provenance sourced from at least 9 different breeding stock companies, including most of the major genetic suppliers to the commercial industry. The pyramidal distribution of breeding stock in the swine industry is arguably a point of vulnerability that could lead to rapid dissemination of emerging pathogens throughout the industry, particularly for agents that typically do not cause disease in pigs (Wales et al. 2013). However, our observations suggest that MRSA are not widely disseminated in major breeding stock populations in the USA at present. Furthermore, we found no clear association of MLST lineages of *S. aureus* with genetic suppliers, as multiple lineages were found among all genetic suppliers with more than one herd studied, and multiple MLST lineages were present in many herds.

An unexpected finding of the study was that no MRSA isolates could be detected in any of the 38 herds sampled. Based on zero positives among 38 herds in the study sample, the upper 95% confidence limit for herd prevalence, using exact binomial confidence intervals, is 9.25%. Higher herd prevalences (25 to 30%) have been reported in geographically restricted studies in the USA and Canada (Khanna et al. 2008; Molla et al. 2012; Frana et al. 2013). However, larger and more geographically representative studies in both these countries reported herd prevalence of the order of 10% (Weese et al. 2011b; Smith et al. 2013). Recent studies of occupationally exposed people in the USA also indirectly point to the likelihood that MRSA prevalence may be substantially lower in the US swine industry than in many European countries. In two small studies in North Carolina, MRSA prevalence was 7% in workers in both intensive livestock operations (3/41) and antibiotic free operations (3/42) (Rinsky et al. 2013), and one of 22 (4.5%) workers was positive in a longitudinal study (Nadimpalli et al. 2015). A large study in Iowa found only 4 of 163 (2.5%) participants with current pig contact to be MRSA positive, which did not differ from prevalence (2.8%; 26 of 939) in participants without pig contact (Wardyn et al. 2015). These prevalences of MRSA in US swine workers were only marginally higher than the 1.5% estimated for the general US population (Gorwitz et al. 2008), but are an order of magnitude below MRSA prevalences reported in swine workers in community based studies in pig dense regions of Germany and the Netherlands (Cuny et al. 2009; van Cleef et al. 2010; Bisdorff et al. 2012). Similarly, longitudinal studies in the Netherlands and USA indicate MRSA is much less prevalent in swine veterinarians in the USA (9%) than the Netherlands (44%), although both groups had comparable high prevalences (64%, 72%) of *S. aureus* in nasal

swabs (Verkade et al. 2013; Sun et al. 2015a). More definitive estimation of the herd prevalence of MRSA in the USA will require formal random sampling of the commercial swine industry. If such studies were to be pursued, based on current information we suggest they be designed with an expected prevalence of no more than 15%, and with the realization that positive herds may be clustered geographically (Frana et al. 2013). Globally, the predominant lineages found in studies of MRSA in swine populations have been ST398 (most European and some North American studies), ST9 (most Asian studies) and ST5 (some North American studies). The current findings confirm that ST9, ST398, and ST5 MSSA are widely distributed in the US commercial swine population. Although diversity of spa types was evident within these lineages, each lineage was dominated by one spa type (ST9-t337; ST398-t034; ST5-t002) and these collectively comprised 54% of all *S. aureus* isolated. Perhaps unsurprisingly, t034 and t002 have been the predominant spa types among ST398 and ST5 MRSA found in pigs in North America, and 2 ST9-t337 MRSA isolates have also been reported (Khanna et al. 2008; Molla et al. 2012; Frana et al. 2013; Smith et al. 2013). Furthermore, ST9-t337 and ST398-t034 also constituted the majority of *S. aureus* isolates from 50 small pig farms in Connecticut (Osadebe et al. 2013), and from pigs sampled at agricultural fairs in two Midwestern states (Dressler et al. 2012), suggesting a general predominance across multiple segments of the domesticated swine population. The complete absence of enterotoxin genes A-E among the isolates tested is consistent with another recent investigation of *S. aureus* in pigs (Normanno et al. 2015) and suggests foodborne enterotoxicosis with these toxin types associated with consumption of pork products is most likely to result from post-harvest contamination with toxigenic *S. aureus*.

It is well established that particular lineages of *S. aureus* are more adapted to certain host species, and early observers of staphylococcal diversity among animal species posited that the host environment is probably the selective factor which determines the biological properties in host adapted staphylococci (Oeding et al. 1972; Lowder et al. 2009; Moodley et al. 2012). Although 3 MLST lineages accounted for almost all isolates in the original 36 study herds, it is likely that we underestimated diversity both within and among herds by sampling only one anatomical site in just 20 pigs per herd (Linhares et al. 2015). To date, the relatively common occurrence of ST5 MRSA and MSSA isolates in the swine reservoir appears to be unique to North America (Khanna et al. 2008; Molla et al. 2012; Frana et al. 2013; Smith et al. 2013), although there are several reports of ST5 MRSA or MSSA from pigs in other countries (European Food Safety Authority. 2009; Fall et al. 2012; He et al. 2013). Unlike the ST398 and ST9 lineages, which to date have played a negligible role in MRSA epidemiology of humans in the USA, the ST5 lineage has been a major component of hospital and community associated MRSA and MSSA infections worldwide (Miko et al. 2013). While often deemed a 'human adapted' lineage in some studies of swine, an international study of 48 isolates from broilers in 8 countries found a majority were ST5 that exhibited specific genetic changes related to host adaptation (Lowder et al. 2009). Recently, swine associated ST5 MRSA isolates in the USA were also found to differ markedly from ST5 human clinical isolates, suggesting that swine adapted ST5 isolates, like swine adapted ST398, may have reduced virulence for humans (Hau et al. 2015).

It is evident that multiple lineages of *S. aureus* can be endemic in pig populations, and their relative prevalences vary geographically and probably temporally (Espinosa-

Gongora et al. 2014). Given this diversity, the penchant to designate the ST398 lineage of *S. aureus* to be uniquely and synonymously 'livestock associated' is untenable and should be discouraged. More importantly with respect to elucidating the human health implications of ST398 in livestock, in several countries distinct human-adapted ST398 variants have been shown to circulate in the community, independent of livestock reservoirs, and cause human clinical MSSA infections (Uhlemann et al. 2012; Chroboczek et al. 2013). Consequently, the tendency to casually infer zoonotic origin of human *S. aureus* infections based simply on MLST lineage or spa type is imprudent, particularly in the absence of known livestock contact (Davies et al. 2011). On the other hand, indirect transmission of *S. aureus* of apparent livestock origin has led to human clinical infections in some countries, although the pathways of transmission in such cases are undetermined (Lekkerkerk et al. 2015). Conversely, dismissing the possibility of an animal origin of isolates based solely on MLST or spa type is equally questionable as variants of certain subtypes (e.g., ST5, ST15, and ST72) have been documented in pigs in multiple countries (EFSA,. 2009). Essential steps towards more nuanced inference and understanding of the nature of livestock reservoirs of *S. aureus* are to widen the focus of research from MRSA towards more holistic studies of *S. aureus* populations in animals, more comprehensive genotyping of isolates of animal and human origin, and to advance the understanding of genotypic determinants of host adaptation and virulence in the context of interspecies transmission (Guinane et al. 2011; Uhlemann et al. 2012; Viana et al. 2015).

**Table 2.1. Overview of *Staphylococcus aureus* prevalence in the USA**

| Type | Year | Pig Source (age) | Location | # of herd sampled | | Herd Prevalence | Animal Prevalence | Spa types (or sequence type) | MLST | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| Commercial | 2009 | 9, 12, 15, 18, 21, and 24 weeks | IA, IL | 2 | **MRSA** | 50% (1/2) | 49% (147/299) | NR | ST398 | (Smith et al. 2009) |
| | 2011 | Small, Medium , Large size of pigs on USDA standard | CT | 35 | **MRSA** | 14% (5/35) | 3% (8/259) | t008, t007, t011 | ST8, ST398 | (Osadebe et al. 2013) |
| | | | | | MSSA | 43% (15/35) | 30% (85/259) | t337, t034, t334, t4529 t8760, t1166 | NR | |
| | 2012 | Various ages | IA | 40 | **MRSA** | 30% (12/40) | 18% (34/194) | t002, t034, t548 | ST398, ST5 | (Frana et al. 2013) |
| | 2012 | Market age | OH | 10 | **MRSA** | 50% (5/10) | 3% (7/240) | t034, t337 | ST398, ST9 | (Molla et al. 2012) |
| | 2013 | 6 and 18 weeks | IL(+) IA(+) MN, NC, OH | 45 | **MRSA** | 9% (4/45) | 5% (50/1085) | t034, t002, t337, t571 t3446, t002 | ST398, ST5 ST9 | (Smith et al. 2013) |
| | 2015 | From farrowing to Finish | MN | 2 | MSSA | 100% (2/2) | 91.1% (175/192) | t034, t337, t7331, t2462, t3446, t001, t571, t1255, t526 | ST398, ST5, ST9 | (Linhares et al. 2015) |
| | **Total** | | | 134 | **MRSA** | 20% (27/134) | 9% (246/2269) | | | |
| Others | 2011 | Backyard-raised pigs | MI | 50 | **MRSA** | 2% (1/50) | 4% (2/53) | N/A | ST5 | (Gordoncillo et al. 2012) |
| | 2012 | Statefairs A and B | MN, IA | N/A | **MRSA** | | 2% (2/103, Fair B) | t3075, t337 | ST398 ST2136 | (Dressler et al. 2012) |

**Table 2.2. MLST and spa types of *S. aureus* (n=1200) isolated from pigs in 38 herds**

| | | Number of isolates (%) | Number of herds |
|---|---|---|---|
| ST9 | t337 | 384 (32) | **25** |
| | t3446 | 73 (6) | 5 |
| | t2498 | 59 (5) | 3 |
| | t2315 | 23 (2) | 1 |
| | t1334 | 14 (1) | 1 |
| | t2462 | 3 (0.3) | 2 |
| | unknown 1 | 2 (0.2) | 1 |
| | t10494 | 2 (0.2) | 1 |
| | unknown 2 | 1 (0.1) | 1 |
| | unknown 3 | 1 (0.1) | 1 |
| | t3232 | 1 (0.1) | 1 |
| ST398 | t034 | 152 (13) | **12** |
| | t571 | 138 (12) | 4 |
| | t1255 | 34 (3) | 1 |
| | t899 | 30 (3) | 2 |
| | unknown 4 | 27 (3) | 1 |
| | t5838 | 19 (2) | 1 |
| | t14581 | 16 (1) | 1 |
| | unknown 5 | 12 (1) | 1 |
| | t1419 | 12 (1) | 1 |
| | t011 | 10 (1) | 3 |
| | t11374 | 6 (0.5) | 1 |
| | t11241 | 4 (0.3) | 1 |
| | t2582 | 3 (0.3) | 2 |
| | t11744 | 2 (0.2) | 1 |
| | t2582 | 1 (0.1) | 1 |
| | t1793 | 1 (0.1) | 1 |
| | t5883 | 1 (0.1) | 1 |
| | t5462 | 1 (0.1) | 1 |
| ST2007 | t8314 | 5 (0.4) | 1 |
| ST1 | t127 | 7 (0.6) | 1 |
| ST5 | t002 | 117 (11) | **9** |
| | t570 | 22 (2) | 3 |
| | t242 | 13 (1) | 1 |
| | t306 | 4 (0.4) | 1 |

*Repeat succession of unknown types: Unknown1 (r07r16r23r23r02r12r17r23r02r34), Unknown2 (r07r16r16r16r23r23r02r12r23r02r34), Unknown3 (r07r16r16r23r02r12r23r02r34), Unknown4 (r08r475r2r25r2r25r34r34r25), unknown5 (r07r16r23r23r02r23r02r34).

**Table 2.3. Prevalence of *Staphylococcus aureus* in pigs by herd (n=36) characteristics**

| | % Herds (n) | Median herd Prevalence % | Prevalence Pig % (n) | Prevalence ratio Pig (95% CI)* |
|---|---|---|---|---|
| **Herd size** | | | | |
| ≤3,000 | 47 (17) | 85 | 74 (253/344) | Ref |
| >3,000 | 47 (17) | 95 | 77 (273/355) | 1.05 (0.96 - 1.14) |
| Missing | 6 (2) | . | . | . |
| **Type of farm** | | | | |
| Nursery | 61 (22/36) | 60 | 66 (299/452) | Ref |
| Finishing | 17 (6/36) | 90 | 98 (120/122) | 1.49 (1.39 - 1.59) |
| Wean to Finish | 14 (5/36) | 95 | 78 (82/105) | 1.181 (1.05 - 1.33) |
| Farrow to Finish | 8 (3/36) | 100 | 95 (57/60) | 1.44 (1.32 – 1.57) |
| **Pig source** | | | | |
| Single | 81 (29/36) | 90 | 74 (439/595) | Ref |
| Comingle (2 sources) | 11 (4/36) | 90 | 79 (63/80) | 1.07 (0.94 - 1.21) |
| Comingle (≥ 2 sources) | 8 (3/36) | 95 | 88 (56/64) | 1.19 (1.07 - 1.32) |
| **Geographic** | | | | |
| Midwest † | 81 (29/36) | 90 | 75 (446/597) | Ref |
| Non-Midwest ‡ | 19 (7/36) | 100 | 79 (112/142) | 1.056 (0.958 - 1.163) |
| **Sampling age (Weeks)** | | | | |
| 4-5 | 42 (15/36) | 60 | 61 (186/305) | Ref |
| 6-8 | 25 (9/36) | 90 | 79 (147/185) | 1.73 (1.52 – 1.97) |
| 9-12 | 19 (7/36) | 95 | 85 (125/147) | 1.85 (1.63 - 2.10) |
| >12 | 14 (5/36) | 100 | 98 (100/102) | 2.14 (1.91 - 2.38) |

* Pooled data not adjusted for clustering by herd
†Midwest : IA, IL, IN, MI, MN, NE, SD
‡Non-midwest : AL, NC, PA, TX

**Supplement table 2.1 *Spa* types and MSLT types of MSSA and MRSA isolates from 38 swine farms**

| Farm | STATE | SOURCE | N pigs | N MRSA | N MSSA | MSSA | | | | | MRSA | Spa types |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ST398 | ST9 | ST5 | ST1 | ST2007 | ST398 | |
| **1** | IN | VETERINARIAN | 20 | 0 | 11 | 0 | 22 | 0 | 0 | 0 | 0 | t3446, t337 |
| **2** | IL | VETERINARIAN | 21 | 0 | 4 | 0 | 5 | 3 | 0 | 0 | 0 | t002, t2498 |
| **3** | NE | VETERINARIAN | 20 | 0 | 20 | 0 | 25 | 15 | 0 | 0 | 0 | t337, t570 |
| **4** | NE | VETERINARIAN | 20 | 0 | 19 | 23 | 1 | 13 | 0 | 0 | 0 | t034, t242, t2582, t337 |
| **5** | MN | VETERINARIAN | 25 | 0 | 13 | 18 | 2 | 4 | 0 | 0 | 0 | t899, t337, t306 |
| **6** | IL | VETERINARIAN | 20 | 0 | 20 | 1 | 38 | 1 | 0 | 0 | 0 | t2498, t337, t002, t3446, t2582, t2462, unknown2 and 3 |
| **7** | IL | VETERINARIAN | 20 | 0 | 16 | 0 | 30 | 0 | 0 | 0 | 0 | t3446, t337 |
| **8** | MN | VETERINARIAN | 20 | 0 | 9 | 5 | 3 | 2 | 0 | 0 | 0 | t034, t011, t002, t337 |
| **9** | MI | VETERINARIAN | 20 | 0 | 20 | 40 | 0 | 0 | 0 | 0 | 0 | t034, t1793 |
| **10** | IA | POSITIVE CONTROL | 20 | 20 | 20 | 40 | 0 | 0 | 0 | 0 | 20 | T034, t11374 |
| **11** | IA | VETERINARIAN | 20 | 0 | 20 | 4 | 36 | 0 | 0 | 0 | 0 | t11241, t337 |
| **12*** | AL | VETERINARIAN | 20 | 0 | 8 | 0 | 22 | 0 | 0 | 0 | 0 | t2498 |
| **13** | MN | VETERINARIAN | 20 | 0 | 6 | 1 | 10 | 0 | 0 | 0 | 0 | t337, t5462 |
| **14** | SD | VETERINARIAN | 20 | 0 | 18 | 16 | 12 | 0 | 0 | 0 | 0 | t337, t571 |
| **15** | IN | VETERINARIAN | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| **16** | PA | VETERINARIAN | 20 | 0 | 20 | 2 | 3 | 34 | 0 | 0 | 0 | t002, t034, t337 |
| **17** | TX | VETERINARIAN | 20 | 0 | 7 | 14 | 0 | 0 | 0 | 0 | 0 | t034 |
| **18** | NC | VETERINARIAN | 22 | 0 | 17 | 0 | 31 | 0 | 0 | 0 | 0 | t337 |
| **19** | NC | VETERINARIAN | 20 | 0 | 20 | 1 | 0 | 39 | 0 | 0 | 0 | t002, t5883 |
| **20** | MN | VETERINARIAN | 22 | 0 | 21 | 33 | 2 | 1 | 0 | 5 | 0 | t571,t14581,t8134, t10494, t570 |
| **21** | MN | VETERINARIAN | 20 | 0 | 3 | 0 | 6 | 0 | 0 | 0 | 0 | t337 |
| **22** | IN | VETERINARIAN | 25 | 0 | 24 | 12 | 30 | 0 | 0 | 0 | 0 | t337, unknown5 |
| **23** | MN | VETERINARIAN | 20 | 0 | 12 | 19 | 0 | 0 | 0 | 0 | 0 | t5838 |
| **24** | IL | VETERINARIAN | 20 | 0 | 16 | 0 | 30 | 0 | 0 | 0 | 0 | t337 |
| **25** | IN | VETERINARIAN | 20 | 0 | 20 | 0 | 38 | 0 | 0 | 0 | 0 | t337 |
| **26** | MN | VETERINARIAN | 20 | 0 | 18 | 0 | 29 | 7 | 0 | 0 | 0 | t3446, t337, t002 |
| **27** | MN | VETERINARIAN | 20 | 0 | 17 | 27 | 0 | 6 | 0 | 0 | 0 | t002, unknown4 |
| **28** | IN | VETERINARIAN | 20 | 0 | 18 | 22 | 4 | 10 | 0 | 0 | 0 | t002,t034, t337, unknown1 |
| **29** | IN | VETERINARIAN | 22 | 0 | 22 | 0 | 44 | 0 | 0 | 0 | 0 | t337 |
| **30** | NC | VETERINARIAN | 20 | 0 | 20 | 0 | 40 | 0 | 0 | 0 | 0 | t337, t3232 |
| **31** | IA | VETERINARIAN | 22 | 0 | 22 | 18 | 22 | 0 | 0 | 0 | 0 | t011, t034, t337, t3446, t899 |
| **32** | MN | VETERINARIAN | 20 | 0 | 5 | 10 | 0 | 0 | 0 | 0 | 0 | t034 |
| **33** | IA | VETERINARIAN | 20 | 0 | 13 | 12 | 14 | 0 | 0 | 0 | 0 | t1419, t337 |
| **34** | IL | VETERINARIAN | 20 | 0 | 19 | 1 | 16 | 21 | 0 | 0 | 0 | t002, t034, t1334, t570,t2462 |
| **35** | IA | VETERINARIAN | 20 | 0 | 20 | 35 | 2 | 0 | 0 | 0 | 0 | t011, t337, t571 |
| **36** | NC | VETERINARIAN | 20 | 0 | 20 | 39 | 0 | 0 | 0 | 0 | 0 | t034, t11744, t1255 |
| **37*** | MN | EDUCATIONAL | 34 | 0 | 34 | 75 | 0 | 0 | 0 | 0 | 0 | t034, t571 |
| **38** | MN | EDUCATIONAL | 30 | 0 | 27 | 1 | 46 | 0 | 7 | 0 | 0 | t034, t127, t2315, t337 |

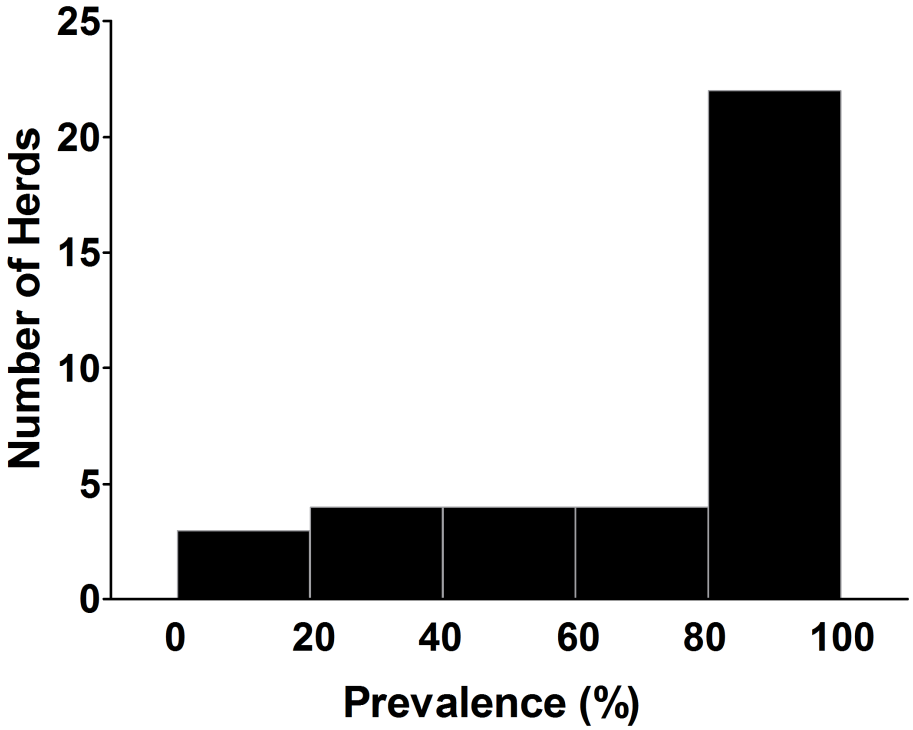



**Figure 2.1. Histogram of prevalence of *Staphylococcus aureus* in 38 herds.**

# Chapter 3. Longitudinal Study of *Staphylococcus aureus* Colonization and Infection in a Cohort of Swine Veterinarians in the United States.

## Introduction

Working and living in close contact with domestic animals facilitates bidirectional interspecies transmission of microbiota, be they innocuous commensals or potential pathogens. Concerns about the importance of animal reservoirs of antibiotic resistant pathogens have been heightened by the unveiling of healthy livestock as reservoirs of methicillin-resistant *Staphylococcus aureus* (MRSA) in many parts of the world (Voss et al. 2005; Khanna et al. 2008; Nemati et al. 2008; Van den Eede et al. 2009; Graveland et al. 2010; Smith et al. 2013; Chuang and Huang, 2015). While most research has focused on the ST398 lineage of livestock associated MRSA that predominates in Europe, it is evident that several genotypes of *S. aureus* are adapted to livestock, and their relative prevalence varies geographically and among livestock species (Chuang and Huang, 2015; Alba et al. 2015; Feltrin et al. 2015; Sun et al. 2015b).

In developed countries, approximately 20 to 30% of healthy people harbor *S. aureus* in the nasal cavity, among other anatomical sites, and nasal colonization is associated with elevated risk of clinical infections (Sivaraman et al. 2009; den Heijer et al. 2013). The most recent (2003-2004) national data for the USA indicate that 28.6% and 1.5% of the general community harbor *S. aureus* and MRSA, respectively, in their nasal cavities (Gorwitz et al. 2008). *S. aureus* colonization in the human population is not random, but is heterogeneously distributed across subsets of the population which have been classified as 'permanent', 'intermittent' or 'non' carriers, although the criteria for defining permanent carriage vary among studies (Wertheim et al. 2005; Muthukrishnan et al. 2013). Bacterial, host, microbiome and other environmental factors may influence the likelihood and duration of nasal colonization of humans with *S. aureus* (Frank et al.

2010; Sollid et al. 2014; Zipperer et al. 2016). However, more detailed longitudinal studies of the dynamics of nasal carriage and bacterial genetic diversity are necessary to better understand this phenomenon (Muthukrishnan et al. 2013). Given that particular lineages of *S. aureus* are known to be host-adapted to certain avian or mammalian species (Guinane et al. 2010; Lowder and Fitzgerald, 2010), and that subtle genomic changes can alter host tropism (Viana et al. 2015), regular exposure of people to *S. aureus* of animal origin is likely to further complicate the poorly understood biology of nasal staphylococcal colonization.

*S. aureus* is considered part of the normal bacterial flora of pigs (Linhares. 2013), and numerous studies have reported that people working with live pigs (farmers, veterinarians, abattoir workers and their families) are at elevated risk of being culture positive for *S. aureus* and MRSA. Notably the predominant genotypes detected have been endemic in the animals with which people have contact (Voss et al. 2005; Armand-Lefevre et al. 2005; Cuny et al. 2009; van Cleef et al. 2010; Bisdorff et al. 2012; Verkade et al. 2013; Frana et al. 2013; Fang et al. 2014). Researchers have typically used enrichment protocols and have reported prevalence of culture positivity, but rarely quantitative data on numbers of organisms present. *S. aureus* appear to be among the most numerous bacteria in bioaerosols of swine barns (Gibbs et al. 2006; Friese et al. 2012), therefore discriminating between transient contamination of superficial anatomical sites (e.g., upper airways or skin) and sustained colonization is problematic, particularly for workers with regular animal contact. Several short term studies have indicated that transient contamination may be the most common outcome in people after short term

exposure to MRSA positive swine herds (VAN DEN Broek et al. 2009; van Cleef et al. 2011; Frana et al. 2013). To date there have only been 2 substantial longitudinal studies of MRSA and *S. aureus* colonization in occupationally exposed swine workers, both in the Netherlands. A study of 110 farm workers sampled 6 times over a year (3 times in one week, then every 4 months) reported that 38% were 'persistent nasal carriers' of MRSA, but the possibility of repeated exposure and recontamination could not be eliminated (van Cleef et al. 2015). Overall, 79% of nasal swabs were positive for *S. aureus* and 63% for MRSA. A study of 137 swine veterinarians sampled 5 times over a 2 year period (quarterly for 12 months, then one year later) classified 13% of subjects to be persistently colonized with MRSA based on consistent molecular typing of isolates (Verkade et al. 2013). Overall, 72% and 44% of nasal swabs were positive for *S. aureus* and MRSA respectively. The rather different estimates (38% vs, 13%) of permanent carriage reported in these 2 studies, despite rather similar overall prevalence of culture positivity, may be an artifact of the different sampling protocols and/or experimental subjects (farmers vs. veterinarians).

Fundamental questions remain about the capacity for *S. aureus* lineages disseminated to animals to both colonize and cause disease in humans. Veterinarians are likely to be a more informative group than farmers for elucidating long term colonization patterns following interspecies exposure as they typically are exposed to multiple herds rather than a single animal population. The goal of this study was to analyze long term patterns of *S. aureus* (including MRSA) colonization in an intensively sampled cohort of swine veterinarians.

**Materials and methods**

The specific aims of the study were to describe the frequency and duration of positive *S. aureus* and MRSA nasal cultures in a cohort of veterinarians having regular contact with varied populations of commercial swine in the USA, and to characterize the genotypes of the isolates detected. The intensity of sampling (monthly for 18 months) was designed to enable more detailed understanding of *S. aureus* colonization patterns than in previous studies of swine workers. All procedures were approved by the Institutional Review Board of the University of Minnesota (1111M06583) and by the Institutional Biosafety Committee (1406-31632H).

Recruitment of study participants

Participants for the study were recruited at the annual meeting of the American Association of Swine Veterinarians (AASV) in Denver, CO in 2012. Eligible veterinarians were members of the AASV who were US residents and typically had regular (e.g. twice per week) professional contact with pigs. A total of 71 veterinarians provided written consent to be research subjects, of which 68 subsequently participated in sample collection. Two participants withdrew during the course of the study (one due to emigration, one due to leaving swine practice), yielding a final cohort of 66 veterinarians who completed the longitudinal sampling protocol. Participants resided in 15 US states (IA, IL, IN, MI, MN, NE, SD, TX, OK, AL, MO, PA, NC, SC, OH), predominantly in the major swine producing regions of the Midwest and Southeast.

Sample submission and survey data

Regular communication with study subjects was maintained by email throughout the course of the study. Collection materials were mailed to the participants who were given written instructions for self-collection of nasal swabs, as well as an instructional video via YouTube. Starting in July 2012, participants were contacted by monthly email and requested to collect and submit a nasal swab via mail. The email message included a link to an on-line survey (http://www.surveymonkey.com) for veterinarians to provide information related to recent pig contact (e.g., time since last pig contact, hours worked in the previous week, number of farms visited in the previous week), and events of physical injury and selected health events (occurrence of skin or soft tissue infections, or confirmed staphylococcal infections) occurring in the month preceding sampling. To encourage compliance, sample collection after the email request was done at the convenience of the participants during the course of their work, and follow-up emails were sent to non-responders to encourage response rates. This process was repeated monthly until the study was terminated. A one-time cross-sectional sampling was performed on 41 available subjects who attended the 2014 AASV meeting in Dallas, TX to determine quantitative bacteriology of *S. aureus*.

Bacteriology

Samples were refrigerated on arrival at the University of Minnesota, and processed in 3 to 4 batches each month as samples typically were received over a 10-14 day period. The dates of arrival and processing were recorded to enable evaluation of the impact of delay in processing (from date of sample collection) on the observed data. For the quantitative

bacteriology samples, all were collected and processed as one batch within 24 hours of collection.

Isolation of *S. aureus* was performed using the methods described previously (Linhares et al. 2015). Nasal swabs were double enriched in Mueller-Hinton broth (BBL$^{TM}$, MD, USA) supplemented with NaCl (6.5%) and in Phenol-Red Mannitol broth (BBL$^{TM}$, MD, USA) supplemented with 4ug/ml Oxacillin (Sigma-Aldrich, MO, USA). Broths showing a color change to yellow were selected for inoculation on chromogenic agar plate (BBL CHROM agar MRSA, MD, USA) and Factor plate (Veterinary Diagnostic Laboratory, University of Minnesota, MN, USA) to culture MRSA and *S. aureus*, respectively. Two colonies per sample were collected for further characterization. DNA was extracted from one colony on the plate with 19.5µl 10mM Tris-HCl and 0.5µl Lysostaphin (both Sigma-Aldrich, MO, USA) at 37°C for 30 min. PCR was used to detect the *mecA* gene and perform *spa* typing. The primers for the *mecA* gene were [F: 5' GTA GAA ATG ACT GAA CGT CCG ATA A 3', R: 5' CCA ATT CCA CAT TGT TCG GTC TAA 3'] , and the *spa* gene [F: 5' AGA CGA TCC TTC GGT GAG C 3', R: 5' GCT TTT GCA ATG TCA TTT ACT G 3' ]. PCR master mix (USB HotStart-IT FideliTaq, affymetrix, CA, USA) was used to amplify DNA under the following conditions : 95°C for 2min, 94°C for 30s, 55°C for 30s, 72°C for 1min with 30 cycles and 72°C for 10 min. All PCR products were visualized on 1% agarose gel with SYBR Safe dye in 1X TAE buffer (Tris-Acetate-EDTA, Thermo Fisher Scientific Inc., MA USA) for 40min at 200 V.

Quantitative bacteriology

Forty-one nasal swabs collected from AASV meeting were transported in one batch on ice to the laboratory and placed in 1ml Mueller-Hinton broth tubes within 24 hours. The tubes were vortexed thoroughly for 1 min and 10-fold dilutions were prepared from 100ul broth (up to $10^{-4}$). 100ul from each dilution was placed using a spreader on a Factor plate and incubated at 37 °C for 18-22 hours prior to counting. Observers were blinded to the carrier status of subjects, and *S. aureus* counts based on colony morphology and hemolysis were calculated as CFU/swab.

Molecular typing and analysis

All selected *S. aureus* isolates were subtyped using *spa* typing (Harmsen et al. 2003). After amplification of the *spa* gene, PCR products were cleaned up with Illustra Exoprostar, (GE Healthcare Bio-sciences, PA, USA) then submitted to the University of Minnesota Genomics Center to obtain gene sequences. After aligning sequences using Sequencher 5.1 software (Gene Codes Corporation, MI, USA), each sequence was submitted to the Ridom *spa* typing database (http://spa.ridom.de/index.shtml). Multi-locus sequence typing (MLST) of *S. aureus* was performed following methods reported previously (Enright et al. 2000). Briefly, seven housekeeping genes (carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferrase (*pta*), triose-phosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*)) were amplified and sequenced. Specific allelic numbers of each isolate and sequence type were obtained via the MLST database of *S. aureus* (http://saureus.mlst.net). MLST typing was performed selectively and not on all isolates. At least one isolate from all *spa* types detected was also evaluated by MLST.

Definition of carrier status

In line with previous studies (Wertheim et al. 2005; Muthukrishnan et al. 2013; Verkade et al. 2013), we classified subjects by carrier status to be non-carriers (NC), intermittent carriers (IC), or permanent carriers (PC). A carrier index (range 0 to 1) was defined as the proportion of sampling events that yielded a *S. aureus* (including MRSA) isolate. Non-carriers were subjects that were never positive for *S. aureus* (including MRSA). Intermittent carriers were subjects that were culture positive on at least one occasion and had a carrier index of < 0.8. Permanent carriers were subjects with a carrier index of 0.8 or greater. The cut-off of 0.8 for the carrier index was based on *post hoc* evaluation of the frequency distribution of the carrier index and was considered conservative (i.e., biased against false positive misclassification of permanent carriage). PC subjects were further classified as true permanent carriers (TPC) if a single *spa* type of *S. aureus* was recovered at all positive sampling events.

PCR testing for the *scn, chp, sak*, and enterotoxin A to E genes

A subset of 116 isolates was purposively selected for further genetic characterization for selected genes. The selection framework identified 4 categories of veterinarians 1) TPC subjects colonized with 1 *spa* type; 2) PC subjects colonized with more than 1 *spa* types 3) IC subjects 4) IC subjects colonized with MRSA on at least one occasion. For TPC subjects (category 1), the first and last isolates obtained were selected for each veterinarian. Among PC subjects (category 2) where the detected genotype changed over time, 2 isolates from each *spa* type detected were chosen up to 4 isolates per veterinarian (i.e., 2 predominant *spa* types). For IC subjects (category 3), only the predominant *spa*

type for each veterinarian was selected. Both MRSA and MSSA were detected from 4 IC subjects during the study period (category 4). For these 4, one MRSA isolate and the predominant methicillin susceptible *spa* type were selected. A total of 24 MRSA and 92 MSSA were thus included for PCR detection of the *scn, chp, sak* and enterotoxin A to E genes (*sea, seb, sec, sed, see*) following the methods previously described (Mehrotra et al. 2000). Briefly, primers for the *scn, chp* and *sak* were [F: 5'- TGA GGC ACA AGC TAG CAC AAG CT-3', R: 5'-TGA AGT TGA TAT TTT GCT TCT GAC ATT TTC-3'], [F: 5'- TTT ACT TTT GAA CCG TTT CCT AC-3', R: 5'-TGC ATA TTC ATT AGT TTT TCC AGG-3'] and [F: 5'-TGA GGT AAG TGC ATC AAG TTC A-3', R: 5'-CCT TTG TAA TTA AGT TGA ATC CAG G-3'], respectively. Annealing temperatures for the *scn, chp* and *sak* were 63°C, 51.5°C and 53°C, respectively. PCR testing for enterotoxin genes A-E followed the same protocol published previously (Sun et al. 2015b). All PCR products were electrophoresed on 1% agarose gel stained with SYBR Safe dye in 1X TAE buffer (Tris-Acetate-EDTA, Thermo Fisher Scientific Inc., MA USA) for 40min at 200 V and visualized on a UV transilluminator. ATCC 700698 (Mu3), ATCC 700699 (Mu50) and ATCC 25904 (Newman) were used as positive controls for *scn, chp* and *sak* genes, respectively, and ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), 90-S-1025 (SED), ATCC 27664 (SEE) were used as positive control strains for enterotoxin genes A to E. Another subset of isolates (MRSA isolates of the ST5 genotype) was evaluated more comprehensively as part of a wider study of ST5 MRSA linked to pigs in the USA which was reported separately (Hau et al. 2015)

Comparison with *S. aureus* isolates from veterinarians and from pigs in the USA

The prevalence and genotypic characterization of a geographically diverse sample of *S. aureus* collected from pigs in the USA was recently published (Sun et al. 2015b). The majority (36 of 38) of farms included in that study were served by 36 veterinary participants of the current study, providing a congruent time-space window to underpin the comparison of genotypes detected in US pigs and swine veterinarians. *Spa* types from the current study and from the study of pigs were categorized to be 'shared' (if detected in both species), 'swine only' or 'human only'. Minimal spanning tree (MST) for clustering of *spa* types was constructed using the Bionumerics 7.1 (Applied Maths, SintMartens-Latem, Beligum).

Statistical analysis

Univariate analysis was performed to estimate the association between culture positivity of *S. aureus* and working activities related with occupational exposure using R studio (Version 0.99.892). For this analysis, the persistent carriers were excluded due to the likelihood of culture positive regardless of risk factors. The risk factors included were following: last contact with pigs (Categorized variable, 0 : same day, 1 : within 1 day, 2: within 2 days, 3 : within 3 days, 4 : more than 3 days) , hours of pig contact (Continuous) and the number of farms visited in the previous week (Continuous). In addition, wearing a mask while they visit farms, the history of injuries by livestock or other soft tissue infection were also evaluated.

The quantitative assessment of *S. aureus* was compared of $\log_{10}$CFU (colony forming unit) between intermittent carriers and persistent carriers.

**Results**

Two veterinarians withdrew from the study due to emigration and altered work circumstances respectively, leaving a cohort of 66 swine veterinarians were enrolled in the study and provided monthly nasal swabs. One veterinarian stopped working with swine after 7 months, but completed the sampling protocol and was retained in the study. Compliance with swab submission and survey completion was over 99% for both nasal swab submission (1,179/1,188) and survey submission (1,177/1,188). The median interval between sample collection and sample processing was 4 day (IQ range : 3-5days, range : 1-35days) and was not associated with the likelihood of culture positivity (p=0.986). Overall, *S. aureus* was detected in 63.7% (757/1188) of nasal swab samples (yielding 1356 *S. aureus* isolates characterized) and MRSA in 9.5% (113/1188) samples (yielding 213 MRSA isolates characterized). The monthly apparent prevalence of *S. aureus* ranged from 58% to 82%, while apparent prevalence of MRSA ranged from 6 to 15%, and there was no indication of seasonal or longer term trends in prevalence over the course of the study (Figure 3.1). MRSA was detected at least once during the study in 18 (27%) of subjects.

At the individual level, the proportion of positive sampling events ranged from 0% (one veterinarian) to 100% (18 veterinarians) and was clearly bimodally distributed (Figure 3.2). In univariate analyses, the likelihood of a culture positive result in intermittent carriers was negatively associated with the interval between the last contact with pigs and

collection of the sample (P =0.001) and positively associated with the hours of pig contact (P=0.009) and the number of farms visited (P=0.019) in the previous week. Three subjects reported having a staphylococcal infection during the study (2 MSSA, 1MRSA), all of which were described as localized and did not lead to hospitalization or time off work. This corresponds with an incidence of 3.4 cases per 1000 person months. Of the 66 subjects, 31 (47%) were classified as intermittent carriers and 34 (52%) were classified as permanent carriers of *S. aureus*. Based on consistent detection of a specific *spa* type at all culture positive samplings, 14 (21%) veterinarians were classified as true permanent carriers (TPC). The TPC group included the veterinarian who ceased working with pigs in February of 2013 but remained positive with ST398/t034 (methicillin susceptible) for the remaining 10 months. The majority (60%) of MRSA isolations were from 4 PC subjects who were positive for MRSA (all ST398) on at least 15 occasions. One of the PC MRSA subjects, who consistently harbored ST398/t034 MRSA, worked exclusively in one production system where this *spa* type occurs at high prevalence, and which was used as a MRSA positive control farm in the related swine study (Sun et al. 2015b).

The one-time quantitative assessment of 41 AASV members sampled in 2014, included both PC and IC veterinarians. PC veterinarians (18 of 22; 82%) were more likely to be nasal culture positive than IC (4 of 19, 21%). Culture positive swabs from PC subjects harbored approximately 2 logs more *S. aureus* per swab than positive swabs from IC subjects. *Spa* typing of isolates from this non-selective procedure yielded the same *spa*

type found in other months for all TPC subjects, and the same *spa* type detected in the prior month for all but 2 PC subjects.

Overall, the *S. aureus* isolates were distributed among 27 *spa* types within 8 MLST sequence types (Table 3.1). Three sequence types (ST398, ST5, ST9) constituted over 84% of all *S. aureus* isolates. Over the course of the study, ST398 isolates were detected at least once in 63 (83%) veterinarians; ST5 isolates in 43 (65%) veterinarians, ST9 in 29 (56%) veterinarians; and other MLST types in 8 (12%) veterinarians. The 3 predominant MLST types (ST398, ST5, ST9) were all isolated at least once from 23 (35%) subjects. Within each of these 3 MLST types, a single *spa* type (t034, t002, t337 respectively) constituted approximately 70% of isolates. Two of these sequence types, ST398 (80.8%) and ST5 (14.1%), accounted for almost 95% of all MRSA isolates, with remainder being ST8/t008, a common human MRSA variant that is unlikely to be of swine origin.

The patterns of detection of specific *spa* types over time varied enormously from highly consistent presence of individual *spa* types in TPC subjects, to very inconsistent patterns with multiple *spa* types detected in individual veterinarians over time (Figure 3a, 3b). It was not uncommon (n = 71) for more than one *spa* type to be detected from a veterinarian at a single sampling event. It is also notable, that in some of the PC and IC subjects a single *spa* type was detected over multiple consecutive months, but thereafter other *spa* types were detected.

**IEC genes and enterotoxin genes**

Approximately 10% (12/116) of the tested isolates were positive for IEC genes (Table3.2). Only one isolate (t2196, ST8) was positive for the *scn* and *sak* genes. The majority of isolates positive for IEC genes were *spa* types likely to be of human origin apart from the t011 (ST398), t5883 (ST398) and t002 (ST5) isolates. Three isolates tested positive for enterotoxin genes. Isolates of *spa* types t062 (ST5) and t2196 (ST8) were positive for the *etd* encoding enterotoxin D and a t338 (ST30) isolate was positive for the *eta* gene encoding enterotoxin A.

**Comparison with *S. aureus* isolates from veterinarians and from pigs in the USA**

*Spa* types from the current study and a previous pig study (Sun et al. 2015b) were categorized as 'shared' (if detected in both species), 'swine only' or 'human only'. Thirteen *spa* types were shared and accounted for 83% and 92% of entire isolates from veterinarians and pigs, respectively. Twenty *spa* types were found only among swine isolates while 14 *spa* types were identified only among veterinary isolates (Table 3.3). A minimal spanning tree (MST) analysis for clustering of *spa* types from the swine and veterinary studies was constructed (Figure 3.4). As expected, isolates were clustered together by sequence type. ST9 isolates were more likely to be found from pig isolates while *spa* types belonging to ST398 and ST5 were relatively more frequent among isolates from veterinarians.

**Discussion**

Our observations provide new insight into the influence of occupational exposure to swine on human nasal carriage of *S. aureus*. Consistent with several studies of people working with swine, the high prevalence of *S. aureus* recovered from nasal cultures reflects the increased exposure to *S. aureus* that occurs in livestock environments (Bisdorff et al. 2012; Smith et al. 2013; van Cleef et al. 2014). The overall *S. aureus* prevalence (63%) in swine veterinarians is approximately double that estimated for the overall US population (Gorwitz et al. 2008), but very similar to that reported (72%) in a study of Dutch swine veterinarians (Verkade et al. 2013). The prevalence of MRSA (9%) was also higher than reported in the US population (1.5%), but was substantially lower than in the Dutch swine veterinarians (44%), which likely reflects the lower prevalence of MRSA in the US swine industry relative to the Netherlands (Smith et al. 2013; Sun et al. 2015b; Dierikx et al. 2016).

If animal contact did not result in transmission of *S. aureus* to humans, it would be expected that one-quarter to one-third of nasal swabs from US veterinarians would be positive, and that the isolates would all of human origin. However, a substantial majority (>84%) of *S. aureus* isolates in this study were deemed likely to be of swine origin based on several criteria. A parallel study of 38 US swine farms, sampled by a subset of the veterinarians in the current study, found that three MLST sequence types (ST9, ST398, ST5) constituted over 99% of swine isolates, with *spa* types t337, t034, and t002 predominating (Sun et al. 2015b). The same set of sequence types and *spa* types were similarly predominant among the veterinarians, generally lacked the IEC genes, and were

predominantly (85%) tetracycline resistant (Sun et al. 2016b). The absence of the IEC genes, together with tetracycline resistance, has been used previously to differentiate isolates of human and animal origin (Stegger et al. 2013; Cuny et al. 2015) and our data suggest that *S. aureus* acquired from swine may largely displace *S. aureus* of human origin in the nasal flora of swine veterinarians. However, given that more than one *spa* type was often detected in individual samples, and only 2 isolates per sample were categorized, it is possible that carriage of relatively low numbers of human *S. aureus* by veterinarians went undetected (Votintseva et al. 2014), and that animal contact added to, rather than displaced human *S. aureus*. Regardless, the data suggest that animal exposure may alter the composition of the nasal *S. aureus* populations of swine workers. Any resultant impact on risk of clinical infection will depend on the relative persistence, transmissibility, and pathogenicity of *S. aureus* of swine origin compared with human adapted variants.

The primary goal of this study was to understand the persistence of *S. aureus* of swine origin in occupational groups that are in intimate contact with pigs. Several previous studies of livestock workers have examined this question, with varying outcomes (Armand-Lefevre et al. 2005; Khanna et al. 2008; Graveland et al. 2010; van Cleef et al. 2011; Bisdorff et al. 2012; Smith et al. 2013; van Cleef et al. 2014). Most studies have focused on MRSA alone, and the frequency and duration of sampling has varied widely. A common obstacle to inference has been the inability to differentiate repeated contamination of the nasal mucosa from true persistent colonization (Goerge et al. 2015). This is particularly the case for farmers who typically are repeatedly exposed to the same herds (and therefore same *S. aureus* populations) over time (van Cleef et al. 2014). We

specifically chose to study veterinarians because they generally visit multiple farms and therefore should be exposed to more diverse *S. aureus* populations. We also included both MSSA and MRSA to obtain a comprehensive assessment of interspecies exchange and persistence of *S. aureus*.

Longitudinal studies of nasal carriage of *S. aureus* in humans typically classify subjects as persistent carriers, intermittent carriers, and non-carriers (Muthukrishnan et al. 2013; Ritchie et al. 2016). It is believed that a subset (usually of the order of 20%) of healthy people is persistently colonized with *S. aureus* (WILLIAMS. 1963; Wertheim et al. 2005), and this is associated with higher risk of *S. aureus* clinical infections (von Eiff et al. 2001; Verhoeven et al. 2014). Both host genetic factors and microbial factors may play a role in determining duration of carriage (Sollid et al. 2014), and it was recently reported that presence of *S. lugdunensis* in the nose may suppress *S. aureus* populations (Zipperer et al. 2016). Currently, there is no accepted consensus for defining persistent carriers, and categorization of individuals will be influenced by study design (particularly the frequency, duration and sampling), and the cut-off (carrier index) used to define carriage status (Ritchie et al. 2016). We employed a post-hoc epidemiological approach to establish a cut-off (carriage index >0.8) to define persistent carriage. The same criterion has been employed in previous studies (Muthukrishnan et al. 2013; Ritchie et al. 2016). The bimodal distribution of the frequency of culture positive samples among veterinarians (Figure 3.2) suggests that even in environments with high exposure to *S. aureus* of animal origin, individual host characteristics are likely important determinants of the persistence of colonization. This inference is further supported by the substantially higher numbers of *S. aureus* recovered from nasal swabs from PC compared with IC

veterinarians, which is consistent with quantitative studies performed in humans both with (van Cleef et al. 2014) and without (Verhoeven et al. 2012) known livestock association.

The proportion of persistent carriers (52%, 34/66) was considerably higher, and the proportion of non-carriers (<2%, 1/66) considerably lower, than reported in people without livestock contact (Muthukrishnan et al. 2013; Ghasemzadeh-Moghaddam et al. 2015; Ritchie et al. 2016). This may reflect unusually frequent exposure to *S. aureus* that can occur in intensive swine facilities (Bos et al. 2016). Very similar rates of apparent persistent carriage of *S. aureus* were also observed in recent longitudinal studies of swine farmers (52%, (van Cleef et al. 2014)) and swine veterinarians (47%, (Verkade et al. 2013)) in the Netherlands, although the sampling protocols and criteria for defining persistence in those studies differed.

The significant associations observed between those that are culture positivity and time since last pig contact, hours of pig contact in the previous week, and number of sites visited is consistent with previous studies and indicates that occupational exposures result in transient contamination or short term colonization (Graveland et al. 2011; van Cleef et al. 2011; Frana et al. 2013). The variability of *spa* types detected over time in the IC group (Figure 3.3b) also attests to multiple exposures of swine veterinarians to diverse *S. aureus* populations over time. Although early research of persistent carriage in humans suggested that people were colonized over the long term by a single *S. aureus* variant, more recent studies indicate that *S. aureus* variants are often replaced over time (Muthukrishnan et al. 2013; Ghasemzadeh-Moghaddam et al. 2015; Ritchie et al. 2016).

Ritchie (2015) sampled 122 healthy young adults weekly over 13 weeks and described 3 patterns of persistent carriage, being continuous carriage of a single *spa* type; an abrupt change from one *spa* type to another; and periods of co-carriage with two *spa* types. Although 'strain turnover' was observed in both intermittent and persistent carriers in that study, the majority (63%) of PC subjects were colonized by a single *spa* type. We made similar observations in the veterinary cohort where all 3 patterns of PC were observed, and a substantial proportion (41%; 14 of 34 PC) of the PC veterinarians were classified as 'true persistent colonization' due to the repeated presence of a single *spa* type over 18 months. This slightly lower proportion (41% vs. 63%) of TPC could be an artifact of the longer sampling period (18 months vs. 13 weeks) providing more opportunity for strain turnover, or the additional exposure to *S. aureus* variants that occurs in the livestock environment. Use of sub-typing methods more discriminatory than *spa* typing would provide greater certainty that subjects were indeed colonized by single variants, which was indicated in one study using whole genome mapping of isolates from 16 Dutch veterinarians (Bosch et al. 2015). Although it is not possible to eliminate the possibility of repeated reacquisition of the same variant from pigs by veterinarians, this is considered unlikely over an extended period unless they were exposed to homogeneous swine populations harboring few *S. aureus* variants. However, repeated reacquisition can be eliminated for one subject in our study who remained culture positive for ST398-t034 MSSA for 9 months after leaving swine practice. Current evidence suggests that a substantial proportion of swine veterinarians become colonized with *S. aureus* of swine origin for at least 18 months to 2 years (Verkade et al. 2013), and harbor substantial numbers of these organisms. Furthermore, although several studies indicate that ST398

MRSA are less transmissible among people than MRSA of human origin, transmission from veterinarians to their families, who also may become persistently infected, has been clearly demonstrated (Verkade et al. 2014; Bosch et al. 2015).

The fact that some swine workers consistently harbor substantial numbers of *S. aureus* with genotypes consistent with swine origin has implications regarding occupational health. However, the human health consequences of livestock associated *S. aureus* are not well defined, and available information is largely limited to ST398 MRSA. In the USA, to date *S. aureus* of animal origin appear to have had negligible impact on human health. The reported incidence of *S. aureus* infections in this study (3.4 per 1000 person months, or 4% per veterinary-year) is similar to that reported in a study of Iowa residents (2.7 per 1000 person months). Furthermore, *spa* types linked to livestock represented only 1% of *S. aureus* isolates, and 0.24% of MRSA isolates, from human clinical infections in the pig dense state of Iowa (Nair et al. 2016). In contrast, a substantial study in Denmark showed increased likelihood of infection with specific livestock associated MRSA, but not increased MRSA infection risk overall, in people living in pig dense areas (Larsen et al. 2015). Foodborne transmission was considered unlikely, and transmission from swine workers into the broader community is a plausible explanation. However, despite the substantial exposure to *S. aureus* of swine origin that occurs in intensive production environments, there are remarkably few reports of medically significant infections occurring in swine workers (Omland and Hoffmann, 2012; Goerge et al. 2015). To date, we are unaware of any studies demonstrating increased risk of clinical *S. aureus* infections in livestock workers. A recent prospective study of swine farm workers in Holland found that carriage of ST398 MRSA was not associated with

elevated risk of infections, healthcare contact, or measures of reduced quality of life (VAN Cleef et al. 2016). Somewhat surprisingly, the small numbers of fatal human cases of ST398 infection have occurred in people without known livestock contact, but who were generally medically compromised (Nielsen et al. 2016).

Even though there are a small number of fatal human cases of ST398 infections, it is important to understand the interchange of *S. aureus* between humans and animals and the implications for the transfer of resistance elements. At present the risk seems limited, continued measures are necessary to understand this exchange of bacteria and promote antibiotic stewardship "best practices".

**Table 3.1. Numbers (%) of spa types of *S. aureus* and MRSA isolated from swine veterinarians, by MLST type**

| Sequence type | *Spa* type | *S. aureus* (n=1356) | MRSA (n=213) |
|---|---|---|---|
| ST398 | t034 | 462 (34.1) | 116 (54.5) |
| | t571 | 71 (5.2) | 0 |
| | t011 | 63 (4.6) | 12 (5.6) |
| | t337* | 20 (1.5) | 0 |
| | t3446* | 19 (1.4) | 0 |
| | t1250 | 8 (0.6) | 0 |
| | t2330 | 6 (0.4) | 44 (20.7) |
| | t2876 | 24 (1.8) | 0 |
| | t7160 | 13 (1.0) | 0 |
| | t1255 | 3 (0.2) | 0 |
| | | **650 (51.8)** | **172 (80.8)** |
| ST5 | t002 | 238 (17.6) | 27 (12.7) |
| | t045 | 69 (5.1) | 0 |
| | t062 | 19 (1.4) | 0 |
| | t242 | 0 | 3 (1.4) |
| | t570 | 12 (0.9) | 0 |
| | t856 | 3 (0.2) | 0 |
| | | **341 (25.1)** | **30 (14.1)** |
| ST9 | t337* | 178 (13.1) | 0 |
| | t2498 | 47 (3.5) | 0 |
| | t10494 | 11 (0.8) | 0 |
| | t3446* | 9 (0.7) | 0 |
| | t1334 | 3 (0.2) | 0 |
| | t1430 | 2 (0.1) | 0 |
| | | **289 (18.4)** | **0** |
| ST8 | t008 | 0 | 11 (5.1) |
| | t2196 | 18 (1.3) | 0 |
| ST30 | t338 | 4 (0.3) | 0 |
| | t363 | 1 (0.1) | 0 |
| ST72 | t126 | 30 (2.2) | 0 |
| ST278 | t330 | 22 (1.6) | 0 |
| ST2007 | t8314 | 1 (0.1) | 0 |

* Two sequence types were identified within t337 and t3446 isolates

**Table 3.2. *S. aureus* isolates testing positive for IEC and enterotoxin A-E genes.**

| | Month* | ID | MRSA | *spa* type (ST) | *scn* | *sak* | *chp* | ET |
|---|---|---|---|---|---|---|---|---|
| 1 | 04 | 24 | MSSA | t126 (ST72) | + | + | + | |
| 2 | 04 | 57 | MSSA | t330 (ST278) | + | + | + | |
| 3 | 04 | 61 | MSSA | t062 (ST5) | + | + | + | D |
| 4 | 06 | 19 | MSSA | t5883 (ST398) | + | + | + | |
| 5 | 07 | 05 | MSSA | t338 (ST30) | + | + | + | A |
| 6 | 09 | 44 | MSSA | t2196 (ST8) | + | + | - | D |
| 7 | 12 | 61 | MSSA | t062 (ST5) | + | + | + | |
| 8 | 12 | 61 | MSSA | t011 (ST398) | + | + | + | |
| 9 | 16 | 57 | MSSA | t330 (ST278) | + | + | + | |
| 10 | 17 | 41 | **MRSA** | t008 (ST8) | + | + | + | |
| 11 | 17 | 66 | MSSA | t002 (ST5) | + | + | + | |
| 12 | 18 | 66 | MSSA | t002 (ST5) | + | + | + | |

* Month of sampling from month 1 to month 18.

**Table 3.3. *Spa* type comparison between swine and veterinary isolates**

| Shared | Swine only | Human only |
|---|---|---|
| t002 | t899 | t008 |
| t011 | t5883 | t045 |
| t034 | t5838 | t062 |
| t10494 | t5462 | t1250 |
| t1255 | t3232 | t126 |
| t1334 | t306 | t1430 |
| t242 | t2582 | t2196 |
| t2498 | t2462 | t2330 |
| t337 | t2315 | t2876 |
| t3446 | t1793 | t330 |
| t570 | t14851 | t338 |
| t571 | t1419 | t363 |
| t8314 | t11744 | t856 |
| | t11374 | t922 |
| | t11241 | |
| | unknown1 | |
| | unknown2 | |
| | unknown3 | |
| | unknown4 | |
| | unknown5 | |

*Repeat succession of unknown types: Unknown1 (r07r16r23r23r02r12r17r23r02r34), Unknown2 (r07r16r16r16r23r23r02r12r23r02r34), Unknown3 (r07r16r16r23r02r12r23r02r34), Unknown4 (r08r475r2r25r2r25r34r34r25), unknown5 (r07r16r23r23r02r23r02r34).

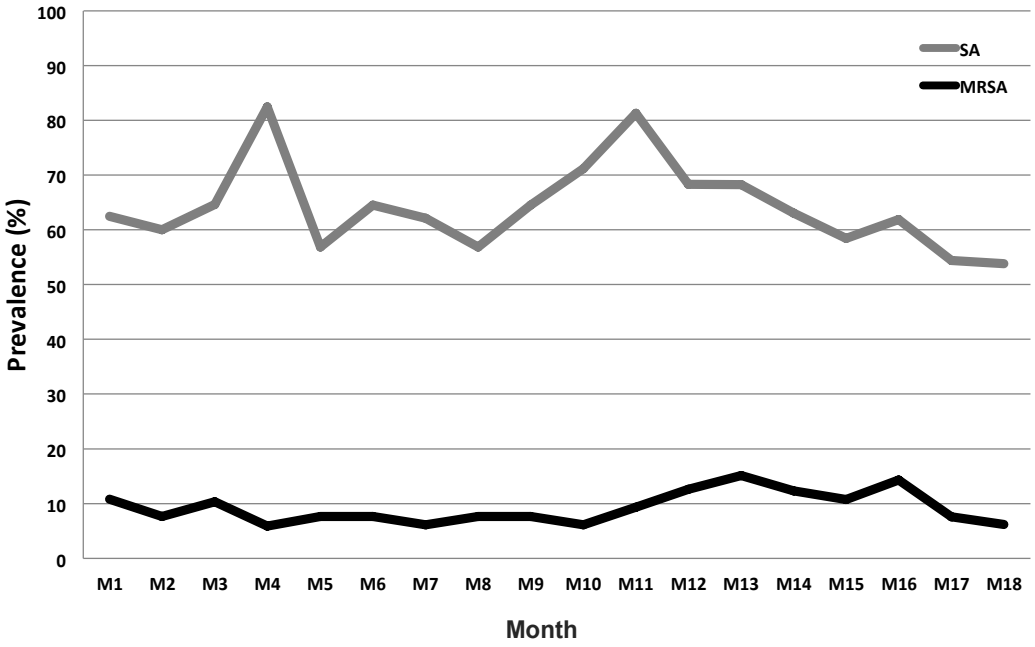



**Figure 3.1. Proportion of *S. aureus* and MRSA positive nasal swabs from a cohort of swine veterinarians sampled monthly from July 2012 to December 2013**

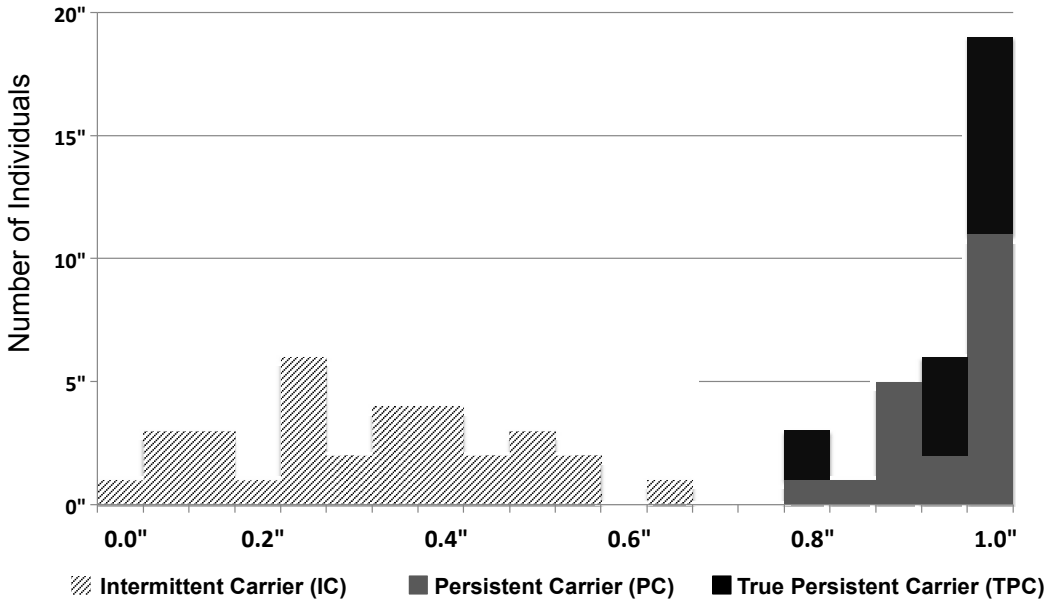
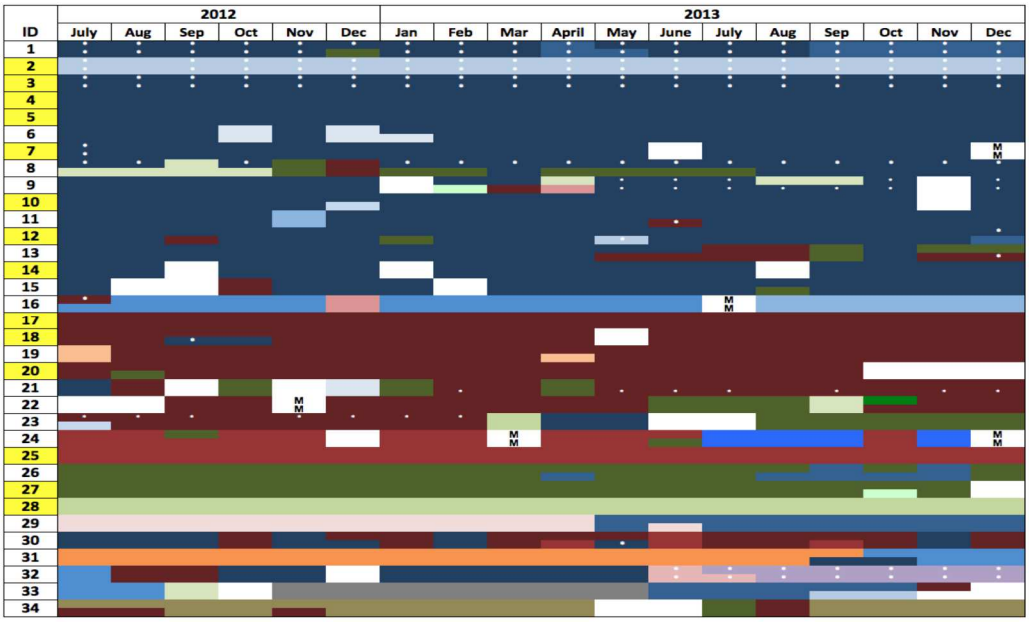



**Figure 3.2. Histogram of the proportion of sampling events yielding a *S. aureus* isolate ("Carrier index")**

a) Persistent carriers (PC)




b) Intermittent carriers (IC)

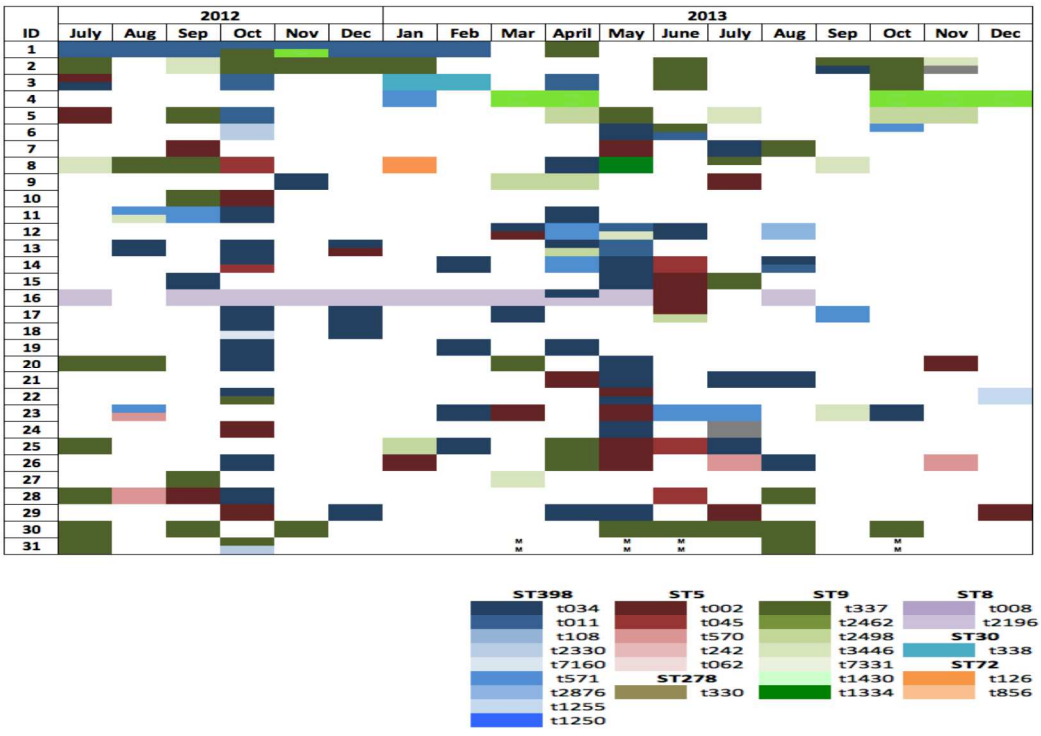



**Figure 3.3. Patterns of detection of *S. aureus spa* types in veterinarians categorized as permanent carriers (a) and intermittent carriers (b). Missing samples are indicated as 'M', white spaces indicate culture negative events; white dots signify methicillin resistant isolates (typically 2 isolates typed per month). Yellow boxes in (a) indicate true persistent carriers. Colors also reflect the MSLT type of the major sequence types being ST398 (blue shades), ST5 (red shades), and ST9 (green shades)**

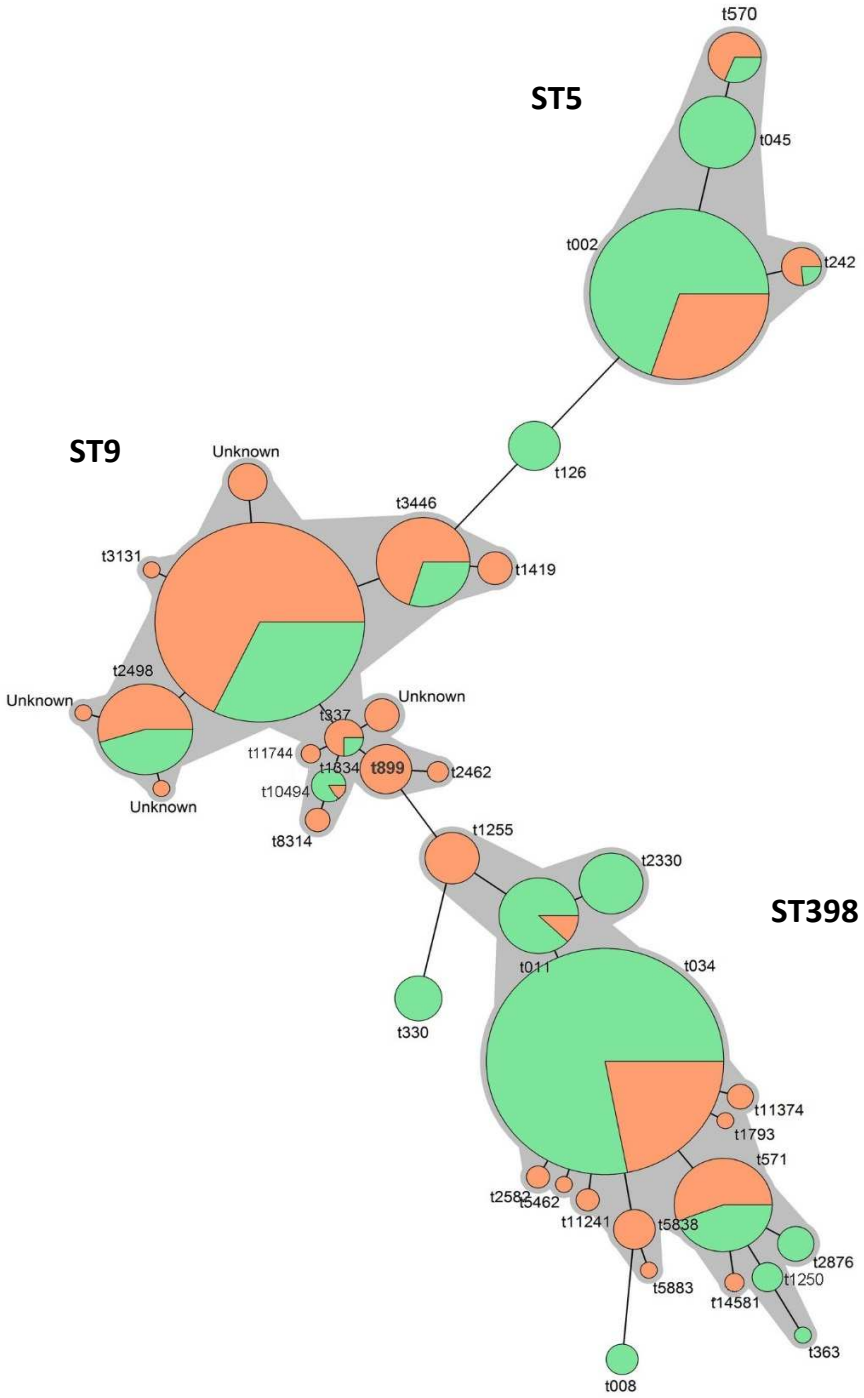



**Figure 3.4 Genetic relatedness of *S. aureus* isolates from swine (n=1193) and veterinarians (n=1659). Each node in this minimum spanning tree depicts one of 38 *spa* types identified from swine and swine veterinarians. The size of circles denotes the number of isolates. Swine isolates and vet isolates are color coded with orange and green, respectively.**

# Chapter 4. Antimicrobial Resistance of *Staphylococcus aureus* Isolated from Swine and Swine Veterinarians in the USA

## Introduction

Over the past decade, the recognition that specific variants of methicillin resistant *Staphylococcus aureus* (MRSA) are prevalent in livestock in many countries has drawn attention to the human health implications of this reservoir, particularly for people working with livestock (van Cleef et al. 2014; Chuang and Huang, 2015; Smith. 2015). The first lineage of 'livestock associated' MRSA (LA-MRSA), identified in pigs in the Netherlands, was a previously unrecognized sequence type (ST), ST398 (Voss et al. 2005). Subsequently, wider research showed that other STs of MRSA are also associated with pigs, including ST9 in Asia (Chuang and Huang, 2015) and ST5 in North America (Khanna et al. 2008; Frana et al. 2013). Available data indicate that the prevalence of MRSA in swine herds in the North America is considerably lower than in many European countries including Denmark and the Netherlands (EFSA,. 2009; Weese et al. 2011b; Smith et al. 2013). However, methicillin susceptible (MSSA) variants of three predominant sequence types (ST398, ST9, ST5) identified among LA-MRSA variants globally comprise the majority of commensal *S. aureus* isolates in commercial pigs in the USA (Linhares et al. 2015; Sun et al. 2015b).

The emergence of LA-MRSA has become a centerpiece of debate about the contribution that antibiotic use in food animals makes to the prevalence of human clinical infections of people with antibiotic resistant bacteria. Although some causal role of antibiotic use in livestock in the pathways of emergence of LA-MRSA in livestock is arguably self-evident and generally assumed, epidemiological evidence of such relationships is not readily apparent (Lassok and Tenhagen, 2013). Notably, selective pressure from non-antibiotic factors such as metals (zinc) and disinfectants may also be implicated in the

emergence of particular MRSA genotypes in humans and animals indicating complex, and likely heterogeneous, biological processes (Cavaco et al. 2010; Nair et al. 2014; Slifierz et al. 2015).

Studies of swine workers in the USA have reported colonization of MRSA similar, or only marginally higher, than reported in the general US population (Wardyn et al. 2015; Nadimpalli et al. 2015), and much lower than reported in swine workers in Germany and the Netherlands (Bisdorff et al. 2012; Osadebe et al. 2013; van Cleef et al. 2014).

Because of the understandable bias towards investigation of MRSA and not MSSA in livestock populations, the epidemiology of the latter remains relatively neglected. The aims of this study were to 1) compare antibiotic and zinc resistance phenotypes of *S. aureus* isolates from swine with isolates from nasal swabs of swine veterinarians 2) evaluate the relationships between resistance phenotypes of swine isolates and recent antibiotic exposure on farms and 3) determine the presence of immune evasion cluster genes as potential markers of host adaptation of *S. aureus* to pigs or humans (Schijffelen et al. 2010).

**Materials and methods**

Sample selection

a) Swine isolates

*S. aureus* isolates (n = 128, including 2 MRSA) were purposively selected from isolates obtained in 2013-2015 in a prior study in which 739 nasal swabs were collected from

growing pigs in 36 herds located across 11 states in the USA (Sun et al. 2015b). The genomic profiles of all isolated *S. aureus* and MRSA were characterized using *spa* typing and multilocus sequence typing (MLST), and 34 *spa* types within 4 sequence types (ST9, ST398, ST5 and ST2007) were identified. Purposive selection was performed to attempt to maximize the diversity among of the subset of isolates included for the current study. Herds were classified into 5 groups based on the numbers of *spa* types identified in each herd (Figure 4.1). Briefly, for herds with only one *spa* type found, two isolates were randomly chosen per herd. For herds with 2 to 4 *spa* types found, a maximum 4 isolates were selected including at least one isolate for each *spa* type. For herds (n=4) with 5 or more *spa* types, one isolate of each *spa* type was included.

b) Veterinary isolates (vet isolates)

Veterinary isolates were obtained in a previous longitudinal study of 66 US swine veterinarians in 15 states (a subset of 36 of these had each sampled a single client herd to obtain the swine samples described above). Based on patterns of nasal swab positivity for *S. aureus* in monthly samples over 18 months, the veterinarians were categorized as Persistent carriers (PC) or Intermittent carriers (IC). For PC subjects, 2 isolates per person were included if they had been culture positive with only a single *spa* type. If more than 2 *spa* types had been isolated, up to 4 isolates per subject were included to represent all *spa* types. For IC subjects, the predominant *spa* type was included for each subject unless they were culture positive for MRSA, in which case one MRSA isolate and one isolate of the most common MSSA *spa* type were both included. A total of 113 vet isolates, including 21 MRSA isolates, were selected by this process.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed at the Veterinary Diagnostic Laboratory (University of Minnesota, MN, USA). Testing was performed according to the manufacturer's instructions using the Sensititre® BOPO6F (Trek Diagnosis system, OH, USA) system with a panel of 17 antibiotics – ampicillin(AMP), ceftiofur(XNL), chlortetracycline(CTC), clindamycin(CLI), enrofloxacin(ENR), florfenicol(FFR), gentamicin(GEN), neomycin(NEO), oxytetracycline(OXY), penicillin(PEN), spectinomycin(SPE), sulphadimethoxine(SDM), tiamulin(TIA), tilmicosin(TIL), trimethoprim/sulphamethoxazole(SXT), tulathromycin(TUL) and tylosin(TYL). *Staphylococcus aureus* ATCC 29213 was included as a quality control strain. For most antibiotics, determination of susceptibility was based on the breakpoints of the Clinical Laboratory Standards Institute(CLSI, VET01S-Ed3, Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 3rd Edition). Due to the lack of approved CLSI breakpoints for tulathromycin, tylosin and ceftiofur, breakpoints (epidemiological cutoffs) were determined based on the MIC distributions the isolates tested. Breakpoints of >16mg/L were determined for tulathromycin and tylosin resistance and >1 mg/L for ceftiofur. For all antibiotics, isolates with MIC values in intermediate range (I) were included with the susceptible group for analysis.

Zinc susceptibility testing

Phenotypic susceptibility to zinc was tested using an agar dilution assay. Muller Hinton agar plates supplemented with zinc chloride (Sigma-Aldrich) were prepared and adjusted to pH 5.5. The concentrations of zinc chloride ranged from 1 mM to 16 mM (two fold dilutions plus the 12 mM concentration). Ten microliters of an overnight culture medium were inoculated onto plates and incubated for 18 ± 2 hours at 37°C. After incubation, resistance was determined by the presence of growth at 2mM concentration of zinc, based on previous studies (Aarestrup et al. 2010; Cavaco et al. 2010).

Genotypic resistance to zinc was evaluated by PCR for the *czrC* gene. The methods of sample preparation for DNA extraction and amplification were as previously described (Cavaco et al. 2010; Sun et al. 2015b). Briefly, primers used were: forward 5' - TAGCCACGATCATAGTCATG-3' and reverse 5' - ATCCTTGTTTTCCTTAGTGACTT-3' (Integrated DNA Technologies, IA) and the expected size of PCR product was 655 bp. PCR master mix (USB HotStart-IT FideliTaq, affymetrix, CA, USA) was used to amplify DNA under the following conditions : 95°C for 2min, with 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1min, followed by 72°C for 10 min. PCR products were visualized in 1.5% agarose gel with SYBR dye in 1X TBE buffer (Tris-Borate-EDTA,Thermo Fisher Scientific Inc., MA USA) for 40min at 200 V.

Immune evasion cluster (IEC) genes

To identify the presence or absence of components of the IEC, single locus PCR for the *scn, sak* and *chp* genes was performed. The primers and positive controls used were as previously described (Hau et al. 2016). The expected sizes of PCR products were 224,

403 and 404 bp for *scn, sak* and *chp,* respectively. The amplified products were visualized as described above.

Antibiotic use in swine herds

Veterinarians who collected pig nasal swabs were requested to provide information on current and recent use of antibiotics in the pigs sampled. The information included the classes of antibiotics being administered, and the route of administration (feed, water or injection) at the time of sampling the herd, or if used previously in the same group of pigs. For analysis, exposure to an antibiotic class was considered positive at the farm level if that class of antibiotics was used currently or previously in the group sampled, irrespective of route of administration.

Statistical analysis

Prevalence of resistance was calculated as the proportion of isolates tested that had an MIC that exceeded the respective breakpoint. Chi square and Fisher's exact test were used to compare the proportions of resistant isolates between different sequence types within host species or between isolates from different host species. An alpha value of <0.008 was used to determine statistical differences in resistances between swine and vet isolates using the Bonferroni correction for multiple comparisons.

To evaluate co-selection between phenotypic resistance patterns of isolates, associations between all potential pairs of antibiotics were analyzed using Fisher's exact test adjusted with the Bonferroni correction for multiple comparisons. Spectinomycin was excluded because all swine *S. aureus* isolates were resistant to this antibiotic. Susceptibility to

penicillin and ampicillin was uniformly consistent within isolates, as was resistance to chlortetracycline and oxytetracycline. Therefore, for analysis these pairs of antibiotics were grouped together as "AMP" or "TET". A total of 14 antibiotics (pig isolates) and 12 antibiotics (vet isolates) were analyzed yielding 105 and 78 sets of comparisons, respectively. The lower number of comparisons with vet isolates was due to uniform resistance to macrolide antibiotics (tylosin, tilmicosin and tulathromycin). The alpha values of 0.00047 (0.05/105 comparisons in pig isolates) and 0.00064 (0.05/78 comparisons in vet isolates) were used to determine statistical significance using the Bonferroni correction for multiple comparisons.

The relationships between antibiotic exposure and overall antibiotic resistance in swine isolates were assessed quantitatively with an antimicrobial resistance index (ARI). For each isolate, an antibiotic resistance index (ARI) was calculated as the proportion of antibiotics tested to which the isolate was phenotypically resistant. ARI and the proportion of isolates resistant to that specific antibiotic or class were not normally distributed (Shapiro-wilk normality test) to be compared with antibiotic exposure, so the analysis were performed non-parametrically with the Mann-Whitney test using R studio (Version 0.99.892). A dendrogram to visualize the relatedness between isolates based on the patterns of antibiotic resistance was constructed using UPGMA method in Bionumerics ver.7.1 software (Applied Maths, Austin, TX), without including duplicate patterns from the same farms.

**Results**

The proportions of the major MLST types in swine samples (ST9 48%; ST398 37%; ST5 15%) tested for susceptibility closely reflected the overall target population (ST9 48%; ST398 35%; ST5 16%, respectively). Similarly, distribution of sequence types in the vet samples (53% ST398, 24% ST5 and 20% ST9) corresponded closely with the distribution of sequence types among all available vet isolates (52% ST398, 24% of ST5 and 19% of ST9).

The absence of MRSA among the swine herds sampled, apart from one positive control herd, has been reported previously (Sun et al. 2015b). All *S. aureus* isolates from swine were resistant to spectinomycin (Table 4.1), and the majority of isolates showed resistance to chlortetracycline and oxytetracyline (93% and 95%), clindamycin (75%), ampicillin and penicillin (both 72%). The lowest prevalences of antibiotic resistance were observed for trimethoprim/sulphamethoxazole (4%), ceftiofur (6%) and enrofloxacin (9%). Overall the prevalence of resistance tended to be higher among swine than veterinary isolates except for penicillin, ceftiofur and enrofloxacin. Like swine isolates, resistance among vet isolates was most common to spectinomycin (97%), penicillin (90%) and tetracyclines (85%). However, significant differences ($P < 0.008$) in prevalence of resistance were found between swine and vet isolates for penicillin, ceftiofur, florfenicol, and clindamycin.

Resistance to specific antibiotics was associated with MLST sequence type. ST398 had significantly higher prevalence of resistance to tetracycline in vet isolates and lower resistance to penicillin in swine isolates. ST5 in swine isolates were more likely to be

resistant to ceftiofur than ST9 and ST398, and a similar trend was shown in vet isolates. Resistance to neomycin and florfenicol was more commonly observed in ST5 than ST9 or ST398 in both species. ST9 isolates were significantly more resistant to sulphadimethoxine but less likely to be resistant to macrolide antibiotics than ST398 and ST5 regardless of host species.

Distributions of MIC values of *S. aureus* isolates

Similar MIC distributions of isolates for 17 antibiotics were observed for the swine and vet isolates (Table 4.2), although the MIC90 of ceftiofur was higher for veterinary (4mg/L) than swine isolates (1mg/L). For most antibiotics included, bimodal distributions were observed, and the breakpoints defined by CLSI generally corresponded with potential epidemiological cut-offs based on the observed data.

Dendrogram based on resistance phenotypes

The dendrogram created using UPGMA clustering (Unweighted Pair Group Method with Arithmetic Mean) illustrates the broad diversity of resistotypes detected with the presence of two major groups defined by resistance to macrolide antibiotics (point in figure 4.3). The most common resistance profile (6%, 15/241) observed was SPE-CTC-OXY-AMP-FFR(I)-XNL but the large number (n=118) of resistotypes detected indicates wide diversity of resistance in *S. aureus* associated with swine in the USA. There was no apparent association of resistotype with sequence type or with host species.

Associations between resistance phenotypes

To assess evidence for potential co-selection of resistance, associations between resistances to all pairs of antibiotics were screened using a conservative P value to adjust for multiple comparisons (Figure 4.2). There were 5 pairs of antibiotics (CLI-Macrolides, CLI-TIA, FFR-NEO, CLI-FFR, and FFR-Macrolides) for which strong and statistically significant associations were observed with both swine and vet isolates. For another 5 paired comparisons (FFR-ENR, FFR-NEO, NEO-TIA, TIA-ENR and ENR-GEN) the associations were significant only for vet isolates, although similar trends of a positive association were seen among the swine isolates. TET and SDM resistances were negatively associated among vet isolates ($p=0.03$), while among swine isolates there was a small, but non-significant, positive correlation.

Associations between antibiotics uses and antibiotic resistance

Information on current and recent antibiotic exposure was obtained from 35 herds. All groups sampled had been exposed to at least one antibiotic, with the most commonly used compounds being tetracyclines, tiamulin, ceftiofur, and carbadox (Table 4.3). Neither carbadox nor tiamulin is classified as medically important antibiotics by the US. Food and Drug Administration. For several antibiotic classes (penicillin, tetracyclines, macrolides) phenotypic resistance was observed in more than 50% of isolates from pigs that had not been exposed to those respective antibiotics. Spectinomycin resistance was ubiquitous among swine isolates, although none of the farms reported current or prior administration of this antibiotic to the groups of animals sampled. However, current or recent use was significantly associated with higher prevalence of resistance for tetracyclines, macrolides (resistance to tulathromycin), and sulfonamides although a

strong effect (prevalence ratio >2) was only found for sulfonamides (Table 4.3). Current or previous exposures of pigs to tetracyclines ($P < 0.01$), enrofloxacin ($P = 0.05$), or ceftiofur ($P = 0.05$) was significantly associated with ARI.

Zinc resistance

Phenotypic zinc resistance (> 2mM ZnCl) was observed in 17% (22/128) and 27% (30/113) of swine isolates and vet isolates, respectively. Of the 22 zinc resistant swine isolates, only two (9%) were positive for the *czrC* gene, and were ST398-t034 MRSA isolates from 2 pigs on the MRSA positive control farm. In contrast, 43% (13/30) of phenotypically zinc resistant vet isolates were positive for the *czrC* gene, all of which were ST398 MRSA isolates (8 t034, 5 t2330). Testing positive for the *czrC* gene was strongly associated with the ST398 MRSA genotype ($p=0.000004$). However, a majority of isolates (91% of swine, 73% of vet isolates) that were phenotypically zinc resistant lacked the *czrC* gene, suggesting other mechanisms of zinc resistance may exist.

Testing for human related immune evasion cluster (IEC) genes

A total of 241 isolates from both pigs and swine veterinarians were tested for presence of IEC genes (Table 4.4). No swine isolate was positive for any of the three genes *scn, sak* and *chp*, and 91% of vet isolates were similarly negative. One t008 (ST8) MRSA isolate was positive for the three genes, and one t2196 (ST8) was positive for the *scn* and *sak* genes. Several genotypes detected in veterinarians (ST72-t126, ST278-t330, ST30-t338) carried IEC genes, but these genotypes were not present among the swine isolates.

**Discussion**

Key observations in the current study include the high prevalence and diversity of antibiotic resistance phenotypes in both swine and veterinary *S. aureus* isolates, and the overall similarity in genotypes and resistance patterns observed in isolates from both host species. The predominance of the same 3 sequence types among isolates from both species, the similarities in resistance phenotypes for most antibiotics, and the scarcity of the IEC genes (a marker of host adaption to humans) among veterinary ST398, ST9, and ST5 isolates are consistent with frequent transmission of *S. aureus* between pigs and people. This concurs with a substantial body of research indicating that transmission of *S. aureus* from pigs to people occurs routinely in farm environments (Armand-Lefevre et al. 2005; van Cleef et al. 2014). Furthermore, long term colonization (for at least 2 years) with livestock associated *S. aureus* (LA-SA) occurs in a substantial minority of occupationally exposed individuals (Verkade et al. 2013; van Cleef et al. 2014; Walter et al. 2016; Sun et al. 2016a). Potential human health impacts of this regular occupational exposure may manifest directly through the capacity of these organisms to be transmitted among people and cause infections, or indirectly if they serve as reservoirs of antibiotic resistance determinants that are transferable to human pathogens.

Understanding of the direct human health risks posed by *S. aureus* in swine remains incomplete and is mostly derived from studies of ST398 MRSA. It is clearly established that ST398 MRSA of livestock origin can cause clinical infections in people, including a small number of fatal cases in medically compromised patients (Schaumburg et al. 2012; Nielsen et al. 2016). However, reports of medically significant clinical infections in

healthy swine workers remain rare despite substantial exposure (Declercq et al. 2008; Denis et al. 2009; Omland and Hoffmann, 2012; Bosch et al. 2016a) and ST398 LA-MRSA are less transmissible among people than MRSA of human origin (van Rijen et al. 2008; Bootsma et al. 2011; Wassenberg et al. 2011). In regions where ST398 MRSA are prevalent in swine, population-based estimates (cases per 100,000 people per year) of the incidence of clinical infections are: 2 clinical infections, 0.38 invasive infections, and 0.04 bacteremia cases in the Netherlands (Wulf et al. 2012; van Cleef et al. 2013) and 0.25 and 0.7 clinical infections in pig dense regions of Denmark (Omland and Hoffmann, 2012; Larsen et al. 2015). In contrast, in the USA the CDC estimated 31.8 invasive cases and 6.3 fatal cases of MRSA per 100,000 people in 2005 (Klevens et al. 2007), and data available to date indicate that livestock associated ST398 MRSA constitute a minimal (<0.3%) fraction of human MRSA infections in Canada and the USA (Golding et al. 2010; Nair et al. 2016). The overall incidence and severity of LA-MRSA infections appears to be low relative to MRSA of human origin (Kock et al. 2011; Bosch et al. 2016a), and the scarcity of recognized human virulence factors, including the IEC genes, among livestock adapted ST398 MRSA has been a consistent finding (van Belkum et al. 2008; Schijffelen et al. 2010; Argudin et al. 2011; Hallin et al. 2011; Jamrozy et al. 2012). Similarly, ST5 MRSA primarily isolated from swine in the USA uniformly lacked the IEC genes and appear to be phylogenetically distinct from ST5 MRSA isolates causing clinical infections in the USA (Hau et al. 2015).

The potential human health significance of MSSA isolates of swine origin has been largely neglected due to the priority given to research into LA-MRSA. It is important to

note that distinct human and swine variants of ST398 are recognized, and the former may constitute a substantial component of human MSSA infections in some populations without the involvement of livestock (Uhlemann et al. 2013; van der Mee-Marquet et al. 2013). Therefore attribution of source of infection based on MLST lineage alone is ill-advised. Several studies of occupationally exposed and non-exposed people in swine dense regions of the USA reported low prevalences of LA-MRSA but an elevated prevalence in swine workers of MSSA that were deemed to be multiple drug resistant (MDRSA) and likely of swine origin (Frana et al. 2013; Rinsky et al. 2013; Wardyn et al. 2015). However, none of these studies reported data on antibiotic resistance of swine MSSA isolates. Our findings, from a geographically diverse sample of US herds, confirm that commercial swine populations represent a substantial reservoir of multiple drug resistant *S. aureus* that can be transmitted to humans. It is important to note that *S. aureus* is considered normal flora of swine (Sun et al. 2015b), and therefore it is likely that occupational exposure to this reservoir and consequent colonization of swine workers with LA-SA has been endemic for at least several decades, albeit without previous recognition of associated clinical disease in any swine producing country. We are unaware of evidence indicating that people occupationally exposed to swine are at increased overall risk of MSSA or MRSA infections (versus colonization). There is strong evidence of increased likelihood of infection with specific LA-MRSA in people in pig dense areas, but increased MRSA infection risk overall was not observed (Larsen et al. 2015). The uniform absence of IEC genes in swine isolates in the current study suggests that swine associated MSSA, akin to LA-MRSA, may harbor a limited array of putative virulence factors contributing to human disease, a hypothesis we are exploring in

ongoing studies. Recent data from the major swine producing state of Iowa indicate that *spa* types linked to livestock represented only 1% of isolates from human clinical *S. aureus* infections, of which only 2 cases were categorized as invasive (Nair et al. 2016). Collectively, evidence to date indicates that the direct health impact of exposure to LA-SA is a non-zero, but likely low, risk of clinical infection.

Indirect risks to human health posed by LA-SA as reservoirs of antibiotic resistance determinants that could be transferred to other human pathogens are clearly plausible and probably non-zero, but remain unquantified. Clearly absence of evidence is not evidence of absence, and the task of demonstrating and quantifying the complex series of events implicit in such hypothesized pathways of resistance transfer and disease among host species is not trivial (Robinson et al. 2016). A more pragmatic direction for research may be to define factors, including specific practices of antibiotic use, that are causally associated with the emergence and occurrence of resistant LA-SA, and the extent to which changing these factors might reduce the prevalence of resistance and thereby mitigate potential risks to human health. However, several findings in this study highlight challenges that researchers may confront in pursuing this objective. These include the high prevalence of resistance to multiple antibiotics, the broad diversity of resistotypes, statistical evidence of potential cross-selection of resistance phenotypes, and that resistance patterns varied with MLST type.

Many antibiotics approved for use in swine (e.g., penicillin, tetracyclines, sulfonamides) have been available in the USA since the 1950s, and the prevalence of resistance to these

compounds likely reflects the cumulative effect of decades of use in the overall industry as much, or more so, than recent or current use in study populations (Tadesse et al. 2012). Although tetracycline exposure was significantly associated with its resistance phenotype in this study, the observed strength of the effects was modest (prevalence ratio < 2) due to the high prevalence of resistance in unexposed groups. In contrast, exposure to sulfonamides was strongly associated with resistance (prevalence ratio of 12), as resistance was uncommon in isolates from unexposed groups. Cross-selection has the potential to confound studies between antibiotic use and resistance, and in this study may have contributed to the frequent presence of resistance in the absence of recent exposure to the respective antibiotics. The use of alpha values corrected for multiple comparisons reduced the likelihood that the observed associations may be spurious, but the genetic basis underlying the observed associations remains to be explored. Some previous studies have reported the common occurrence and genetic basis of spectinomycin resistance in LA-MRSA (Jamrozy et al. 2014), and have identified specific genes such as the *erm* and *cfr* genes that mediate resistance to multiple antibiotic classes in both ST398 and ST9 isolates from animals (Kehrenberg et al. 2009; Peeters et al. 2015). High prevalences of multiple resistance among ST398 MRSA has led to suggestions that it could reflect a particular capacity of this lineage to acquire multiple resistance determinants. However, in this study, of predominantly MSSA variants, overall resistance was comparable among the 3 MLST genotypes, with no suggestion among US isolates that the ST398 genotype had any unique propensity to acquire resistance elements. However, the occurrence of some specific resistance phenotypes did vary among MLST types, which may reflect some clonal spread. The relative importance of

clonal dissemination rather than selection pressure from antibiotic use in the emergence of multiple resistant bacteria is rarely elucidated although both mechanisms can be expected to play some role at a broad population level (Davis et al. 2002). More comprehensive evaluation of the association between resistance determinants and genotypes in *S. aureus* in animals is warranted, both within and across geographic regions.

Solving the ongoing mysteries surrounding the temporospatial emergence of LA-MRSA globally has been hampered by knowledge deficits of the baseline genotypes and antibiotic resistance patterns of the staphylococcal flora of food animals prior to the emergence of methicillin resistance. It is apparent that, together with some component of clonal spread, complex interactions among multiple factors, including antibiotics, metals and disinfectants could be involved (Aarestrup et al. 2010; Slifierz et al. 2015; Argudin et al. 2016). The concept that any single causal factor has predominantly driven the emergence of LA-MRSA at a broad epidemiological scale is probably fanciful, and would require strong differential susceptibility to that factor between MRSA variants and pre-existing commensal MSSA flora. Some hypotheses that have been advanced include the use of 'nontherapeutic' antibiotics (Rinsky et al. 2013; Neyra et al. 2014), tetracycline use (de Neeling et al. 2007), and cephalosporin use (Dorado-Garcia et al. 2015). Notably, ST398 MRSA emerged to become highly prevalent in Danish pigs almost a decade after elimination of growth promotant antibiotics in 2000. Furthermore, in Denmark differential susceptibility of ST398 MRSA and MSSA for resistance to zinc, but not several antibiotics including tetracyclines, raised the possibility that increased use

of dietary zinc as an alternative to antibiotics may have been one factor contributing to the emergence of LA-MRSA in that country (Aarestrup et al. 2010; Cavaco et al. 2011). The location of the *czrC* gene encoding zinc resistance on the SCC*mec* cassette provides a likely direct genetic mechanism linking zinc resistance to methicillin resistance, particularly in type V SCC*mec* elements that predominates among ST398 LA-MRSA in Europe. It was recently reported that the *czrC* gene was uniformly absent from ST5 LA-MRSA associated with swine in the USA, which predominantly harbored SCC*mec* type III and type IV elements. In contrast, 25% of ST5 MRSA from humans with no swine contact harbored the *czr*C gene and 95% of isolates were SCC*mec* type II (Hau et al. 2016).

The high prevalence in US swine of multidrug resistant MSSA isolates (which lack the SCC*mec* element that harbors the *mecA* gene conferring methicillin resistance), may have played a paradoxical role in the unexpectedly low prevalence of LA-MRSA reported in swine in the USA (Smith et al. 2013; Sun et al. 2015b). In most Asian countries, LA-MRSA isolates have been predominantly ST9, and the predominant SCC*mec* types (III, IVb, V, untypeable) have varied among countries (Chuang and Huang, 2015). The diversity of SCC*mec* types among LA-MRSA globally, together with 'baseline' antibiotic resistance patterns in MSSA swine flora, may have contributed to the differential emergence of ST398 (Europe), ST9 (Asia), and ST5 (North America) genotypes of swine associated LA-MRSA on different continents.

**Table 4.1. Prevalence of resistance to 17 antibiotics among common sequence types of *S. aureus* isolated from pigs and swine veterinarians in the USA**

| Class | Antibiotic | Total | | Swine isolates (n=128) | | | Veterinary isolates (n=113) § | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pig | Vet | ST5 | ST9 | ST398 | ST5 | ST9 | ST398 |
| | | n=128 (%) | n=113 (%) | n=19(%) | n=41 (%) | n=68 (%) | n=24 (%) | n=17 (%) | n=65(%) |
| β-Lactam | Penicillin/Ampicillin | 92 (72) | 101 (90)* | 19 (100) [a] | 40 (98) [a] | 33 (49) [b] | 23 (96) | 17 (100) | 57 (88) |
| | Ceftiofur | 8 (6.3) | 40 (35)* | 4 (21) [a] | 1 (2.4) [b] | 3 (4.4) [b] | 11 (46) | 3 (18) | 24 (37) |
| Tetracycline | Chlortetracycline | 119 (93) | 96 (85) | 18 (95) | 35 (85) | 66 (97) | 21 (88) [x] | 7 (41) [y] | 65 (100) [z] |
| | Oxytetracycline | 121 (95) | 96 (85) | 18 (95) | 36 (88) | 67 (99) | 21 (88) [x] | 7 (41) [y] | 65 (100) [z] |
| Aminoglycoside | Spectinomycin | 128 (100) | 109 (97) | 19 (100) | 41 (100) | 68 (100) | 23 (96) | 16 (94) | 64 (98) |
| | Gentamicin | 22 (17) | 17 (15) | 6 (32) | 3 (7.3) | 13 (19) | 3 (13) | 3 (18) | 9 (14) |
| | Neomycin | 48 (38) | 26 (23) | 14 (74) [a] | 21 (51) [a] | 13 (19) [b] | 15 (63) [x] | 3 (18) [y] | 7 (11) [y] |
| Amphenicol | Florfenicol | 69 (54) | 36 (32)* | 16 (84) [a] | 27 (66) [a] | 26 (38) [b] | 14 (58) [x] | 3 (18) [y] | 16 (25) [y] |
| Lincosamide | Clindamycin | 96 (75) | 64 (57)* | 16 (84) | 26 (63) | 54 (79) | 15 (63) | 5 (30) | 43 (66) |
| Pleuromutilin | Tiamulin | 59 (46) | 32 (28) | 8 (42) | 23 (56) | 28 (41) | 5 (21) | 4 (24) | 22 (34) |
| Quinolone | Enrofloxacin | 12 (9.4) | 14 (12) | 0 | 5 (12) | 7 (10) | 3 (13) | 2 (12) | 7 (11) |
| Sulfonamide | Sulphadimethoxine | 35 (27) | 14 (12) | 2 (11) [a] | 20 (49) [b] | 13 (19) [a] | 1 (4) [x] | 8 (47) [y] | 1 (1.5) [x] |
| | Trimethoprim/ Sulfamethoxazole | 5 (3.9) | 2 (1.8) | 0 | 3 (7.3) | 2 (2.9) | 0 | 0 | 0 |
| Macrolide | Tilmicosin | 71 (56) | 57 (50) | 13 (68) [ab] | 15 (37) [a] | 43 (63) [b] | 15 (63) [x] | 2 (12) [y] | 39 (60) [x] |
| | Tulathromycin | 73 (57) | 58 (51) | 13 (68) [ab] | 15 (37) [a] | 45 (66) [b] | 15 (63) [x] | 2 (12) [y] | 40 (62) [x] |
| | Tylosin | 70 (55) | 58 (51) | 13 (68) [ab] | 15 (37) [a] | 42 (62) [b] | 15 (63) [x] | 2 (12) [y] | 40 (62) [x] |

* significant difference between pig and vet isolates using p value ($p<0.008$) adjusted for multiple comparisons

a, b unlike superscripts indicate significant differences within rows (swine)

x, y, z unlike superscripts indicate significant differences within rows (veterinary)

§ Sequence types other than ST5, ST9 and ST398 excluded due to small sample size.

**Table 4.2. The distribution of minimum inhibitory concentrations (MIC) in pig and veterinary *S. aureus* isolates. Concentrations tested varied by antibiotic, and untested concentrations are indicated in grey. Single and double lines indicate intermediate and resistant cut-offs respectively.**

**(a) Swine isolates**

| | Antimicrobial concentration | | | | | | | | | | | | MIC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | $MIC_{50}$ | $MIC_{90}$ |
| AMP | | 36 | 4 | 4 | 16 | 6 | 11 | 51 | | | | | 4 | 16 |
| PEN | 36 | 0 | 3 | 3 | 5 | 11 | 70 | | | | | | 8 | 8 |
| XNL | | 0 | 6 | 114 | 6 | 2 | 0 | | | | | | 1 | 1 |
| CTC | | | 6 | 1 | 0 | 0 | 121 | | | | | | 8 | 8 |
| OXY | | | 4 | 3 | 0 | 0 | 121 | | | | | | 8 | 8 |
| SPE | | | | | | | 0 | 0 | 0 | 128 | | | 64 | 64 |
| GEN | | | | 102 | 2 | 0 | 2 | 22 | | | | | 1 | 16 |
| NEO | | | | | | 93 | 0 | 25 | 10 | | | | 4 | 16 |
| FFR | | 0 | 0 | 0 | 3 | 56 | 69 | | | | | | 8 | 8 |
| CLI | | 24 | 6 | 2 | 0 | 0 | 12 | 84 | | | | | 16 | 16 |
| TIA | | | 27 | 19 | 2 | 3 | 8 | 6 | 3 | 60 | | | 16 | 64 |
| ENR | 43 | 61 | 8 | 4 | 12 | | | | | | | | 0.25 | 1 |
| SDM | | | | | | | | | | | 97 | 31 | 128 | 256 |
| SXT | | | | 124 | 4 | | | | | | | | 1 | 1 |
| TIL | | | | | | 44 | 9 | 4 | 0 | 71 | | | 64 | 64 |
| TUL | | | | 0 | 4 | 32 | 17 | 2 | 0 | 73 | | | 64 | 64 |
| TYL | | | 0 | 30 | 19 | 7 | 2 | 0 | 70 | | | | 32 | 32 |

**(b) Veterinary isolates**

| | Antimicrobial concentration | | | | | | | | | | | | MIC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | $MIC_{50}$ | $MIC_{90}$ |
| AMP | | 12 | 4 | 12 | 15 | 16 | 21 | 33 | | | | | 4 | 16 |
| PEN | 12 | 0 | 4 | 5 | 10 | 6 | 76 | | | | | | 8 | 8 |
| XNL | | 2 | 3 | 68 | 19 | 17 | 4 | | | | | | 1 | 4 |
| CTC | | | 8 | 9 | 0 | 0 | 96 | | | | | | 8 | 8 |
| OXY | | | 2 | 10 | 5 | 0 | 96 | | | | | | 8 | 8 |
| SPE | | | | | | | 0 | 0 | 4 | 109 | | | 64 | 64 |
| GEN | | | | 95 | 0 | 0 | 1 | 17 | | | | | 1 | 16 |
| NEO | | | | | | 87 | 4 | 18 | 4 | | | | 4 | 16 |
| FFR | | 0 | 0 | 0 | 3 | 74 | 36 | | | | | | 4 | 8 |
| CLI | | 41 | 4 | 3 | 1 | 1 | 4 | 59 | | | | | 16 | 16 |
| TIA | | | 29 | 31 | 2 | 1 | 13 | 4 | 1 | 32 | | | 1 | 64 |
| ENR | 37 | 48 | 1 | 13 | 14 | | | | | | | | 0.25 | 2 |
| SDM | | | | | | | | | | | 109 | 14 | 128 | 256 |
| SXT | | | | 111 | 2 | | | | | | | | 1 | 1 |
| TIL | | | | | | 48 | 6 | 2 | 0 | 57 | | | 64 | 64 |
| TUL | | | | 0 | 21 | 27 | 7 | 0 | 1 | 57 | | | 64 | 64 |
| TYL | | | 0 | 38 | 13 | 4 | 0 | 0 | 58 | | | | 32 | 32 |

**Table 4.3. Antibiotics (AB) administered to groups of pigs on the farms sampled and proportions of *S. aureus* isolates resistance to those compounds**

| | Used on Farm (n=35[#]) | | Proportion resistant ¶ | | |
|---|---|---|---|---|---|
| Antibiotic (resistance tested) | Yes | No | Farm that used AB | Farm that didn't use AB | P value[§] |
| Penicillin | 7 | 28 | 0.90 (19/21) | 0.66 (71/107) | 0.07 |
| Chlortetracycline | 29 | 6 | 0.95 (104/110) | 0.83 (15/18) | 0.03* |
| Oxytetracycline | 29 | 6 | 0.93 (106/110) | 0.83 (15/18) | 0.01* |
| Pleuromutilins (Tiamulin) | 14 | 21 | 0.42 (21/50) | 0.44 (34/78) | 0.28 |
| Gentamicin | 8 | 27 | 0.25 (7/28) | 0.15 (15/100) | 0.27 |
| Neomycin | 8 | 27 | 0.36 (10/28) | 0.38 (38/100) | 0.34 |
| Macrolide (Tilmicosin) | 4 | 31 | 0.42 (8/19) | 0.58 (63/109) | 0.39 |
| Macrolide (Tylosin) | 4 | 31 | 0.37 (7/19) | 0.58 (63/109) | 0.34 |
| Draxxin (Tulathromycin) | 4 | 31 | 0.93 (14/15) | 0.04 (5/113) | 0.03* |
| SXT | 5 | 30 | 0.19 (3/16) | 0.02 (2/112) | 0.01* |
| Fluoroquinolones (Enrofloxacine) | 5 | 30 | 0.04 (1/24) | 0.11 (11/104) | 0.35 |
| Cephalosporin (Ceftiofur) | 13 | 22 | 0.08 (4/51) | 0.05 (4/77) | 0.39 |
| Spectinomycin | 0 | 35 | 0 (0/0) | 1 (128/128) | N/A |
| Carbadox | 10 | 25 | N/A | N/A | N/A |

¶ was calculated as a proportion which comprised the number of isolates being resistant over a total isolates used for antibiotic susceptibility test on each farm.

§ P value was driven from the proportion of isolates resistant to that specific antibiotic by individual farms to compare between AB use farm and non-AB use farm using Mann-Whitney test.

N/A : not applicable due to no panel for the antibiotic tested
# a total of 35 were included in this analysis since one farm was negative for *S. aureus*

**Table 4.4. *S. aureus* veterinary isolates PCR positive for immune evasion cluster genes**

| | Month* | Vet ID | MRSA | spa type (ST) | scn | sak | chp |
|---|---|---|---|---|---|---|---|
| 1 | 04 | 24 | MSSA | t126 (ST72) | + | + | + |
| 2 | 04 | 57 | MSSA | t330 (ST278) | + | + | + |
| 3 | 06 | 19 | MSSA | t5883 (ST398) | + | + | + |
| 4 | 07 | 05 | MSSA | t338 (ST30) | + | + | + |
| 5 | 09 | 44 | MSSA | t2196 (ST8) | + | + | - |
| 6 | 12 | 61 | MSSA | t062 (ST5) | + | + | + |
| 7 | 12 | 61 | MSSA | t011 (ST398) | + | + | + |
| 8 | 17 | 41 | **MRSA** | t008 (ST8) | + | + | + |
| 9 | 17 | 66 | MSSA | t002 (ST5) | + | + | + |
| 10 | 17 | 61 | MSSA | t011 (ST398) | + | + | + |

*Month indicates the sampling months ranged from month 1 to month 18.

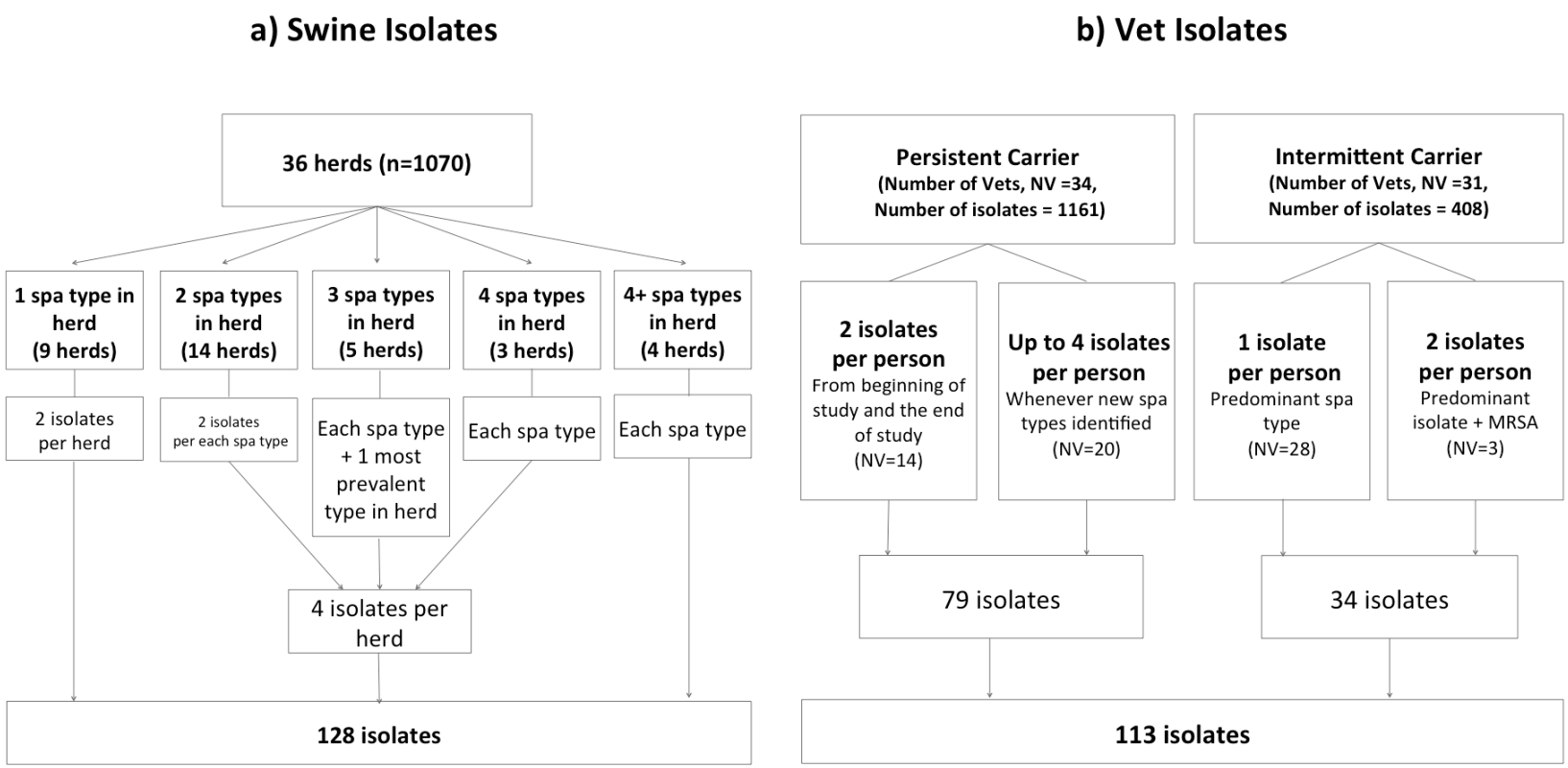



**Figure 4.1. Flow chart for purposive selection *S. aureus* of subset isolates from previous studies. (a) Swine isolates (b) Veterinary isolates**

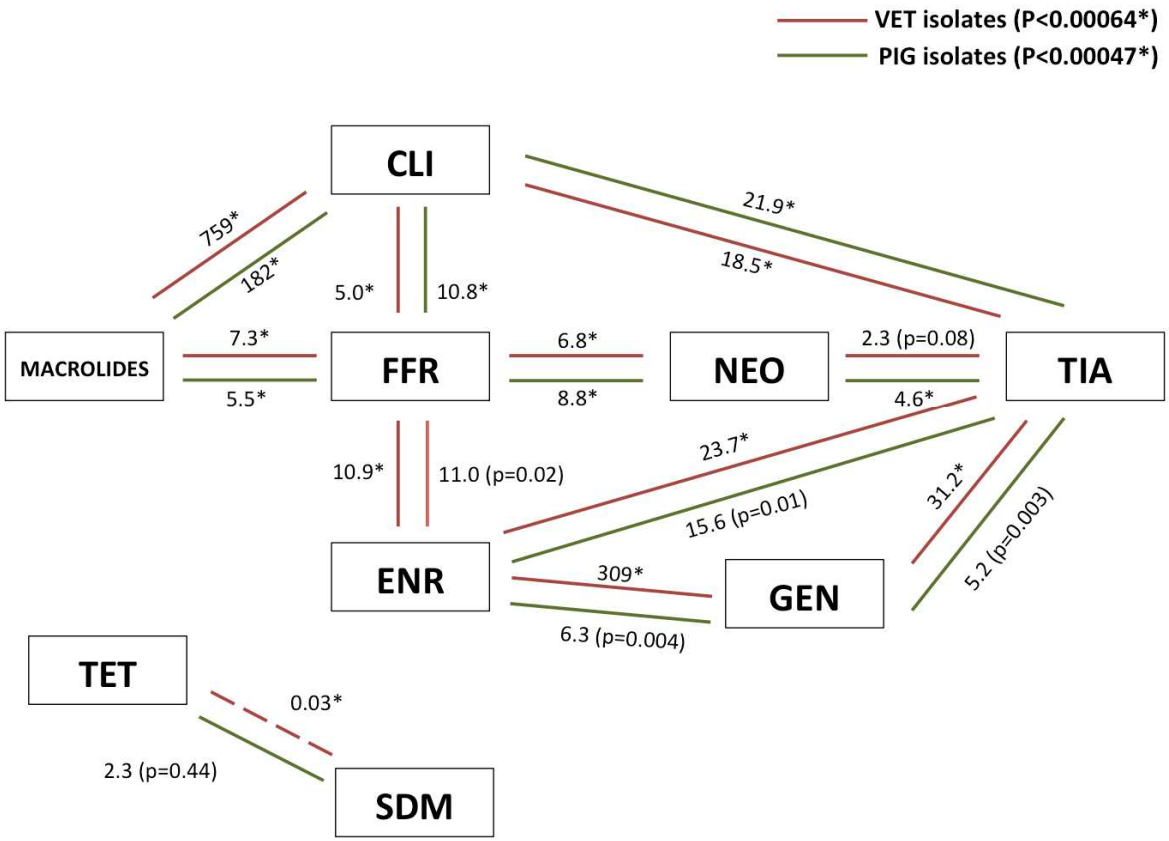



**Figure 4.2. The strength association (odds ratios) of resistance for pairs of antibiotics. Green lines and red lines indicate pig isolates and veterinarian isolates, respectively. The dotted line indicates a negative association between resistance phenotypes. Asterisks indicate statistical significance based on p values adjusted for multiple comparisons. The abbreviations of antibiotics were described in table 4.2**

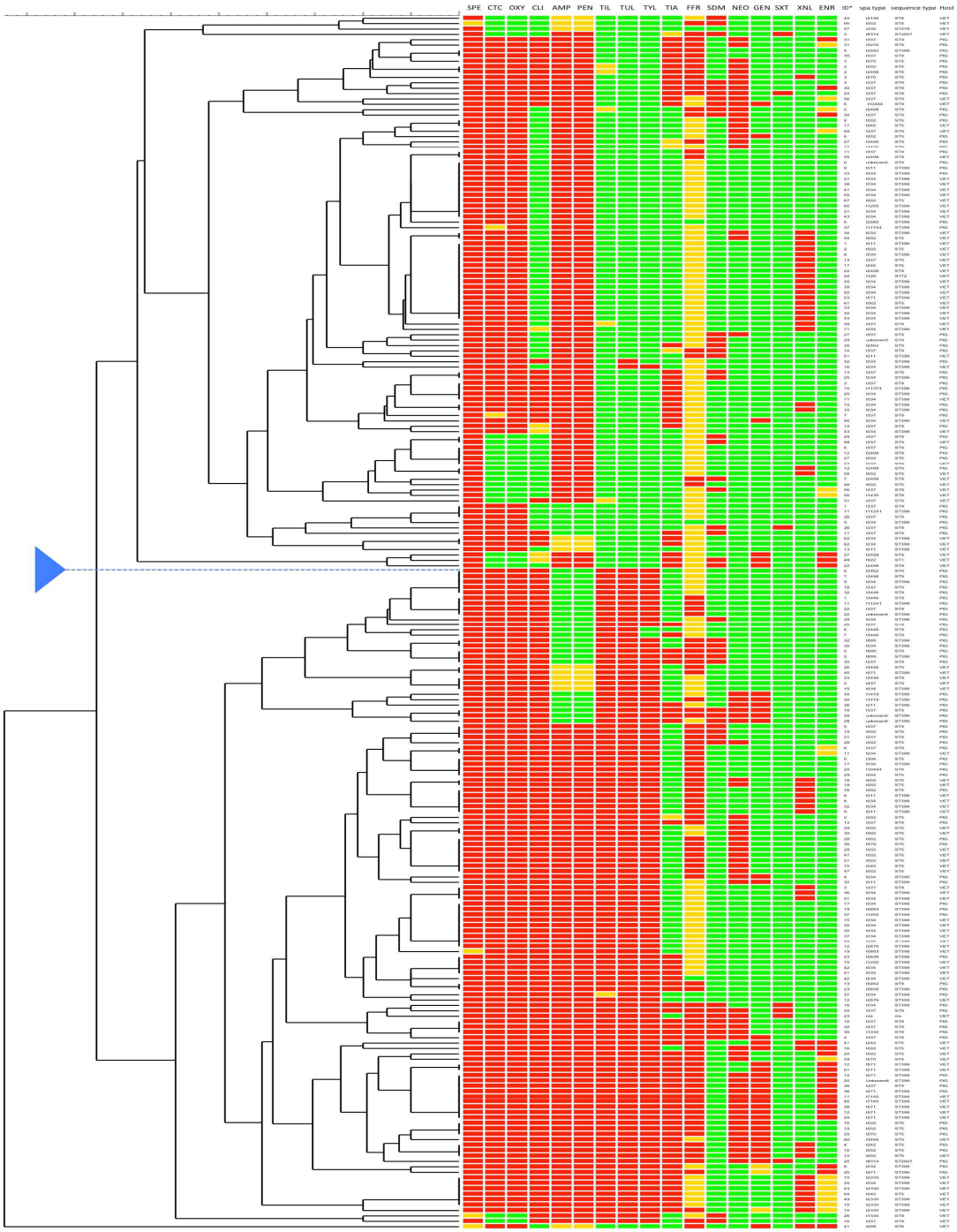



**Figure 4.3. The dendrogram of the isolates based on the antibiotic resistance profiles. Red, yellow and green colors indicate resistant, intermediate and susceptible. ID* in swine and vet isolates represents Farm ID and individual vet ID, respectively.**

# Chapter 5. Genomic Characterization of *Staphylococcus aureus* at the Swine-Human Interface

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a commensal organism that commonly colonizes the skin, nose and throat of humans and pigs, as well as a variety of other mammalian and avian species (Sung et al. 2008; Linhares et al. 2015). It is a sporadic and unimportant pathogen of pigs, but is a major opportunistic pathogen of humans. In particular, methicillin resistant *S. aureus* (MRSA) is among the organisms of greatest concern in the current crisis of antibiotic resistance in human medicine (Centers for Disease Control. 2013). Negligible research had been conducted on *S. aureus* in swine prior to 2004 when a previously unknown lineage (ST398) of MRSA was found to be prevalent in pigs and people in the Netherlands (Voss et al. 2005; Armand-Lefevre et al. 2005). Subsequent research soon showed that ST398 MRSA could be detected in multiple livestock species and in many countries (EFSA,. 2009; Smith and Pearson, 2011). To date, the bulk of research on *S. aureus* in pigs has focused on ST398 MRSA, mostly in northern Europe. However, it is evident that other genotypes of MRSA may be prevalent in pigs in other regions, particularly ST9 MRSA in Asia and ST5 MRSA in Canada and North America (Khanna et al. 2008; Frana et al. 2013; Chuang and Huang, 2015).

The existence of MRSA reservoirs in livestock is of concern for human health, and the capacity of ST398 MRSA to cause clinical infections in people is well established (Smith and Wardyn, 2015). However, significant clinical infections have been rare in farm workers, and in hospital settings ST398 MRSA have been shown to be less transmissible among people than MRSA variants that are common in people (Wassenberg et al. 2011; Bootsma et al. 2011). There is also some evidence to suggest

that ST398 MRSA of animal provenance may be relatively less virulent than MRSA variants that are endemic in human populations (Kock et al. 2011), and several studies have indicated the relative sparsity of known human virulence factors in ST398 MRSA isolates from animals (Schijffelen et al. 2010; Argudin et al. 2011; Hallin et al. 2011; Price et al. 2012; Jamrozy et al. 2012). In contrast, ST398 isolates of animal origin have generally contained more genes encoding antibiotic resistance than MRSA from humans (Jamrozy et al. 2012; Price et al. 2012).

To date there has been little investigation of the virulence and antibiotic genes harbored by methicillin susceptible *S. aureus* (MSSA) in pig populations (Vandendriessche et al. 2014) and few reports of MRSA of other lineages found in pigs such as ST5 (Hau et al. 2015) and ST9 (Yan et al. 2016). We recently characterized the *S. aureus* populations in pigs and swine veterinarians across multiple states of the USA (Sun et al. 2015b; Sun et al. 2016a). In both hosts, 3 lineages (ST5, ST9, ST398) comprised the majority of isolates, and the data indicated that persistent colonization with *S. aureus* of likely swine origin occurred in approximately 20% of veterinarians (Sun et al. 2016a). Furthermore, although MRSA appear to occur at relatively low prevalence in the US swine industry, many *S. aureus* from both sources were phenotypically resistant to several antibiotics (Sun et al. 2016b). To obtain further insight into the epidemiology of *S. aureus* at the swine-human interface in the USA, the goal of this study is to compare ST398, ST5, and ST9 *S. aureus* with respect to the presence of genes encoding virulence factors and antibiotic resistance.

## Materials and Methods

### *S. aureus* isolates

Seventy-six isolates of *S. aureus* (Table 5.1) from preceding studies of swine herds and swine veterinarians in the USA were selected purposively for this study (Sun et al. 2015b; Sun et al. 2016a). Briefly, *S. aureus* and MRSA had been isolated from growing pigs (36 farms in 11 states), or from swine veterinarians (66 veterinarians in 15 states) in the major swine producing states, including Iowa, Minnesota and North Carolina. A subset of isolates for this study was selected mainly to focus on ST9 isolates due to substantial published works in ST398 and ongoing research projects with ST5 isolates (Hau et al. 2015). The 76 selected isolates were comprised of 46 veterinarian isolates and 30 swine isolates. The MLST types of isolates were ST9 (n=47), ST398 (n= 19) and ST5 (n = 9) and ST72 (n = 1). Thirteen and 63 isolates were MRSA and MSSA, respectively.

### Next generation sequencing

Genomic DNA was extracted from overnight cultures in LB (Lysogeny broth, BD Difco™, NJ, USA) using the Qiagen Blood and Tissue Kit (Valencia, CA, USA) following the manufacturer's instructions. Approximately 10ng of extracted DNA per sample was sent to University of Minnesota Genomics Center (Minneapolis, MN, USA). Independent NGS libraries (Nextera DNA Library Preparation Kit, CA, USA) were created for each sample, pooled onto a single lane HiSeq 2500 rapid-run, and 250 bp paired-end reads were generated. The run produced 171,234,468 total reads and 85,960

Mb of sequences, yielding an average of 2.3 million reads per sample. 82% of reads had a quality score (Phred+33) greater than 30 (mean percentile across all samples).

**Read-set filtering**

Raw reads were de-multiplexed and quality control metrics were determined using FastQC (v0.11.2) software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.). All reads were quality filtered and adapter sequences were trimmed using Trimmomatic(v0.33) (Bolger et al. 2014) . The minimum length was set to 125 bp. All reads that contained greater than 70% of any single nucleotide were removed by Prinseq (v0.20.4) (Schmieder and Edwards, 2011). Despite sequencing pure bacterial isolates, we further identified and removed any non-*Staphylococcus aureus* (chromosome or plasmid) reads from our filtered read-set using Kraken software (v0.10.5-beta) (Wood and Salzberg, 2014) with a custom database containing 33,778 bacterial strains (complete genomes, chromosomes, or scaffolds) from the RefSeq database (http://www.ncbi.nlm.nih.gov/books/NBK21101).

**Genome Assembly**

*De novo* genome assembly of our filtered read-set was completed using Megahit (v1.0.4-beta-3-g027c6b6) (Li et al. 2015; Li et al. 2016) with the following assembly options: --no-mercy --min-count 3 --k-min 105 --k-max 225 --k-step 10 --prune-level 3. Initial assemblies were analyzed using QUAST (v3.1) (Gurevich et al. 2013) revealing that the mean number of contigs assembled was 248 (80 min, 903 max) per sample containing a mean of 2,728,240 bp (2,678,477 min, 2,789,826 max) and a mean N50 value of 41,539

bp (4,787 min, 124,222 max). These draft assemblies were improved by remapping all trimmed reads to the assembly using Bowtie 2 (v2.2.4) (Langmead and Salzberg, 2012), bam files were sorted in samtools(v1.2) (Li et al. 2009; Li. 2011), and Pilon (Walker et al. 2014) (v1.16) was used to correct regions of mis-assembly using default settings. Contigs were extended using SSPACE (v3.0) (Boetzer et al. 2011) and the closest reference genome for each sample was discovered by BLASTn comparison against a database of 215 *Staphylococcus aureus* complete chromosomes and 673 plasmids from GenBank, using NCBI_blast+ (v2.2.28) (Camacho et al. 2009). For each isolate, contigs were aligned to its closest reference strain using CAR (Lu et al. 2014) (version July 14, 2014) and MUMer (v3.23) (Kurtz et al. 2004) software, which positioned and orientated contigs into a single scaffold, and gaps between contigs were connected with 'N's. Gap-lengths were reduced using Gapfiller(v1.10) (Boetzer and Pirovano, 2012) and finally SNPs and indels in the draft assembly were corrected by running six iterations of ICORN2(v0.95) software (Otto et al. 2010). Across all samples, mean read coverage was 286x when remapping trimmed reads to completed assemblies. Finally, the completed chromosome and plasmid sequences were annotated with gene and protein information using Prokka (v1.11) (Seemann. 2014).

**Virulence factors and antibiotic resistance genes**

Genes encoding virulence factors and antibiotic resistance were detected using only the filtered read-set and SRST2 (0.1.8) software (i.e. this typing was independent from the genome assembly described above) (Inouye et al. 2014). SRST2 can map short reads from each sample to a database of known gene variants and determine which allele (if

any) is present in the sample. Three *S. aureus* gene databases were used for sequence typing: MLST (Jolley and Maiden, 2010)(downloaded from http:// pubmlst.org, 2016-06-30), the full set including all known and predicted virulence factors (Chen et al. 2016) (downloaded 2016-06-17) and ARGannot (Gupta et al. 2014)(downloaded 2016-06-30). Genes detected in each sample were visualized using a heat map (presence/absence) where samples and genes were clustered using Jaccard distance and the UPGMA agglomerative hierarchical clustering algorithm. This analysis was completed in R (v3.3.0) using the packages: NMF (0.20.6) (Gaujoux and Seoighe, 2010), vegan (2.4-0) (https://CRAN.R-project.org/package=vegan), and RColorBrewer (https://CRAN.R-project.org/package=RColorBrewer) (1.1-2).

**Genomic comparison and phylogenetic analysis**

To assess genetic diversity between ST398 and ST9, nine assembled genomes were selected and compared with LA-MRSA ST398-t034 reference genome (CP003808) using the CG View comparison tool (Grant et al. 2012). To estimate genetic distances, the isolates were aligned using Mauve (ver.2) and SNPs from aligned genomes were extracted and imported into MEGA 7.0 software to generate maximum likelihood phylogenetic tree employing 500 bootstrap replicates.

## Results

No distinguishing differences with respect to virulence and resistance genes were observed among the isolates from the pigs and veterinarians, apart from the presence of genes of the immune evasion cluster in two ST5/t062 isolates and the sole ST72 isolate (Figure 5.1).

### Virulence associated gene profiles

Among 173 putative virulence genes examined, 42 genes were not detected in any of the isolates, including those encoding enterotoxins, Panton-Valentine leukocidin (PVL) or toxic shock syndrome toxin (TSST) (Table 5.4). However, all 76 isolates harbored 77 genes (Table 5.2) belonging to 6 different functional groups with roles in cell attachment, iron regulation and cytotoxin production (alpha, beta, gamma and delta).

The remaining 54 virulence genes were variably distributed and were clustered by sequence type (Figure 5.1). Notably, ST398 isolates possessed fewer virulence genes than ST9 and ST5 isolates (Table 5.3). The 2 isolates of ST398/t571, lacked the *cna* gene encoding collagen adhesion protein which was present in all other ST398 isolates.

Staphylococcal exotoxin-like (SET) genes are located on a large chromosomal region (RD13) containing various combinations of exotoxin-like genes in different isolates (Aguiar-Alves et al. 2006). There were 37 SET genes included in this database (i.e, set1, set2… set 26; set 30…set40). Nineteen SET genes were not detected in any isolates while 18 genes were distributed variably by sequence type (Table 5.3). Each sequence type had a specific combination of *set* genes: set35 for ST9; set15 and set 16 for ST5; and 5 *set* genes (set2, set3, set4, set5, set30) for ST398 indicating the genetic structure of

chromosomal region RD13 might play an important role in holding the evolutionary track of each sequence type.

Among virulence genes listed in Table 5.3, ST5 isolates harbored more genes than other sequence types. The *lukD-lukE* loci encoding leukotoxins were only observed in ST5 isolates regardless of host species. The *fnbB* (fibronectin binding protein B) and *sasG* (surface protein G), one of the major *S. aureus* microbial surface recognizing adhesive matrix molecules (MSCRAMMs) were also found solely in ST5 isolates. ST5/t062 isolates from swine veterinarians showed a unique combinations of virulence genes compared to other isolates, including the presence of the *scn, sak,* and *chp* genes related to human innate immune responses and three different types of enterotoxin genes (*sej, sed, selr*).

**Antibiotic resistance genes**

The ARG-ANNOT database contains 1,689 genes encoding different classes of antibiotic resistance, of which 24 resistance genes were detected in this study. As observed for virulence genes, antibiotic resistance genes were associated with sequence type. The *blaZ, fosB* and *norA genes*, which confer penicillin, fosfomycin and fluoroquinolone resistance, respectively, were present in all ST5 and ST9 isolates. In contrast, *fosB* and *norA* genes were not present in ST398 isolates, and the *blaZ* gene was variably present in this group (Figure 5.2).

The *ermA* and *spc* genes shared identical distributions among ST9 and ST398 isolates (but were absent in ST5) suggesting a likely genetic linkage such as a transposon (Tn554) carrying the *ermA* and *spc* together, or another recombinant form of Tn554. A majority

(78%; 25/32) of ST398 isolates carried *ermA-spc* genes, compared with 29% (10/34) of ST9 isolates.

Three tetracycline resistance genes- *tetK, tetL, tetM* were detected. The distribution of tetracycline resistance genes also differed by sequence type. The *tetL* gene was most common in ST5 (56%; 5 of 9) and ST9 (41%; 14 0f 34) isolates. None of ST5 isolates harbored the *tetK* or *tetM* genes, which were also found at low prevalences of 9% (3/34) and 3% (1/34) of ST9 isolates. In contrast, all ST398 isolates (32/32) carried *tetM*, and *tetK* (38%, 12/32) and *tetL* (9%, 3/32) were also detected.

The *ermA, ermC, lnuA* and *vagA* genes encoding macrolides, lincosamides and streptogramin resistances (MLS resistance), respectively, were all found among ST9 isolates. The most prevalent gene was *lnuA* (34%, 5/34) followed by *ermA* (29%, 10/34), *ermC* (21%, 7/34), and *vgaA* (15%, 5/34). In ST398, *ermA, ermB, ermC* (76%, 6%, 45%) and *lnuA, lnuB* (15%, 12%) occurred at variable prevalence, but the *vagA* gene was not detected.

The *aadD* and *aac6-aph2* genes conferring resistance to aminoglycosides were distributed sporadically across all the lineages but were uniformly absent in ST398/t337 and ST398/t3446 isolates which have not been identified previously.

**Characterization of novel ST398/t337 and ST398/t3446 variants**

Using SRST2 software and WGS data, short reads were mapped to thecurrent MLST database and sequence types of all isolates were confirmed. Unexpectedly, 13 isolates (4 t337, 8 t3446 and 1 t2462) that had been putatively assigned to ST9, based on *spa* typing in earlier studies were determined to be ST398. Three isolates from these novel variants

(NV) were compared in more detail with other ST9 and ST398 isolates. A total of 24,185 SNP sites within the core genome were identified and phylogenetic analysis of core SNPs led to two distinct groups ST398 and ST9 on Figure 5.3 (b). The novel isolates clustered closely with the reference with ST398, with an average 0.0867 nucleotide substitutions per site genetic distance. The overall genomic portraits of NV, ST9 and ST398 isolates compared with ST398/t034 MRSA reference genome (CP003808) were described using CG view mapping software (Figure 5.3(a)). The isolates were compared with BLASTn and a database of 2,703 coding regions from reference genome which showed that ST398/t337 isolates and ST398/t3446 isolates were more similar to the reference genome (ST398/t034) than the other isolates.

**Discussion**

The likelihood that domestic animals could serve as reservoirs of coagulase positive staphylococci, be transmitted to people, and cause clinical infections was recognized more than 50 years ago (MORRISON et al. 1961; RAVENHOLT et al. 1961). Particular variants of *S. aureus* are adapted to individual host species, although the mechanisms underlying host adaptation are not fully elucidated (Sung et al. 2008; McCarthy et al. 2012a; Shepheard et al. 2013). However, it appears that relatively small genetic differences can strongly influence affinity for difference host species, and the transfer of mobile genetic elements may play an important role in host adaptation (Sung et al. 2008; Guinane et al. 2011; Viana et al. 2015).

Understanding of the biology of *S. aureus* and the emergence of MRSA in swine populations globally is incomplete, in part due to the paucity of studies of methicillin susceptible variants that are normal flora of swine (Linhares et al. 2015). Based on profiles of virulence and antibiotic resistance genes, most *S. aureus* isolated from US swine veterinarians were not distinguishable from *S. aureus* from pigs, consistent with numerous studies indicating a high frequency of interspecies transmission in livestock settings (Verkade et al. 2013; Frana et al. 2013; van Cleef et al. 2014). Exceptions in this study were ST5/t062 isolates, and the ST72/t126 isolate, which harbored genes of the immune evasion cluster that is considered a human-specific marker (Cuny et al. 2015). Also, neither of these *spa* types has been reported among several studies of US pigs (Dressler et al. 2012; Linhares et al. 2015; Sun et al. 2015b), suggesting their presence was unrelated to livestock exposure.

The most salient finding in this study was that each MLST lineage carried distinct sets of putative virulence factors, and profiles of antibiotic resistance genes also differed among sequence types. However, apart from SCCmec elements including *mecA*, MRSA and MSSA isolates displayed similar profiles within MLST lineages. This clustering of genetic profiles by MLST lineage is highly unlikely to be an artifact of sampling. The purposive sampling used to select swine herds was designed to achieve broad geographic sampling and included herds of diverse genetic provenance (Sun et al. 2015b). Also, many swine herds, and individual pigs, harbor more than one MLST lineage (Linhares et al. 2015; Sun et al. 2015b), suggesting ample opportunity of genetic exchange among lineages within herds. Selection of the subset of isolates included in this study similarly was designed to avoid inclusion of isolates of the same MLST/*spa* types from a single

herd. The findings indicate that these 3 lineages that are prevalent in swine in the USA have evolved somewhat independently in this species, suggesting a limited effect of horizontal gene transfer of virulence genes among different sequence types.

The *tetM* was uniformly present in ST398 (both MSSA and MRSA) isolates in this study, but present in only one isolate of other sequence types. It has been inferred that ST398 MRSA in pigs resulted from a host jump of MSSA from humans to pigs, followed by acquisition of the *tetM* and *mecA* genes (Price et al. 2012). Similar analyses have yet to be conducted on ST5 or ST9 MRSA. The possibility that these lineages may also have been acquired by pigs via host jumps is plausible but unknown, as is the duration over which these lineages have been part of the commensal flora of in pigs. However, host jumps of *S. aureus* CC97 from cattle to humans has also been inferred (Spoor et al. 2013), therefore the origins and directionality of the ST5 and ST9 lineages in pigs remain unknown.

To date, human infections with *S. aureus* isolates of likely livestock origin, particularly ST398 MRSA, appear to have been extremely uncommon in the USA, even in states with large swine populations (Nair et al. 2016). However, a livestock independent human clade of ST398/t571 among the most common causes of MSSA infections in New York, and was also common in a prison community in the USA (David et al. 2013; Uhlemann et al. 2013). The two ST398/t571 isolates included in this study (one swine and one veterinary) lacked the IEC genes, which is consistent with the animal clade, but also lacked some virulence and antibiotic resistance genes found in other ST398 isolates (*cna, ermA, spc*). Also, the phenicol exporter gene *fexA,* present only in ST398/t571 in this

study, is commonly located on the Tn558 transposon, which is structurally similar to Tn554 carrying *ermA* and *spc*, suggesting replacement by partial genomic exchange via horizontal gene transfer (Kehrenberg and Schwarz, 2006).

Notably, ST398 isolates harbored the least number of virulence genes, while the largest number was present in ST5 isolates (Table 5.3). Previous genomic studies suggested that reduced presence of virulence genes in ST398 *S. aureus* of animal origin reflects avoidance of unnecessary genes encoding human host immune response elements, while the presence of more antibiotic resistance determinants has assisted ST398 adapt to the antibiotic pressures in livestock environments (Price et al. 2012; Uhlemann et al. 2012) . In this study, the presence of multiple resistance genes was common across all lineages, but the distributions of genes differed among lineages. Although MRSA variants of ST398, ST5 and ST9 have been reported in pigs in the USA, MRSA variants do not appear to have become widely established. This is contrary to countries such as Denmark and the Netherlands, where regulation of antibiotics in food animals has been more stringent but ST398 MRSA are almost ubiquitous. There is therefore a real possibility that MRSA will become more prevalent in swine in the USA in the future, and background information of resistance genes in MSSA in swine may be of value in elucidating reasons for the future emergence of any successful lineage. Three tetracycline resistance genes (*–tetM, tetL, tetK)* were detected among the isolates, all of which were phenotypically resistant (>4 mg/L) (Sun et al. 2016b). Recent experimental data indicate that the combined presence of both *tetM and tetK,* linked to the SCCmec cassette, increased the fitness of ST398 MRSA during exposure to sub-lethal

concentrations of tetracyclines, pointing to a role of tetracyclines in the emergence of ST398 MRSA in Europe (Larsen et al. 2016a). However, both genes were found to be present in both ST398 MSSA isolates in the current study, suggesting that tetracycline susceptibility is an unlikely means of differential selection for MRSA variants unless gene expression is affected, which would require analysis of the transcriptome. ST398 isolates in this study more often harbored *tetM* than *tetL* or *tetK,* consistent with European studies (Argudin et al. 2011; Larsen et al. 2016a). This may be due to the presence of transposons containing *tetM* genes (de Vries et al. 2009) but further analysis is necessary to determine the lineage specific mechanisms.

This preliminary study used WGS to describe the occurrence of virulence and antibiotic resistance genes across MLST types that are prevalent in US swine and occupationally exposed people. However, more extensive analysis is needed to obtain more detailed understanding of the epidemiology of these organisms. The method (SRST2) used all high quality short reads for MLST, resistance genes, and virulence factor gene discovery, regardless their origin in the core genome or in mobile genomic elements, which will be further explored using the draft genome assemblies. Some combinations of genes were associated, and are likely to be carried on plasmids or transposons. For example, *aadD-tetL* were uniformly associated, sometimes in combination with *vgaA*. Commonly *tetL* gene is carried on plasmids with other genes (Kadlec et al. 2012) and further investigation of genetic structure of mobile genomic elements is warranted. Comparison with the reference genome revealed several blanks in the map because short read sequencing does not allow closing the genome of isolates. Further efforts to close the genomes are indicated, and may be facilitated by long-read sequencing technology that

creates read longer than 20Kb, or by using Sanger sequencing to amplify unmapped regions of genomes.

Lastly, comparative analyses with more reference genomes of the ST398, ST5 and ST9 types were not performed in this study. Broader analysis may be informative, particularly for ST5 lineage which is prevalent in cases of human infection in hospital and community settings.

**Table 5.1. *Staphylococcus aureus* isolates used in this study**

| ID | Host | spa type | ST original | ST SRST# | mecA | ID | Host | spa type | ST original | ST SRST2 | mecA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | vet | t337 | 9 | 9 | - | 39 | vet | t2498 | 9 | 9 | - |
| 2 | vet | t337 | 9 | 9 | - | 40 | vet | t2498 | 9 | 9 | - |
| 3 | vet | t2498 | 9 | 9 | - | 41 | vet | t2498 | 9 | 9 | - |
| 4 | vet | t2498 | 9 | 9 | - | 42 | vet | t2498 | 9 | 9 | - |
| 5* | vet | t337 | 9 | 9 | - | 43 | vet | t1334 | 9 | 9 | - |
| 6 | vet | t337 | 9 | 9 | - | 44 | vet | t1334 | 9 | 9 | - |
| 7 | pig | t337 | **9** | **398** | - | 45 | vet | t10494 | 9 | 2007 | - |
| 8 | pig | t337 | 9 | 9 | - | 46 | vet | t10494 | 9 | 2007 | - |
| 9 | pig | t337 | **9** | **398** | - | 47 | vet | t1430 | 9 | 9 | - |
| 10* | pig | t337 | **9** | **398** | - | 48 | vet | t034 | 398 | 398 | MRSA |
| 11* | pig | t337 | 9 | 9 | - | 49 | vet | t034 | 398 | 398 | - |
| 12* | pig | t3446 | **9** | **398** | - | 50 | vet | t034 | 398 | 398 | MRSA |
| 13* | pig | t3446 | 9 | 9 | - | 51 | vet | t034 | 398 | 398 | MRSA |
| 14 | pig | t3446 | **9** | **398** | - | 52 | vet | t011 | 398 | 398 | MRSA |
| 15 | pig | t3446 | **9** | **398** | - | 53 | vet | t002 | 5 | 5 | MRSA |
| 16 | pig | t2498 | 9 | 9 | - | 54* | pig | t034 | 398 | 398 | MRSA |
| 17 | pig | t2498 | 9 | 9 | - | 55 | pig | t11374 | 398 | 398 | - |
| 18 | pig | t2498 | 9 | 9 | - | 56* | pig | t034 | 398 | 398 | - |
| 19 | pig | t2498 | 9 | 9 | - | 57 | pig | t034 | 398 | 398 | - |
| 20 | pig | t2498 | 9 | 9 | - | 58 | pig | t5462 | 398 | 398 | - |
| 21 | pig | t2315 | 9 | 9 | - | 59 | pig | t571 | 398 | 398 | - |
| 22 | pig | t1334 | 9 | 9 | - | 60 | pig | t002 | 5 | 5 | - |
| 23 | pig | t2462 | **9** | **398** | - | 61 | pig | t002 | 5 | 5 | - |
| 24 | pig | t2462 | 9 | 9 | - | 62 | vet | t034 | 398 | 398 | MRSA |
| 25 | pig | t3232 | 9 | 9 | - | 63 | vet | t011 | 398 | 398 | MRSA |
| 26 | pig | t10494 | 9 | 2007 | - | 64 | vet | t2330 | 398 | 398 | MRSA |
| 27 | pig | Unk2+ | 9 | 9 | - | 65 | vet | t2330 | 398 | 398 | MRSA |
| 28 | pig | Unk3+ | 9 | 9 | - | 66* | vet | t034 | 398 | 398 | MRSA |
| 29 | vet | t337 | 9 | 9 | - | 67 | vet | t002 | 5 | 5 | - |
| 30 | vet | t337 | 9 | 9 | - | 68 | vet | t002 | 5 | 5 | - |
| 31* | vet | t337 | **9** | **398** | - | 69 | vet | t045 | 5 | 5 | - |
| 32 | vet | t337 | 9 | 9 | - | 70 | vet | t045 | 5 | 5 | - |
| 33 | vet | t337 | 9 | 9 | - | 71 | vet | t062 | 5 | 5 | - |
| 34 | vet | t3446 | **9** | **398** | - | 72 | vet | t062 | 5 | 5 | - |
| 35 | vet | t3446 | **9** | **398** | - | 73 | vet | t011 | 398 | 398 | - |
| 36 | vet | t3446 | **9** | **398** | - | 74 | vet | t126 | 72 | 72 | - |
| 37 | vet | t3446 | **9** | **398** | - | 75 | vet | t571 | 398 | 398 | - |
| 38 | vet | t3446 | **9** | **398** | - | 76 | vet | t034 | 398 | 398 | MRSA |

***** These isolates (9 isolates) were used for phylogenetic analysis.
+ The repeat succession numbers of the isolates are Unknown2 (Unk2 : r07r16r16r16r23r23r02r12r23r02r34) and Unknown3(Unk3: r07r16r23r23r02r12r17r23r02r34), respectively.
# SRST2 : sequence types were confirmed with whole genome data using SRST2 software.

**Table 5.2. The list of the virulence genes harbored by all isolates in this study**

| Category | Virulence factor | Related genes |
|---|---|---|
| **Adherence** | Autolysin | atl |
| | Cell wall associated fibronectin binding protein | ebh |
| | Clumping factor A | clfA |
| | Clumping factor B | clfB |
| | Elastin binding protein | ebp |
| | Extracellular adherence protein/MHC analogous protein | eap.map |
| | Intercellular adhesin | icaA |
| | | icaB |
| | | icaC |
| | | icaD |
| | | icaR |
| | Staphylococcus aureus surface protein | sdrE* |
| | Staphylococcal protein A | spa |
| **Exoenzyme** | Serine V8 protease | sspA |
| | Cysteine protease | sspB |
| | | sspC |
| | N-acetylmuramoyl-L-alanine amidase | sle1 |
| | Lipase | geh* |
| | | lip |
| | Thermonuclease | nuc |
| | Sortase | srtB |
| **Host Immune evasion** | Capsule | cap5H |
| | | cap5I |
| | | cap5J |
| | | cap5K |
| | | cap8A |
| | | cap8B |
| | | cap8C |
| | | cap8D |
| | | cap8E |
| | | cap8F |
| | | cap8L |
| | | cap8M |
| | | cap8N |
| | | cap8O |
| | | cap8P |
| **Iron regulated** | iron-regulated surface proteins | isdA* |
| | | isdB |
| | | isdC |
| | | isdD |
| | | isdE |
| | | isdF |
| | | isdG |
| | | isdH* |
| | | isdI |
| | Siderophore biosynthesis protein | sbnA |
| | | sbnB |
| | | sbnC |
| | | sbnD |
| | | sbnE |
| | | sbnF |
| | | sbnG |
| | | sbnH |
| | | sbnI |
| | Iron-regulated ABC transporter | sirA |
| | siderophore-binding protein | sirB |
| | | sirC* |
| | Staphylococcus aureus siderophore receptor | htsA |
| | | htsC |
| | siderophores staphyloferrin | sfaA |
| | | sfaB |
| | | sfaC |
| | | sfaD |
| **Secretion system** | Type VII secretion system | esaA |
| | | esaB |
| | | essA |
| | | essB |
| | | esxA |
| **Toxin** | Alpha hemolysin | hla |
| | Beta hemolysin | hlb |
| | Gamma hemolysin | hlgA/B/C |
| | Delta hemolysin | hld |
| | Exfoliative toxin type A | eta |
| | S component of leucocidin R | lukS-R |

*99% of isolates carried the genes.

**Table 5.3. The presence or absence of virulence factors by sequence type**

| Virulence factor | Related gene | ST398 | ST5 | ST9 |
|---|---|---|---|---|
| **uncharacterized leukocidin-like protein 2** | SAUSA300_1975 | - | + | + |
| **Extracellular matrix protein-binding protein** | emp | - | + | + |
| **MHC analogous protein** | map | - | + | + |
| **Type VII secretion system (Protein EsaC)** | esaC | - | + | + |
| **Type VII secretion system(Protein EssC)** | essC | - | + | + |
| **Type VII secretion system(Protein EsxB)** | esxB | - | + | + |
| **Fibronectin binding protein** | fnbA | - | + | + |
| **Heme ABC type transporter** | htsB | - | + | + |
| **Enterotoxin G** | seg | - | + | + |
| **Enterotoxin I** | sei | - | + | + |
| **Enterotoxin-like M** | selm | - | + | + |
| **Enterotoxin-like N** | seln | - | + | + |
| **Enterotoxin-like O** | selo | - | + | + |
| **Enterotoxin-like V** | selv | - | + | + |
| **Enterotoxin Yent2** | yent2 | - | + | + |
| **Exotoxin** | set19, set31,set32, set34, set36, set37, set38, set9, set35 | - | + | + |
| **Staphylocoagulase** | coa | + | + | - |
| **Collagen adhesion** | cna* | + | + | - |
| **Exotoxin** | set4, set30, set2, set5 | + | + | - |
| **Leukotoxin D** | lukD | - | + | - |
| **Leukotoxin E** | lukE | - | + | - |
| **Virulence-associated cell-wall-anchored protein** | sasG | - | + | - |
| **Fibronectin binding protein** | fnbB | - | + | - |
| **Serine protease** | splA, splB, splC, splF | - | + | - |
| **Exotoxin** | set15, set16 | - | + | - |
| **Enterotoxin J** | sej | - | #t062 only | - |
| **Enterotoxin D** | sed | - | t062 only | - |
| **Enterotoxin-like R** | selr | - | t062 only | - |
| **Chemotaxis inhibitory protein** | chp | - | t062 only | - |
| **Staphylokinase** | sak | - | t062 only | - |
| **Staphylococcal complement inhibitor** | scn | - | t062 only | - |
| **Exotoxin** | set30, set2, set5 | + | - | - |

***** Only ST398 t571 isolates did not carry the gene among ST398 isolates.
# Only ST5 t062 isolates carried these genes among ST5 isolates.

# Table 5.4. 173 virulence genes that were examined in this study (VFDB database)

| Virulence factors | Related genes | Virulence factors | Related genes | Virulence factors | Related genes |
|---|---|---|---|---|---|
| *Toxin* | | *Adherence* | | *Exoenzyme* | |
| Alpha hemolysin | hly/hla | Autolysin | atl | Cysteine protease | sspB |
| Beta hemolysin | hlb | | aur | | sspC |
| Delta hemolysin | hld | Cell wall associated fibronectin binding protein | ebh | Hyaluronate lyase | hysA |
| Enterotoxin A | sea | Clumping factor A | clfA | Sortase | srtB |
| Enterotoxin B | seb | Clumping factor B | clfB | Lipase | lip |
| Enterotoxin C | sec | Collagen adhesion | cna | | geh |
| Enterotoxin D | sed | Elastin binding protein | ebp | Serine protease | splA |
| Enterotoxin E | see | Extracellular adherence protein/MHC analogous protein | eap/map | | splB |
| Enterotoxin G | seg | Fibrinogen binding protein | efb | | splC |
| Enterotoxin H | seh | Fibronectin binding proteins | fnbA | | splD |
| Enterotoxin I | sei | | fnbB | | splE |
| Enterotoxin J | sej | Staphylococcus aureus surface protein | sasC | | splF |
| Enterotoxin Yent1 | yent1 | | sasG | Serine V8 protease | sspA |
| Enterotoxin Yent2 | yent2 | Intercellular adhesin | icaA | Staphylocoagulase | coa |
| Enterotoxin-like K | selk | | icaB | Staphylokinase | sak |
| Enterotoxin-like L | sell | | icaC | Thermonuclease | nuc |
| Enterotoxin-like M | selm | | icaD | *Host Immune evasion* | chp |
| Enterotoxin-like N | seln | | icaR | | scn |
| Enterotoxin-like O | selo | Ser-Asp rich fibrinogen-binding proteins | sdrC | Capsule | cap5H |
| Enterotoxin-like P | selp | | sdrD | | cap5I |
| Enterotoxin-like Q | selq | | sdrE | | cap5J |
| Enterotoxin-like R | selr | | sdrF | | cap5K |
| Enterotoxin-like U | selu | | sdrG | | cap8A |
| Enterotoxin-like V | selv | | sdrH | | cap8B |
| Exfoliative toxin type A | eta | Serine-rich surface protein | sraP | | cap8C |
| Exfoliative toxin type B | etb | Staphylococcal protein A | spa | | cap8D |
| Exfoliative toxin type C | etc | Iron-regulated surface proteins | isdA | | cap8E |
| Exfoliative toxin type D | etd | | isdB | | cap8F |
| Exotoxin | set1 | | isdC | | cap8G |
| | set2 | | isdD | | cap8L |
| | set3 | | isdE | | cap8M |
| | set4 | | isdF | | cap8N |
| | set5 | | isdG | | cap8O |
| | set6 | | isdH | | cap8P |
| | set7 | | isdI | *Secretion system* | |
| | set8 | Iron-regulated ABC trnasporter | sirA | Type VII secretion system | esxA |
| | set9 | | sirB | | esaA |
| | set10 | | sirC | | essA |
| | set11 | Siderophore biosynthesis protein | sbnA | | esaB |
| | set12 | | sbnB | | essB |
| | set13 | | sbnC | | essC |
| | set14 | | sbnD | | esaC |
| | set15 | | sbnE | | esxB |
| | set16 | | sbnF | | |
| | set17 | | sbnG | | |
| | set18 | | sbnH | | |
| | set19 | | sbnI | | |
| | set20 | Siderophores staphyloferrin | sfaA | | |
| | set21 | | sfaB | | |
| | set22 | | sfaC | | |
| | set23 | | sfaD | | |
| | set24 | Staphylococcus aureus siderophore receptor | htsA | | |
| | set25 | | htsC | | |
| | set26 | | | | |
| | set30 | | | | |
| | set31 | | | | |
| | set32 | | | | |
| | set33 | | | | |
| | set34 | | | | |
| | set35 | | | | |
| | set36 | | | | |
| | set37 | | | | |
| | set38 | | | | |
| | set39 | | | | |
| | set40 | | | | |
| Gamma hemolysin | hlgA | | | | |
| | hlgC | | | | |
| | hlgB | | | | |
| Leukocidin M | lukM | | | | |
| | lukF-like | | | | |
| Leukotoxin D | lukD | | | | |
| Leukotoxin E | lukE | | | | |
| S component of leucodicin R | luk_S-R | | | | |
| Panton-Valentine leukocidin | lukS-PV | | | | |
| | lukF-PV | | | | |
| Toxic shock syndrome toxin | tsst | | | | |

Gene : the genes not detected in any of the isolates

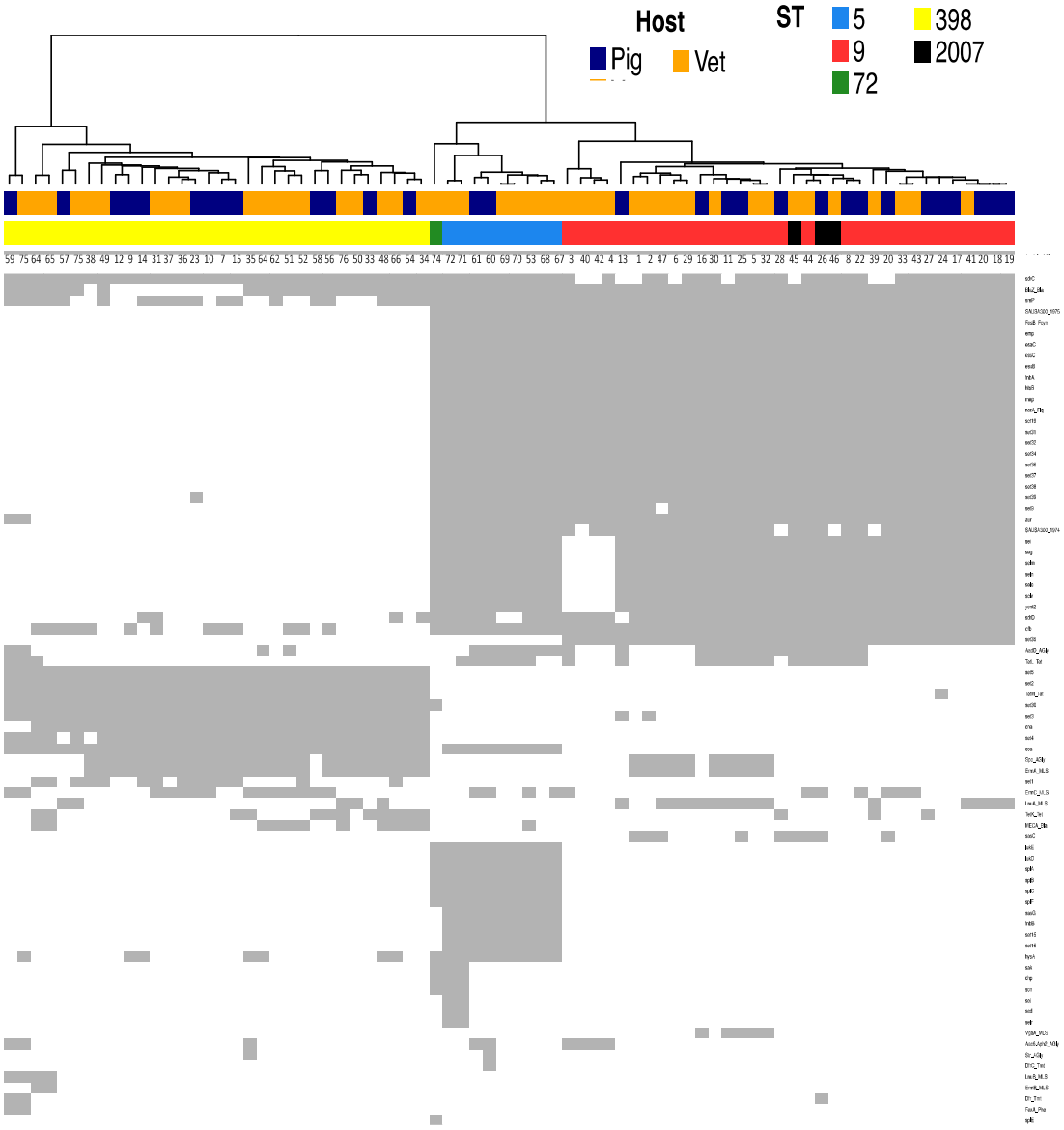

Host
Pig
Vet
ST
5
398
9
2007
72
59 75 64 65 57 75 38 49 12 9 14 31 37 36 23 10 7 15 35 64 62 51 52 58 56 76 50 33 48 66 54 34 74 72 71 61 60 69 70 53 68 67 3 40 42 4 13 1 2 47 6 29 16 30 11 25 5 32 28 45 44 26 46 8 22 39 20 33 43 27 24 17 41 20 18 19


**Figure 5.2. The profile of antibiotic resistance genes in all isolates. The genes – *aph*, *aac3*, *dha1*, *tet38* present in all isolates were not included in this figure. The bold numbers of ST indicate MRSA isolates corresponding with the presence with *mecA* gene.**

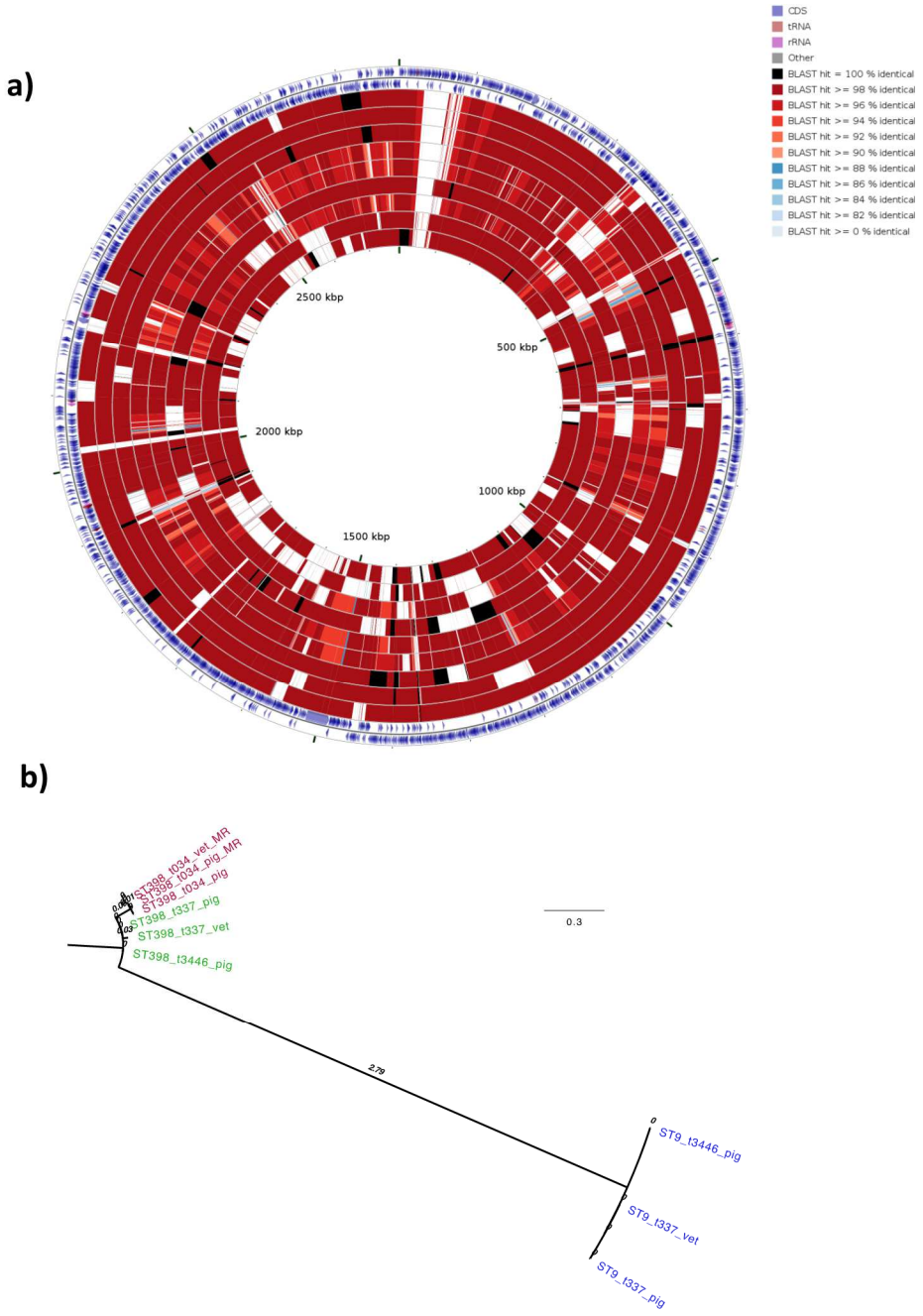



**Figure 5.3. The genetic relatedness of ST398-ST9 lineage with ST9 and ST398. (a) Graphic circular map visualized with reference genome (ST398-t034 MRSA in human, CP003808) located in the outermost ring. The isolates were displayed by following order (from inside circle to outside) : ST398/t034_pig_MRSA, ST398/t034_vet_MRSA, ST9/t3446_pig, ST398_t034_pig, ST9/t337_vet, ST9/t337_pig, ST398/t3446_pig, ST398/t337_pig, ST398/t337_vet. (b) Phylogenetic relationships with 100 bootstrap iterations. The tree was generated using maximum likelihood method based on identified SNPs. All branches were shown over 90%**

**confidence. Isolates were color-coded by sequence type and the numbers on the branch describe number of nucleotide substitution per site.**

# Chapter 6. General Discussion and Conclusions

The goal of this thesis was to advance understanding of the epidemiology of *Staphylococcus aureus* in the US swine industry, particularly in relation to potential human health risks from this animal reservoir. At the initiation of this work in 2012, the information available in the USA was limited to reports on the detection of LA-MRSA in North America, with negligible information reported about methicillin susceptible *S. aureus*. A basic premise for the work undertaken was that in order to understand the epidemiology of LA-MRSA at the livestock-human interface, it is necessary to understand the epidemiology of *S. aureus* generically, rather than solely MRSA variants. Therefore the investigations included the first systematic characterization of the *S. aureus* population in the US swine industry; a longitudinal cohort study focused on understanding the dynamics of *S. aureus* colonization of swine veterinarians; comparison of phenotypic resistance to antibiotics and zinc among isolates for pigs and swine veterinarians; and documentation of the presence of genes encoding virulence factors and antibiotic resistance in *S. aureus* at the swine-human interface.

The most unexpected and significant finding in the prevalence study of *S. aureus* in US swine was the absence of any MRSA isolates in 37 herds of previously unknown status (Chapter 2). The inclusion of a positive control herd of known MRSA status (ST398), in which all 20 pigs tested were MRSA positive, confirmed that this was not a methodology problem, coupled with the detection of MSSA in 77% of all pigs tested. The design of the study was informed by a pilot project on 2 multiple site systems in Minnesota that (Linhares et al. 2015) were also MRSA negative, but where 3 MLST types (ST398, ST9, ST5) accounted for all isolates. The number of herds studies was limited by cost, but the

herds included were both geographically diverse (from 11 states) or diverse genetic backgrounds, although likely biased to larger herds that account for the bulk of the US pork supply. The 95% upper confidence level of 9.3% herd prevalence of MRSA is very low relative to most northern European countries, although substantial intercountry variability occurs (EFSA,. 2009). The reasons why LA-MRSA, although present in the USA since at least 2007 (Smith et al. 2009), have not become more widely disseminated in the US industry remain to be determined. Another salient finding was that the same 3 MLST types found in the pilot study by Linhares et al (2015) constituted all but 2 of the 1,200 *S. aureus* isolated from these herds (Linhares et al. 2015). It is likely that broader sampling of herds may reveal more *S. aureus* variants do occur in US pigs, but it is clear that all three of these lineages are broadly established in the commercial swine population. Also important is the fact that all three of these lineages have been prominent among reports of LA-MRSA globally, albeit with differing geographical distributions (Khanna et al. 2008; EFSA,. 2009; Frana et al. 2013; Chuang and Huang, 2015). The information from this study will provide valuable information MRSA of one or more of these lineages emerge to occur at high prevalence in pigs in the future. A case could be made for intermittent cross-sectional sampling of the US swine population to monitor changes in *S. aureus* and MRSA over time.

A third finding of relevance to public health was that major enterotoxin genes (A-E) were universally absent from a subset of 130 isolates chosen to provide broad representation of farms and genotypes. Staphylococcal enterotoxicosis is not uncommonly associated with pork products, particularly ham. The data suggest that the origin of enterotoxigenic

strains on pork products is unlikely to be the farms, and attention should be focused on prevention of *S. aureus* contamination in downstream sectors of the food supply.

The most substantial project in this thesis was the longitudinal study of a cohort of 66 swine veterinarians who submitted monthly nasal swab samples over 18 months (Chapter 3). Prior to undertaking this work, numerous studies had consistently observed that people with occupational contact with swine (farm workers, veterinarians, abattoir workers) had prevalences of MRSA carriage much higher than observed in the general populations of the corresponding countries due to the presence of LA-MRSA. Elevated risk of exposure to LA-MRSA based on nasal swab cultures was therefore well established, but the biological relevance of this phenomenon were uncertain.
A key question was whether positive nasal cultures represented transient contamination of mucosal surfaces or longer term colonization. Most published studies were either cross sectional (with no insight into persistence), longitudinal but short term of days to weeks, and/or involved farmers who were regularly exposed to pigs prohibiting differentiation of repeated contamination from persistent colonization. The monthly sampling over 18 months is the most extensive sampling yet conducted of an occupational group, and evaluation of both MSSA and MRSA provided broader information than previous studies, but the absence of a non-occupationally exposed control group was a limitation. As anticipated, and consistent with prior studies, a high prevalence of both MSSA (65%) and MRSA (9%) was observed, and the MLST types and *spa* types identified were predominantly consistent with those found in US pigs (Chapter 2). Furthermore examination of the colonization patterns of individual

veterinarians showed a clear bimodal distribution of carriage rate indicating persistent colonization occurred in a subset of veterinarians. More importantly, a single *spa* type was isolated consistently over 18 months in a subset of veterinarians, and this was observed with both MSSA and MRSA isolates, and across all 3 MLST types.

This provides convincing evidence that long term nasal colonization of people can occur with *S. aureus* of apparent livestock origin, although the directionality of transmission is not proven. Other veterinarians that were persistently colonized showed variability in MLST or *spa* types over time, indicating that replacement of genotypes could occur over time. This is consistent with current understanding of the dynamics of *S. aureus* colonization in humans. Even greater variability was seen with intermittently colonized veterinarians, likely reflecting multiple episodes of transient contamination or short term colonization with diverse *S. aureus* isolates. Part way through this study, a similar study of 137 Dutch veterinarians, sampled just 5 times over 2 years, reported similar patterns of colonization, but with a much higher prevalence of MRSA (Verkade et al. 2013).

A further observation in the current study was that only 3 veterinarians (1 with MRSA) reported any clinical infections with *S. aureus*, none of which were medically significant or required time off work. In line with other studies (Larsen et al. 2015; van Cleef et al. 2015; Nair et al. 2016), this suggests that despite considerable exposure risk for occupational groups working with swine, the risk of significant clinical infections with livestock associated *S. aureus* may not be a major concern. However, longer duration studies with larger numbers of subjects is necessary to obtain robust estimates of the incidence of clinical *S. aureus* infection, and possible risk factors, in groups working with swine.

The work in Chapters 2 and 3 provided new insights into *S. aureus* at the animal-human interface in the context of the USA swine industry. It also yielded a large library of *S. aureus* isolates that could underpin further studies relevant to animal and public health issues surrounding LA-MRSA. The emergence of LA-MRSA, and to a lesser extent multidrug resistant *S. aureus* (MDRSA), has been at the center of discussions about the use of antibiotics in food animals and its public health implications. This includes the role that antibiotic use in animals played in the relatively recent emergence of LA-MRSA, which is still unexplained. An important knowledge gap in these discussions is the paucity of information about antibiotic resistance in MSSA isolates in animal reservoirs, in which research was overwhelmingly biased towards MRSA. Such information could be particularly relevant in settings such as the USA where LA-MRSA are yet to occur at high prevalence among swine farms. Therefore the work in Chapter 4 characterized patterns of phenotypic antibiotic resistance among a subset of *S. aureus* isolates obtained from pigs and veterinarians in the preceding work. Salient findings included the general similarity in patterns of resistance for the swine and veterinary isolates, consistent with the common occurrence of interspecies transmission and nasal colonization. Also, resistance patterns were extremely diverse, with the most common resistotype found in only 6% of isolates. This may reflect the cumulative outcome of variable patterns of antibiotic use in pigs over the last 6 decades. However, resistance to several antibiotics was associated with MLST sequence type, a phenomenon also seen with resistance genes in the subsequent Chapter 5. The presence of MLST related resistance genotypes and phenotypes perhaps suggests the influence of historic selection

pressures yielding some resistotypes that have persisted over time in multiple commercial swine herds. This baseline information on resistance in MSSA in a swine population with a low prevalence of MRSA may provide valuable background information if one or more MRSA variants emerge in the US swine population in the near future. It could also provide a baseline for comparison in future studies conducted after the implementation of FDA guidance 213 in January 2017.

The ultimate concern regarding *S. aureus* in the livestock reservoir is the capacity of these organisms to cause clinical disease in humans. It is well established that LA-MRSA and LA-MSSA are capable of causing infections, but medically important infections have overwhelmingly occurred in elderly and medically compromised patients. Potential medical significance could be a function of both the virulence of isolates, and their profiles of resistance to medically important antibiotics. Therefore, the final component of the thesis (Chapter 5) was characterization of the profile of genes encoding virulence factors and antibiotic resistance in a subset of the isolates obtained from pigs and veterinarians (Chapters 2, 3). Again, published information was derived largely from studies of ST398 MRSA in Europe, with scarce information available about MSSA isolates and other lineages in swine. Notable findings were that distinct genetic profiles for both virulence factors and antibiotic resistance genes occurred among the 3 major lineages (ST398, ST5, ST9), and that ST398 isolates bore the least number of virulence genes. It cannot be inferred that this predicts greater or lesser pathogenicity in humans due to the multifactorial nature of disease. In contrast, ST398 isolates harbored less virulence genes than ST9 and ST5 isolates, corresponding with the suggestion that a human to animal host jump of ST398 led to loss of virulence genes and acquisition of

resistance genes. Although the focus of this work was on virulence and antibiotic resistance genes, application of WGS has great potential to further explore *S. aureus* epidemiology and the animal human interface. Future opportunities include core genome analysis of genetic variations among and within sequence type(s), to assess the phylogeny and ancestry of livestock associated *S. aureus*. A clear need for extension of this work is to understand the relationships between phenotypic and genotypic resistotypes. Last, detailed analysis of mobile genetic elements harboring crucial virulence genes and antibiotic resistance genes should give further insight into the complex epidemiology of *S. aureus* both with pig populations and with respect to interspecies transmission.

Overall, over many decades *S. aureus* has proven an agile and formidable adversary to the medical profession. The role of food animal reservoirs in contributing to the public health burden of *S. aureus* clearly cannot be ignored. Although current evidence suggests this is a minor component of *S. aureus* disease in humans in most countries, the epidemiology is clearly dynamic. Therefore, there is a need maintain some focus on the ecology of *S. aureus* at the animal-human interface. This might best be achieved with systematic long term studies that evaluate multiple aspects of these organisms, MSSA as well as MRSA, including shifts in genotypes over time, and particularly changes in antibiotic resistance and virulence factors.

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