RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: MRSA of Pigs and Genetic Analysis of Resistance Linked with *mecA* and *mecC*. - **NPB #14-232**

REVISED

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Industry Summary

Methicillin resistant *S. aureus* (MRSA) has been identified as a contaminant of swine and meat associated with swine. This study set out to examine the prevalence of novel methicillin resistant associated traits in a collection of MRSA recovered from swine, and compared with non-*S. aureus* strains as well as isolates recovered from other production animals and humans. This study also focused on determining if methicillin resistance associated with the novel and emerging recently identified *mecC* resistance gene was occurring in pork production. Isolates identified as methicillin resistant were also assessed for the type of resistance identified in *mecA* positive strains. A total of 1130 isolates were assessed including 659 from production pigs at lairage and slaughter, human isolates (n=150), other production animals (n = 58), *S. aureus* from meat and deli meat samples (n = 75) and non-*S. aureus* isolates recovered from production animals and humans (n = 188) were also included in the analysis. The novel *mecC* gene was not detected in swine production, however variant types of *mecA* resistance were detected suggesting that methicillin resistance in swine may be evolving and changing. This data suggests that continuous monitoring for emerging methicillin resistance is important in assessing new resistances as they emerge as well as understanding the potential sources of these resistance and designing interventions to reduce the risk of resistance entering the food chain. The data also highlights that other species of *Staphylococcus* can be potential sources of methicillin resistance.

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Scientific Abstract

Background: Methicillin-associated resistance in *S. aureus* is encoded by the *mecA* gene, carried on the staphylococcal cassette chromosome *mec* (*SCCmec*) resulting in an altered penicillin binding protein (PBP) () and reduced susceptibility to the β-lactam antibiotics including penicillin. Until now, detection of *mecA* or PBP was considered an indicator of methicillin resistance and confirmation of MRSA. Recently, new strains of MRSA resistant to methicillin but negative for *mecA* and PBP have been recognized.

Materials and Methods: This study examined the prevalence of *mecA* and *mec* variants in a collection of MRSA and non MRSA from swine, pork meat, other production animals, meat and humans for detection of resistance genes associated with methicillin resistance. Isolates examined were identified as methicillin resistant on media containing oxacillin. A total of 717 *S. aureus* and 188 non-*S. aureus* strains from production swine, other production animals, meat and human strains (n=150) were examined for *mec* genes including *mecA*, $mecA_{LGA251}$, *mecA1* and *mecC1* using PCR. Swine isolates were also subtyped for *SCCmec* using a multiplex PCR.

Results: Overall most isolates identified as methicillin resistant were positive for the *mecA* gene (n = 521) however, a small collection (n=35) were identified as $mecA_{LGA251}$ positive, but they could not be confirmed as *mecC* using standard primers. Further typing using *mecA1* and *mecC1* primers detected the *mecA1* variant in nine isolates of both *S. aureus* and non-*aureus* species and one isolate from ovine was positive for the *mecC1* variant. Subtyping *mecA* positive isolates of swine (n = 519) using the *SCCmec* typing scheme found the most common subtypes detected included II, IV and VI. A significant number of isolates however could not be assigned to a subtype using the current scheme and will require further analysis to determine if there are more emerging subtypes not currently identified.

Conclusion: While the emerging *mecC* variant has not been detected in MRSA and non-MRSA of swine to date, our data suggests that there are other emerging novel variants of *mecA* and *mecA1* that would appear to be occurring. These novel variants may also be a source of, or recipient of resistance and could potentially pose a threat to animal or human health.

Introduction

Staphylococcus aureus is an important cause of a wide variety of diseases in humans worldwide including food poisoning, pneumonia, wound and nosocomial infections. Methicillin-resistant *S. aureus* (MRSA) is an increasing cause of health care-associated (HA-MRSA), community-associated (CA-MRSA) and livestock associated (LA-MRSA) infections worldwide. Methicillin resistance is encoded by the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) and encodes an altered penicillin binding protein PBP resulting in reduced susceptibility to β-lactam antimicrobials including penicillin. Until now, detection of *mecA* or PBP was considered an indicator of methicillin resistance and confirmation of MRSA. Recently, recognition of strains that were still resistant to methicillin but negative for *mecA* and PBP have emerged. These have been identified as *mecC* (formerly $mecA_{LGA251}$) and new variants of *mecA* including *mecA1*; *mecC* variant *mecC1* and *mecC sciuri*.

Subtyping of *mecA* resistance in MRSA is also important in identifying new clones of MRSA and determining if new variants are emerging or if specific clones are becoming dominant in various environments. Sub-types of *SCCmec* have been identified on the basis of variations in the J regions within the *SCCmec*; these currently include types I through VI.

Most animals can become colonized with *S. aureus*, and during slaughter contamination of carcasses and meat with *S. aureus* and MRSA may occur. MRSA strains have been isolated from several food production animals including pigs, cattle and poultry, and meat, such as pork, chicken, beef, turkey and lamb.. Here we examined the prevalence of methicillin associated resistance in isolates of MRSA and non-MRSA (other *Staphylococcus* species) recovered from slaughter animals, retail and deli meat, human clinical and healthy strains in an effort to assess the prevalence of *mecA* and non-*mecA* associated resistance.

Objectives of the Project

Our approach for this proposal was to ask the following research questions: ***Are MRSA recovered from swine harboring the new emerging methicillin resistance traits?*** Objective 1 described below will answer this question by examining a collection of MRSA previously recovered from production swine during 2013 (McKean et al unpublished data) and isolates that the Logue lab currently has on hand from an earlier research project (Buyukcangaz et al 2013). The collection contains *S. aureus* isolates from production pigs at lairage and the slaughterline, (McKean project 2013), swine and other animal isolates (ruminant) from a previous study assessing MRSA in animals and retail meats (Buyukcangaz et al 2013), human MRSA strains from clinical cases of blood and wound infections (Logue collection) and *Staphylococcus* species (non-aureus) also recovered from an earlier research project (Logue collection). The second question that this project asks is ***What is the role of mec genes in methicillin resistance?*** Objective 2 proposes to answer this question by subtype and comparative gene analysis of methicillin resistance genes in *S. aureus* isolates designated as MRSA and non-*S. aureus* strains from production pigs, and other animals and humans examined in objective 1.

Objective 2 assessed the genetic relatedness and role of *mecA* in methicillin resistance of *S. aureus* and non-*S. aureus* strains. Current evidence reports *mecC* bearing strains although methicillin resistant are not accurately identified as methicillin resistant because they fail detection using the standard *mecA* PCR protocols.

VII. Materials and Methods

A total of 1058 *S. aureus* and non-*S. aureus* species were examined including isolates recovered from nasal swabs of animals at slaughter, (swine, ovine and bovine); isolates recovered from retail raw and deli meats and isolates obtained from humans (clinical and healthy). All isolates included in this study were previously identified as MRSA (clinical) or were chosen as suspect MRSA based on growth from selective enrichment designed to select for MRSA (all animal swabs and meat samples). Isolates were identified as *S. aureus* or other staphylococcal species using the Senstitre GPID panels (Trek). Isolates were struck to TSA from frozen stock and DNA extracted using the boil prep method. DNA was stored frozen at -20C until use.

All isolates were screened for the presence of *mecA,* and $mecA_{LGA251}$ using primers described by Stegger at al. 2012. Isolates determined to be negative for *mecA* using the primers below (Table 1) were tested for the presence of $mecA_{LGA251}$ using the degenerate primer set.

Isolates that failed to amplify for *mecA* or test positive for $mecA_{LGA251}$ were further screened to detect the presence of *mecC* using a combination of the primers described for *mecC* (138bp) and a second set described by Cuny et al 2011 (304bp). Isolates that were

negative for *mecC* but still positive for *mecA*$_{LGA251}$ were also screened for *mecA1* and *mecC1* as described by Holmes et al 2014 (Table 1).

A second study assessed the subtypes of *mecA* in MRSA of swine. Here a series of 519 isolates were subtyped using the primers and protocols described by Milheirico et al (2007).

SCCmec Typing: *Staphylococcal* cassette chromosome *mec* (SCC*mec*) typing was done on isolates that were positive for the *mecA* gene, using PCR primers designed to amplify genes located in the J region of specific cassettes. Amplified products were run on agarose gels and PCR products and sizes were confirmed following staining with ethidium bromide.

Results

We have completed the major work of this proposal and it has led to new avenues of research that have recently been submitted to the national pork board for consideration as a follow on project assessing the metagenomics of the swine host and its environment.

In summary **Objective 1 set out to assess the prevalence of methicillin resistance genes in MRSA and non *S. aureus* of animals and humans.** *S. aureus* isolates from production pigs at lairage and slaughter (n=596) were included with human (n=150) isolates, *S. aureus* from production animals (n = 76), *S. aureus* from meat and deli meat samples (n = 75) and non-*S. aureus* isolates recovered from production animals and humans (n = 188), were examined. All isolates were stored frozen at -85C and recovered from frozen stock for DNA extraction using standard protocols. Isolates will be amplified for the *mecA* and *mecC* genes using PCR analysis with the following primers: *mecA*F TCCAGATTAACAACTTCACCAGG, *mecA*R CCACTTCATATCCTTGTAACG and *mecC*F GAAAAAAAGGCTTAGAACGCCTC, *mecC*R GAAGATCTTTTCCGTTTTCAGC (Stegger et al 2011) and positive products determined using gel electrophoresis.

Isolates that were positive for either *mecA* were subtyped using the *SCCmec* subtyping scheme (see below) to determine which specific *SCCmec* gene cassettes are present and contributing to methicillin resistance in the test strains. Isolates negative for *mecA* were screened for *mecC* using the *mecA*$_{LGA251}$ primer set. Additional primers for the *mecA1* variant and *mecC1* variant recently described were also included in an effort to identify other sources of the genes responsible for methicillin resistance observed (see table 1)

SCCmec sub-typing. *Staphylococcal* cassette chromosome *mec* (SCC*mec*) typing uses PCR amplification of genes located in the J region of specific cassettes (Milheirico et al 2007; Kondo et al 2007) that are designed to detect 6 different cassette types and their variants. Amplified products will be run on agarose gels and PCR products and sizes confirmed; *SCCmec* types were assigned based on positive PCR reactions.

Objective 2: Current evidence reports *mecC* bearing strains although methicillin resistant are not accurately identified as methicillin resistant because they fail detection using the standard *mecA* PCR protocols. This study also found the same thought may account for

emergence of novel *mecA* variants that could not be detected using standard *mecA* primers alone and resistance to methicillin linked with *mecA* variants could be potentially overlooked. 1) Assess the role of *mecA* or *mecC* in methicillin resistance at the genetic level using comparative analysis of the *mec* genes and 2) Examine non-*S. aureus* strains to determine if methicillin resistance carriage (*mecA* or *mecC*) is similar to that of *S. aureus* species.
The non-*S. aureus* strains that produce PCR products for *mecA1* and *mecC1* variants have been identified and will be sequenced. Comparative analysis of these genes sequences with published *mecA* and *mecC* will be used to understand the evolution and role of the *mec* genes in methicillin resistance.

A number of strains showed potential as possessing the novel $mecA_{LGA251}$ variant but were not *mecC* positive when standard primers were used suggesting we have a collection (n=35) of variant *mecA* from animal hosts with 22 of these found in swine. When analyzed for the *mecA1* and *mecC1* variants we could account for about 10 strains being *mecA1* or *mecC1* positive which still leaves us a collection of 25 that need further sequencing to determine the source of mec resistance. In addition a significant number of the isolates that were positive for *mecA* could not be subtyped (n=36) using the standard typing scheme suggesting that there may be additional variants for *mecA* that are not accounted for and will require further subtyping or sequence analysis.

Prevalence of mecA and variants: Table 2 shows the overall prevalence of *mecA* and $mecA_{LGA251}$ variants among the various isolates tested. Of interest, none of the isolates that were found to be positive for $mecA_{LGA251}$ were *mecC* positive on amplification using either the Stegger or Cuny primers suggesting that there are alternative *mecA* variants in this collection.

Also of note, although the degenerate primers provided the best indication of potential *mecC* being present these primers also tended to amplify among isolates that were found to be *mecA* positive suggesting they may overestimate the prevalence of $mecA_{LGA251}$ and additional sequence confirmation is necessary.

Detection of mecA and mecC variants: Additional PCR on a set of isolates (n = 36) that failed to produce *mecC* were examined for the *mecA1* and *mecC1* variants. 9 isolates appeared to be positive for the *mecA1* variant. These were found in swine, ovine and equine isolates of both *S. aureus* and *S. xylosus* types (Table 3 & Fig 1). One isolate was positive for *mecC1* – a *S. aureus* isolated from an ovine and may be one of the first documented *mecC* variants found in the US.

***SCCmec* Typing:** Table 4 shows some of the data of *SCCmec* types detected among MRSA isolates of swine that possessed the *mecA* gene. In general, three types were most common these included types II, IV and VI; and three type I were identified. However, a significant number (n=172) of isolates cannot be assigned at this time requiring additional analysis and suggesting there are additional sub types of mecA not currently accounted for. Further work is ongoing to assess the strains and determine additional *mec* types that may be present in the animal and animal associated isolates.

Other sources of mecA: additional sources of *mecA* from non-*S. aureus* species found in the course of this study are shown in table 5 and will warrant future exploration to determine the nature of the *mecA* type present. Detection in these isolates supports data that other non-*S. aureus* strains can be potential donors or recipients of resistance.

Table 1: mecA and mec variant primers that were used in this study.

Primers	Gene target	Sequence (5′–3′)	Amplicon size (bp)
mecA P4	*mecA*	TCCAGATTACAACTTCACCAGG	162
mecA P7		CCACTTCATATCTTGTAACG	
mecA $_{LGA251}$ FP	*mecA* $_{LGA251}$	TCACCAGGTTCAAC[Y]CAAAA	356
mecA $_{LGA251}$ RP		CCTGAATC[W]GCTAATAATATTTC	
mecA $_{LGA251}$ MultiFP	*mecA* $_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	718
mecA $_{LGA251}$ RP		CCTGAATC[W]GCTAATAATATTTC	
mecA $_{LGA251}$ MultiFP	*mecA* $_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	138
mecA $_{LGA251}$ MultiRP		GAAGATCTTTTCCGTTTTCAGC	
mecA $_{LGA251}$ F	*mecC*	GCTCCTAATGCTAATGCA	304
mecA $_{LGA251}$ R		TAAGCAATAATGACTACC	
mecA1-spec-F	*mecA1*	TTGAAGAAGCAACAACGCAC	300
mecA1-spec-R		GAACCGTAGTCATCTTTCATGTTG	
mecC Uni-F	*mecC/C1*	GGATCTGGTACAGCATTACAACC	400
mecC Uni-R		TGCTTTAAATCRATMTTGCCG	

Table 2 shows the overall prevalence of the *mecA*$_{LGA251}$ variant when compared to strains that tested negative for the *mecA* trait. Of note however, following screening for the *mecA*$_{LGA251}$ variant none of the strains were positive for *mecC* suggesting that there may be some new emerging resistance subtypes of *mecA* and *mecC* that are still unknown, further sequencing of some of these variants may help to answer these questions, and we anticipate some whole gene and genome analysis in the near future. Among the pork isolates 75 isolates were identified as methicillin resistant but negative for the *mecA* gene and when screened for the *mecA*$_{LGA251}$ variant 22 were positive, ***further screening for mecC failed to detect any mecC in swine.*** Our data do however suggest that there are novel methicillin resistance mechanisms that we have not currently identified and will require additional sequencing of the *mec* genes to determine these new types. As an indicator of some new emerging methicillin resistance traits, we did however identify the *mecA1* gene in 4 isolates from swine, 2 were found in *S. aureus* strains and the other two were detected in a stain of *S. intermedius* and another strain that was NT. Of importance is that both *S. aureus* and non-*S. aureus* strains may be potential sources of novel methicillin resistance genes (Table 3)

Table 2: Prevalence of methicillin-associated resistance in isolates of MRSA from humans and animals.

Isolate source		# of samples tested	# of isolates + for *mecA*	-ve for *mecA*	+ve for $mecA_{LGA251}$
Human	Clinical	101	98	3	2
	Healthy	57	29	28	3
Animal	Swine	598	521	77	22
	Bovine	18	3	15	3
	Ovine	47	12	35	4
	Others	11	2	9	1
Meat	Pork	65	23	42	0
	Beef	22	10	12	0
	Chicken	32	11	21	0
	Deli Chicken	7	0	0	0
	Deli Ham	15	4	11	0
	Deli Turkey	14	4	15	0

Table 3: Detection of novel methicillin resistance traits in swine and animal hosts.

Host	Strain type	N =	*mecA1*	*mecC1*
Swine	*aureus*	20	2	
	intermedius	1	1	
	NT	1	1	
Bovine	*aureus*	1	1	
	xylosus	1	1	
Ovine	*aureus*	1		1
	xylosus	2	2	
Equine	*xylosus*	1	1	
Human	*aureus*	4		
	epidermidis	1		

Another aspect of this work focused on the *mecA* resistance gene in swine and identification of the types and sub-types of the gene present among a collection of 519 swine associated MRSA, most were typeable into at least 6 subtypes, an additional 4 isolates could not be typed as they failed to amplify for any *mecA* associated traits – suggesting loss of the resistance trait and a further 36 could not be typed by the typing scheme used suggesting these may be potential novel *mecA* types. This would not be unusual as newer sub types of *mecA* are emerging and being identified regularly worldwide and some of the later typing schemes in association with gene sequence analysis may allow us to identify these at a later date.

Table 4 SCCmecA sub types detected among swine isolates

SCCmec types detected among *mecA* positive isolates from swine	Type
I	77
II	11
III	1
IV	275
V	0
VI	113
NT	36

When we investigated other sources of methicillin resistance to the food chain and pork from non-*S. aureus* strains we found that non *S. aureus* strains can also be a source of resistance. In table 5, some of the non-*S. aureus* types that were most often associated with *mecA* resistance included *S. epidermidis* and *S. haemolyticus*, which could be a potential source of resistance to other members of the *Staphylococcus* species including *S. aureus*. Methicillin resistance is chromosomally located however there is the potential for the gene to move via recombination events or other mobilizing activities. One final aspect of this work that will be completed in the near future is to evaluate the role of oxacillin and cefoxitin as indicators of methicillin resistance. Since most of our isolates were oxacillin resistant and were confirmed as oxacillin resistant we will correlate the resistance and gene presence with cefoxitin as a potential marker using a microdilution or agar dilution assay to correlate with gene presence.

Table 5 Detection of mecA associated resistance in S. aureus and non-S. aureus.

Strain	+ for *mecA*
S. epidermidis	30/45
S. haemolyticus	3/7
S. hyicus	2/15
S. intermedius	3/16
S. lugdunensis	1/1
S. saprophyticus	2/46
S. schleiferi	2/16
S. warneri	2/11
S. xylosus	4/16
S. aureus	661/837

Discussion

This project has failed to identify the presence of novel *mecC* genes in isolates of MRSA and non-MRSA associated with swine which would suggest that this resistance type is not common in swine and pork meat and may be more associated with ruminants such as bovine and ovine where it was originally identified. Variants of *mecA* were however detected in swine MRSA and non MRSA isolates suggesting that there are new resistance types emerging and further analysis over time would help confirm if these are new *mec* types not previously recorded when full genomic sequences are carried out. Also of concern in this study is the potential for non-*S. aureus* strains to be a source of methicillin resistance for other species of the organism including *S. aureus* and the potential for these non *S. aureus* strains to be a source of resistance for the food chain suggesting that monitoring for resistance is warranted.

Summary

- *mecA* subtypes in swine dominated by types I, IV and VI
- Novel *mecA* and variants identified in swine
- Other *non-S. aureus* sources of *mecA* identified

Recent Grants submitted that are associated with this project

- PI - A Metagenomics Based Approach to Define the Resistome of Swine. National Pork Board $60,000
- PI – Potential Role of Lairage in the Distribution of Extended Spectrum Beta Lactam (ESBL) Antimicrobial Resistance into the Swine Slaughter Line. National Pork Board $60,000

Presentations

Logue, C.M., Cavender, T., Nielsen, D.W., Lima Barbieri, N. (2016) Characterizing Methicillin Resistance in Methicillin Resistant *Staphylococcus aureus* of Swine. Abstract Submitted ASM Microbe meeting 01/16.

Logue, C.M., Cavender, T., Romine, M.G., Velasco, V., Lima Barbieri, N. (2015) Characterization of Methicillin Resistance in *S. aureus* and Other Staphylococci Associated with Production Animals, Meat and Humans. Abstract Submitted for the 4th ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public health Implications. Chicago, IL November 2-5, 2015 Accepted 09/15.

McKean, J.D., Frana, T., **Logue, C.M.**, O'Connor, A.M. (2015) Effect of Lairage on Prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in Market Swine. Presentation, SAFE Pork Meeting, Porto, Portugal Sept 7-10, 2015