RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Prevalence and characterization of *Staphylococcus aureus* in pigs in the USA – **NPB #13-056** Revised

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Industry Summary

Over the last 10 years, concerns have grown about the possible importance of livestock reservoirs as a source of methicillin resistant *Staphylococcus aureus* (MRSA). *S. aureus* is a normal bacterial inhabitant of healthy people as well as many animal species including pigs. However, *S. aureus* is also an important cause of human infections, and among the most significant problems related to antibiotic resistance in human medicine. A small number of studies have confirmed the presence of several variants of MRSA in pigs (types ST398; ST5; ST9) in the USA, but the epidemiology of *S. aureus* in pigs in the US has not been well documented. We conducted a study of 36 farms located in 11 states of the US to describe the prevalence and types of *S. aureus* (including MRSA) in the swine reservoir. One additional farm, previously known to harbor MRSA, was included as a positive control farm. We also determined patterns of antimicrobial resistance and the presence of genes producing toxins that can cause food poisoning in people. Because zinc resistance has been implicated in the emergence of 'livestock associated" MRSA in Europe, we also tested isolates for resistance to zinc and for the presence of a specific gene (czrC) linked to ST398 MRSA in Europe.

Overall, 739 pig nasal swabs were collected, of which 558 (76%) were culture positive for *S. aureus* from 35 of the 36 farms (97%). Except the positive control farm, on which all 20 pigs tested MRSA positive, no MRSA were detected in any of the pigs. Among the 35 *S. aureus* positive farms there was considerable diversity found with 33 spa types detected within 4 MLST sequence types. The most prevalent spa types (sequence type) were t337 (ST9), t034 (ST398) and t002 (ST5) which together accounted for 59% (627 of 1070). Antimicrobial resistance testing showed resistance was most common to spectinomycin (100%), tetracycline (94%), clindamycin (75%) and penicillin (72%), and 89% (116/130) of isolates were resistant to 5 or more antibiotics (multidrug resistance SA, MDRSA). ST398 (t034) MRSA isolates from the positive control farm were positive for the czrC gene, but no other isolates tested were positive.

The most striking finding of the study was that none of the 36 study farms were positive for MRSA. Although much higher prevalence (up to 30%) has been previously reported in smaller, and more geographically limited, studies in the USA, relatively low herd prevalence was also reported in larger and geographically diverse studies in the USA and Canada. Our observations on the czrC gene are also aligned with other studies indicating that zinc and other selective factors (e.g., disinfectants and therapeutic use of cephalosporins) are likely to have

had a much greater role in the emergence of these organisms than growth promotant usage. Our observation that multiple antimicrobial resistant is prevalent in *S. aureus* isolates from pigs is both unsurprising and consistent with other studies. However, current evidence suggests that pig adapted *S. aureus* rarely harbor major virulence factors associated with human disease. Hence the human health significance, if any, of multiple resistance in this group of organisms is yet to be established.

Keywords: MRSA, *Staphylococcus aureus*, antimicrobial resistance, zinc

Scientific Abstract

A cross-sectional study of 36 herds of growing pigs across 11 states was conducted to estimate the prevalence of *S. aureus* (including MRSA), and characterize the isolates with respect to spa type, MLST type, presence of enterotoxin genes, and antimicrobial and zinc resistance. A positive control herd known to harbor MRSA was also included in the study. On each farm, nasal swabs were collected from 20 growing pigs older than 4 weeks of age. Overall, 739 pig nasal swabs were collected, of which 558 (76%) were culture positive for *S. aureus* from 35 of the 36 farms (97%). Except the positive control farm, on which all 20 pigs tested MRSA positive, no MRSA were detected in any of the pigs. Among the 35 *S. aureus* positive farms there was considerable diversity found with 33 spa types detected within 4 MLST sequence types. The most prevalent spa types (sequence type) were t337 (ST9), t034 (ST398) and t002 (ST5) which together accounted for 59% (627/1070) of isolates typed. No isolates carried any of the major enterotoxin genes implicated in foodborne staphylococcal enterotoxicosis. Antimicrobial resistance testing showed resistance was most common to spectinomycin (100%), tetracycline (94%), clindamycin (75%) and penicillin (72%), and 89% (116/130) of isolates were resistant to 5 or more antibiotics (multidrug resistance *S. aureus*, MDRSA). ST398 (t034) MRSA isolates from the positive control farm were positive for the czrC gene, but no other isolates tested were positive. However, 14% of SA (18/130) tested were phenotypically zinc resistance based on a break point of 4mM zinc. The absence of MRSA from all 36 study herds supports accumulating data suggesting the MRSA is less prevalent in US swine farms than in many European countries such as Holland and Denmark. It appears 3 lineages (ST9, ST398, and ST5) predominate among *S. aureus* carried by healthy growing pigs in the USA. Although all isolates were methicillin susceptible, these 3 lineages have to date been those most commonly reported for MRSA isolates from pigs. The absence of major enterotoxin genes suggests that staphylococcal enterotoxicosis associated with pork products is more likely the result of contamination in the harvest and post-harvest segments of the pork supply rather than originating on the farm. Multiple antimicrobial resistance was common, in line with some other recent studies in farm workers and rural residents in the USA. However, current evidence suggests that pig adapted *S. aureus* rarely harbor major virulence factors associated with human disease. Hence the human health significance, if any, of multiple resistance in this group of organisms is yet to be established.

Introduction

Staphylococcus aureus is a bacterium that commonly colonizes the skin and mucosal surfaces of many mammalian and avian species. *S. aureus* is also an opportunistic pathogen and a leading cause of bacterial infections of people, causing a broad spectrum of pathology ranging from minor skin infections to fatal invasive disease. Prior to the recognition that pigs and other livestock species can be reservoirs of methicillin resistant *Staphylococcus aureus* (MRSA), *S. aureus* was considered a relatively unimportant organism in animals. It is now more than a decade since ST398 MRSA was found to be prevalent in pigs and other livestock species in the Netherlands and other countries,(Voss et al., 2005; Smith and Pearson, 2011) yet many questions remain unanswered about the epidemiology and public health impact of *S. aureus* in livestock reservoirs. While ST398 MRSA were initially referred to synonymously as ‘livestock associated MRSA’, the epidemiology of livestock

associated MRSA is now known to be more complex. The predominant genotypes of MRSA in pigs have been found to vary geographically, with ST9 being the predominant lineage in Asia.(Chuang and Huang, 2015) Previous studies of MRSA in pigs in North America have variably reported predominance of ST398 or ST5 variants, and ST9 variants have also been detected (Smith et al., 2009; Molla et al., 2012; Frana et al., 2013; Smith et al., 2013)

Remarkably, given the potential importance of this issue, research on the ecology of generic (non-MRSA) *S. aureus* has been largely ignored, but is arguably an important foundation for understanding the origin and ecology of MRSA variants. In a pilot study of 2 systems in the USA (NPB project 11-016), we observed that *S. aureus* are readily detectable in many anatomical sites of pigs, and that multiple spa types and MLST types coexist on individual farms and even within the same pigs.(Linhares et al., 2015)
The goal of the current study was obtain more geographically representative information about the prevalence and diversity of *S. aureus* and MRSA in growing pigs on commercial farms in the USA. We also characterized the organisms isolated for phenotypic resistance to antimicrobials, and for the presence of genes encoding toxins that cause staphylococcal food poisoning in people.

Objectives

1. Determine the predominant profile of *S. aureus* MLST sequence types and spa types in pigs in the US swine industry
2. Describe the antibiotic resistance patterns of predominant *S. aureus* sub-types (MLST/spa type) in relation to current antimicrobial exposure
3. Determine the prevalence of major *S. aureus* enterotoxin types (A – E) in *S. aureus* isolates from US pigs in relation to sub-types (MLST/spa type).

Materials & Methods

Selection of farms and animals

A cross sectional study was conducted on 36 farms located in 11 states in the USA (Midwest: 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) in University of Minnesota (1303-30452A), and sampling was conducted from June 2013 to November 2014. Farms were selected by swine veterinarians who had participated in a separate longitudinal study of MRSA colonization and infection. Each veterinarian chose one client farm to sample by convenience, and no more than 2 farms were serviced by the same veterinary clinic. The veterinarians were mailed sampling instructions for obtaining nasal swabs samples from 20 growing pigs age 4 weeks or older. Veterinarians also completed a survey with basic information about the farm, including current antimicrobial use. Swabs were mailed to University of Minnesota for processing. The same process was used to collect samples from one positive control farm known to be MRSA positive from previous studies. The sample size of 20 pigs per farm was calculated based on a pilot study in which apparent prevalence of *S. aureus* in nasal swabs of pigs was greater than 60%.(Linhares et al., 2015) Based on the binomial distribution, at an expected prevalence of 60% at least 8 isolates would be anticipated in 97% of herds sampled.

Bacterial isolation and characterization

Isolation of *S. aureus* was performed using standard methods described previously.(Linhares et al., 2015) Briefly, nasal swabs were double enriched in Mueller-Hinton broth (BBL™, MD, USA) supplemented with NaCl (6.5%) and in Phenol-Red Mannitol broth (BBL™, MD, USA) supplemented with 4ug/ml Oxacillin (Sigma-Aldrich, MO, USA). DNA was extracted using 1 colony from the plate with 19.5 ul 10mM Tris-HCl

and 0.5ul Lysostaphin (both Sigma-Aldrich, MO, USA) at 37°C for 30 min. PCR was used to detected 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'] (Integrated DNA Technologies, IA). 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. All PCR products were visualized on 1% agarose gel with SYBR dye in 1X TBE buffer (Tris-Borate-EDTA,Thermo Fisher Scientific Inc., MA USA) for 40min at 200 V.

Molecular typing and analysis

All selected *S. aureus* isolates were subtyped using *spa* typing. PCR products (Illustra Exoprostar, GE healthcare Bio-sciences, PA, USA) from amplification of the *spa* gene were sent to Biomedical genomic center (BMGC, University of Minnesota, MN) to obtain gene sequences. After aligning sequences with Sequencher 5.1(Gene Codes Corporation, MI, USA), each sequence was submitted to Ridom spa typing database. Multi-locus sequence typing (MLST) of *S. aureus* was performed following published methods. 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 (http://saureus.mlst.net).

Detection of enterotoxin genes

DNA was extracted using lysozyme enzyme. 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.() The primer mix to run a single PCR reaction contained 5.5 µL nuclease-free water, 12.5 µL HotStart-It Fideli Taq Master Mix, (Affymetrix), 0.5 µL of each 10 µM forward and reverse enterotoxin primers (for *sea*, *seb*, *sec*, and *see*),1.0 µL of 10 µM *sed* primers, and 1.0 µL of extracted *S. aureus* DNA. DNA amplification was conducted with the following thermal cycling profile: an initial denaturation at 94°C for 2 minutes was followed by 30 cycles of amplification (denaturation at 94°C for 2 minutes, annealing at 56°C for 2 minutes, and extension at 72°C for 1 minute), ending with a final extension at 72°C for 5 minutes.

Antimicrobial and zinc susceptibility testing

For antibiotic susceptibility testing, a panel of 18 antibiotics were used as conducted routinely for gram positive organisms at the University of Minnesota Veterinary Diagnostic Laboratory (spectinomycin, ampicillin, penicillin, chlortetracycline, oxytetracycline, clindamycin, tilmicosin, danofloxacin, sulphadimethoxine, florfenicol, neomycin, tiamulin, gentamicin, enrofloxacin, trimethoprim/sulphamethoxazole, tulathromycin, ceftiofur, tylosin). In addition, zinc susceptibility was evaluated using the agar dilution method at concentrations of 0.5, 1, 2, 4, 8, 16, and 32mM.

Results

Thirty-six farms from 11 states in the USA were sampled, mostly (29 of 36, 81%) in the Midwest region where pig production is concentrated. Herd size ranged from 40 to 12,000 head, and most sites (22 of 36, 61%) were nursery farms. Mixing of pigs from multiple sources was not widely practiced, with 29 farms (81%) receiving pigs from a single source. The age of pigs sampled ranged from 4 to 20 weeks. Overall, 739 pig nasal swabs were collected, of which 558 (76%) were culture positive for *S. aureus*, and positive pigs were detected on 35 of

the 36 farms (97%). However, no MRSA were detected in any of the pigs or farms sampled, apart from the positive control herd on which all 20 pigs sampled were positive for MRSA. The prevalence of *S. aureus* was between 80 and 100% on the majority of positive farms (60%, 21/35), and only 7 farms had a prevalence less than 50%. Prevalence tended to be lower (66% of 305 pigs on 15 farms) in young nursery pigs of 4 to 5 weeks of age, and was highest in pigs older than 12 weeks (98% of 102 pigs on 5 farms).

Among the 35 *S. aureus* positive farms there was considerable diversity found with 33 spa types detected within 4 MLST sequence types (Table 1). The most predominant spa types (sequence type) were t337 (ST9), t034 (ST398) and t002 (ST5) which together accounted for 59% (627 of 1060) of isolates typed. Only one isolate belonged to the fourth MLST type (ST 2007). Seven spa types (t3232, t2582, t5883, t1793, t5462, unknown 2 types) detected only once were closely related to the predominant spa type on the respective farm. On 9 farms, all *S. aureus* isolates were of homogeneous spa type (6 ST9 and 3 ST398). On others, multiple spa types were found, and 8 different spa types were isolated on one farm. However, the diversity within farm did not show any relation with pig genetic source, geographic location, herd size or sampling age.

Enterotoxin gene PCR and antimicrobial susceptibility testing was conducted on a subset of 130 isolates. These were chosen to ensure maximum diversity by choosing at least one isolate of each spa type detected on each farm. All isolates tested were negative by PCR for enterotoxin genes A, B, C, D, and E. Antimicrobial susceptibility testing (Figure 1) showed resistance was most common to spectinomycin (100%), tetracycline (94%), clindamycin (75%) and penicillin (72%), and 89% (116/130) of isolates were resistant to 5 or more antibiotics (multidrug resistant *S. aureus*, MDRSA). ST398 (t034). Observed patterns of resistance were extremely diverse, with 55 different patterns found among the 130 isolates. MRSA isolates from the positive control farm were positive for the czrC gene, but no other isolates tested were positive. However, 14% of SA (18/130) tested were phenotypically zinc resistance based on a break point of 4mM zinc.

At the time of sampling, no antimicrobials were being administered in the feed on 14 farms, and a further 2 farms were using either carbadox or tiamulin (neither considered medically important). Of antimicrobials being used in feed, the most common were tetracyclines (19 farms) followed by tiamulin (14 farms), with both being used in combination on 12 farms. No farms were using spectinomycin by any route, yet all isolates tested were resistant to this compound. No antimicrobials were being used in the water on 30 (83%) farms, and there was a trend ($P = 0.07$) to less use of antimicrobials in feed or water at sites other than nurseries (8 of 13 non-nursery farm not using antimicrobials vs. 6 of 23 nurseries).

Discussion

The most striking finding of the study was that none of the 36 study farms were positive for MRSA, and exact binomial confidence intervals show the 95% upper limit for herd prevalence to be 9.7%. Although much higher prevalence (up to 30%) has been previously reported in smaller, and more geographically limited, studies in the USA,(Molla et al., 2012; Frana et al., 2013) , relatively low herd prevalence was also reported in larger and geographically diverse studies in the USA and Canada.(Weese et al., 2011; Smith et al., 2013) Several studies of occupationally exposed people in the USA are also consistent with the possibility that prevalence of MRSA may be substantially lower in the US swine industry than in many European countries.(Rinsky et al., 2013; Wardyn et al., 2015) The apparently low MRSA prevalence in pigs in the USA, where antimicrobial growth promotants will be available until 2017, raises further questions about whether growth promotant usage had any material influence on the emergence of livestock associated MRSA globally. Our observations on the czrC gene are also aligned with other studies indicating that zinc and other selective factors (e.g., disinfectants and therapeutic use of cephalosporins) are likely to have had a much greater role in the emergence of these organisms than growth promotant usage.(Aarestrup et al., 2010; Cavaco et al., 2011; Moodley et al., 2011; Dorado-Garcia et al., 2015; Slifierz et al., 2015) Our observations that multiple antimicrobial resistance is prevalent in *S. aureus* isolates from pigs is both unsurprising and consistent with other studies. This

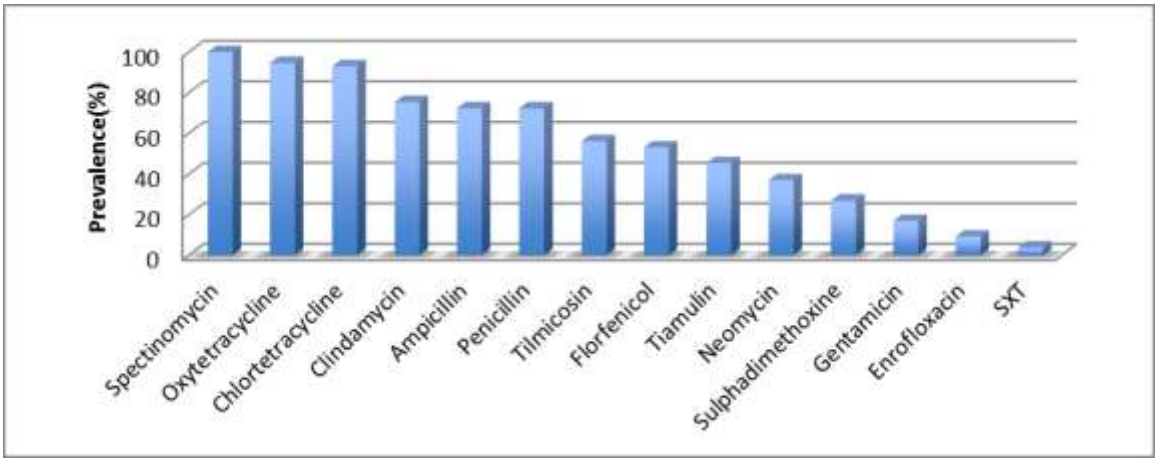
phenomenon is probably not new, and the total absence of awareness or concern (in the pre-ST398 era) about *S. aureus* about as an occupational risk suggests that human health risks of MDRSA in livestock are unlikely to be of great importance. Regardless, it is increasingly likely that in a relative vacuum of MRSA in pigs in the USA that the phenomenon of multiple drug resistant MSSA will attract more attention and may require efforts in both risk assessment and risk communication to quell the concerns of the general public. This shift in emphasis is already clear in recent publications of occupationally exposed people in which MRSA has been infrequent and multiple resistance in other *S. aureus* has been prevalent.(Rinsky et al., 2013; Wardyn et al., 2015) The apparent absence of major enterotoxin genes in the isolates tested suggests that these genes may be very uncommon among *S. aureus* in the swine reservoir. However, episodes of staphylococcal food poisoning linked to pork products (particularly ham) are not uncommon in the USA. Our data suggest that contamination of pork products with enterotoxigenic variants of *S. aureus* may primarily originate in the downstream sectors of the pork supply chain rather than from animals on the farm.

Table 1: Genotypic characterization of *S. aureus* from 36 US swine farms (n=1070)

MLST type	Spa type	Number of isolates (%)	Number of farms
ST9	t337	360 (34)	**23**
	t3446	73 (7)	5
	t2498	59 (6)	3
	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	150(14)	**11**
	t571	64(6)	3
	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(0.6)	1
	t11241	4(0.4)	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(0.5)	1
ST5	t002	117(11)	**9**
	t570	22(2)	3
	t242	13(1)	1
	t306	4(0.4)	1

*Repeat succession of unknown types: Unknown 1 (r07r16r23r23r02r12r17r23r02r34); Unknown 2 (r07r16r16r16r23r23r02r12r23r02r34); Unknown 3 (r07r16r16r23r02r12r23r02r34); Unknown 4 (r08r475r2r25r2r25r34r34r25); unknown5 (r07r16r23r23r02r23r02r34).

Figure 1: Prevalence of antimicrobial resistance among 130 *S. aureus* isolates to 18 antimicrobial compounds (SXT = sulphamethoxazole/trimethoprim)



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