

Title: Prevalence of zinc resistance encoded by the *czrC* gene in US swine-associated MRSA ST5 strains - **NPB 15-145**

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Industry Summary: Livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) draws concern from the public health community because in some countries these organisms may represent the largest reservoir of MRSA outside hospital settings. The emergence and prevalence of LA-MRSA sequence type (ST) 398 isolates in Europe has been attributed in part to the in feed use of zinc as an antidiarrheal agent. The gene encoding zinc resistance is located on the same segment of DNA that also contains the gene encoding resistance to the antibiotic methicillin. This segment of DNA is referred to as the staphylococcal cassette chromosome *mec* (SCC*mec*) element and it is easily transferred from one *S. aureus* bacterial strain to another. Since the gene encoding zinc resistance and the gene encoding methicillin resistance is collocated on the SCC*mec* element, it has been suggested that the use of in feed zinc as an antimicrobial has the potential to contribute to the emergence and spread of MRSA in swine by increasing the selective pressure to maintain the SCC*mec* element in isolates from pigs. In this study we identified the prevalence of the *czrC* gene and phenotypic zinc resistance in US swine associated LA-MRSA ST5 isolates, MRSA ST5 isolates from humans with no swine contact, and US swine associated LA-MRSA ST398 isolates. Our data demonstrate that the prevalence of zinc resistance in US swine associated LA-MRSA ST5 isolates was significantly lower than the prevalence of zinc resistance in MRSA ST5 isolates from humans with no swine contact, swine associated LA-MRSA ST398 isolates, and previous reports describing zinc resistance in other LA-MRSA ST398 isolates. Collectively our data suggests selection pressure thought to be associated with application of zinc in feed is not playing a role in the prevalence and persistence of LA-MRSA ST5 in the US swine population. Additionally, our data indicate that zinc resistance is more associated with MLST lineage suggesting a potential link between genetic lineage and carriage of resistance markers.

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Scientific Abstract: Zinc resistance in livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) sequence type (ST) 398 is primarily mediated by the *czrC* gene co-located with the *mecA* gene, encoding methicillin resistance, within the type V SCC*mec* element. Because *czrC* and *mecA* are located within the same mobile genetic element, it has been suggested that the use of in feed zinc as an antidiarrheal agent has the potential to contribute to the emergence and spread of MRSA in swine through increased selection pressure to maintain the SCC*mec* element in isolates obtained from pigs. In this study we report the prevalence of the *czrC* gene and phenotypic zinc resistance in US swine associated LA-MRSA ST5 isolates, MRSA ST5 isolates from humans with no swine contact, and US swine associated LA-MRSA ST398 isolates. We demonstrate that the prevalence of zinc resistance in US swine associated LA-MRSA ST5 isolates was significantly lower than the prevalence of zinc resistance in MRSA ST5 isolates from humans with no swine contact, swine associated LA-MRSA ST398 isolates, and previous reports describing zinc resistance in other LA-MRSA ST398 isolates. Collectively our data suggests selection pressure thought to be associated with application of zinc in feed is not playing a role in the prevalence and persistence of LA-MRSA ST5 in the US swine population. Additionally, our data indicate that zinc resistance is more associated with MLST lineage suggesting a potential link between genetic lineage and carriage of resistance markers.

Introduction: *Staphylococcus aureus* commonly colonizes the skin and mucosal surfaces of mammalian and avian species and is present in the anterior nares of 20-30% of healthy humans (1). *S. aureus* is also a major opportunistic human pathogen with diverse clinical manifestations ranging from mild skin and soft tissue infections to severe systemic infections and fatal sepsis. Prior to the availability of antibiotics, fatality rates for human cases of *S. aureus* bacteremia were estimated at 80% (2). Increased access to antibiotics has reduced the case fatality rate of *S. aureus* bacteremia to around 20-30% (2), but the capacity of *S. aureus* to acquire resistance to antibiotics has made multi-drug resistant strains a major public health concern (3).

Methicillin resistant *S. aureus* (MRSA) was first reported in 1961 (4) and rapidly became endemic in hospitals (hospital associated MRSA or HA-MRSA) in many countries. During the 1990s, an increasing number of MRSA infections were occurring in persons with no known risk factors for HA-MRSA infection (5). These infections developed in healthy members of the general community and were termed community associated MRSA or CA-MRSA. *S. aureus* is considered a clonal organism, and genotypes associated with hospital infections typically differed from those associated with community infections as well as varying geographically (6).

Although MRSA was first reported in food animals (dairy cattle) in 1972 (7), animal reservoirs were not considered to play a significant role in MRSA epidemiology until 2004, when an atypical MRSA variant was detected in three people in the Netherlands and attributed to their residence on a swine farm (8). These initial isolates were unable to be typed by pulse field gel electrophoresis using the *SmaI* restriction digestion due to a variation in methylation by the type I restriction modification system. Multilocus sequence typing (MLST) revealed these isolates belonged to a novel sequence type (ST) 398 (9). This genotype was found to be widespread in the Dutch pig industry and present in other animal species including cattle, poultry, and horses (1, 10, 11). Subsequent research has revealed a more complex epidemiology, and the predominant genotypes of MRSA found in swine vary geographically. In most Asian countries, ST9 variants are most common (12, 13), while in the USA and Canada both ST398 and ST5 MRSA appear to be relatively common with ST9 MRSA being detected sporadically (14-17).

Resistance to tetracycline antibiotics has been almost universal in *S. aureus* isolates from pigs. Additionally, a prominent feature of LA-MRSA ST398 isolates from Europe and North America is the high prevalence (61-74%) of zinc resistance seen in swine associated isolates (18-20), relative to isolates from veal calves (42%) or humans (48%) (19, 21). Zinc resistance in these MRSA isolates has been attributed to the co-localization of the *czrC* gene, encoding zinc and cadmium resistance, on the type V SCC*mec* element, which

contains the *mecA* gene, conferring methicillin resistance. A strong correlation between phenotypic zinc resistance and the presence of *czrC* has previously been reported, with 99% of zinc resistant MRSA ST398 carrying the *czrC* gene (21). Zinc supplementation over 2400 ppm (versus the minimum nutritional requirement of 100-165 ppm) is commonly fed for 5-10 days to weaned pigs to control post-weaning diarrhea. Since *czrC* and *mecA* are co-located on the SCC*mec* element, it has been suggested that the use of high concentrations of zinc in feed may have contributed to the emergence and spread of MRSA in swine by increasing selection pressure to maintain the SCC*mec* element in swine associated LA-MRSA isolates. (20, 22-24).

While many reports have been published detailing the prevalence of zinc resistance in LA-MRSA ST398 and ST9 isolates, little to no information exists regarding the prevalence of zinc resistance in LA-MRSA ST5 isolates (21). Here we report the prevalence of zinc resistance in US swine associated LA-MRSA ST5 isolates and compare it with the prevalence exhibited by MRSA ST5 isolates obtained from humans with no swine contact, US swine associated LA-MRSA ST398 isolates, and previous studies reporting zinc resistance in LA-MRS ST398 isolates.

Objectives:

The objective of this study was to measure the prevalence of the *czrC* gene and phenotypic zinc resistance in US swine associated LA-MRSA ST5 isolates, MRSA ST5 isolates from humans with no swine contact, and US swine associated LA-MRSA ST398 isolates.

Materials and Methods:

Isolate Acquisition

Swine associated LA-MRSA ST5 cultures were isolated from swine (n = 38), the environment within swine facilities (n = 26), and persons with short-term (n = 9) and long-term (n = 9) swine contact. These isolates were provided by Iowa State University and the University of Minnesota (14). Clinical isolates from humans with no swine contact were obtained from the University of California Irvine (n = 64) (25) and the University of California San Francisco (n = 9). Swine associated LA-MRSA ST398 cultures obtained from Iowa State University were isolated from swine (n = 8) or the environment within swine facilities (n = 6) (14). Isolates were MLST and spa typed prior to acquisition.

Zinc Susceptibility Testing

Minimum inhibitory concentration (MIC) to zinc chloride was determined by agar dilution as described by Aarestrup and Hasman (26). Briefly, plates of Mueller Hinton agar with an adjusted pH of 5.5 were supplemented with zinc chloride in two-fold dilutions with concentrations ranging from 0.25-16 mM. The isolate *Salmonella enterica* subspecies enterica serovar Typhimurium ATCC 14028 was used as a positive control and *S. aureus* ATCC 29213 and ATCC 43300 were used as negative controls. An MIC \geq 4 mM was used as the cutoff to designate resistance in accordance with the result of the positive control.

***czrC* PCR Testing**

Presence of the *czrC* gene was determined by PCR using previously reported primers and protocol (19). Briefly, PCR was carried out in a MJ Research PCT-200 DNA Engine thermocycler (GMI, Ramsey, MN) using 50 ng of purified genomic DNA from the appropriate strains and forward primer 5'-TAGCCACGATCATAGTCATG-3' and reverse primer 5'-ATCCTTGTTTTCCCTTAGTGACTT-3'. Reaction mixtures included 0.4 μ M primers, 1 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μ l of 10 \times buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM MgCl₂, and 200 μ M deoxynucleoside triphosphates (dNTPs) in a final volume of 50 μ l. Cycling conditions were 2 min at 95°C, 30 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 1.5 min, and a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light.

SCC*mec* Typing

SCC*mec* typing was completed using previously designed primer sets (Table 1) (27-32). Briefly, PCR was carried out in a MJ Research PCT-200 DNA Engine thermocycler (GMI, Ramsey, MN) using 50 ng purified genomic DNA from the appropriate strains and reaction mixtures included 0.4 μ M primers, 1 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μ l of 10 \times buffer II (100 mM Tris-HCl, pH 8.3, 500 mM

KCl), 2.5 mM MgCl₂, and 200 μM deoxynucleoside triphosphates (dNTPs) in a final volume of 50 μl. PCR for the *ccrA* and *ccrB* genes was a multiplex reaction with cycling conditions of 2 min at 95°C, 10 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 1.5 min, followed by 25 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension step of 72°C for 7 min. PCR of the *ccrC* gene used cycling conditions of 2 min at 95°C, 30 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 2 min, and a final extension step of 72°C for 7 min. PCR of the *mec* element genes was completed with cycling conditions of 2 min at 95°C, 30 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 2 min, and a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light.

Statistical Analysis

Comparisons between isolates from humans with no swine contact and swine associated isolates were completed using Fisher's exact test using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results:

Prevalence of the *czrC* gene

czrC-specific PCR demonstrated that none of the tested swine associated MRSA ST5 isolates (0/82) harbored the *czrC* gene (Table 2). In contrast, all LA-MRSA ST398 isolates (14/14) tested harbored the *czrC* gene. The prevalence of *czrC* in LA-MRSA ST5 isolates associated with swine was significantly lower than swine associated LA-MRSA ST398 isolates ($p < 0.0001$) (Table 2), and also lower than that reported for other LA-MRSA ST398 isolates ($p < 0.0001$) (21). Over one-fifth (16/73, 22%) of MRSA ST5 isolates obtained from humans with no swine contact contained the *czrC* gene (Table 2). The prevalence of the *czrC* gene among MRSA ST5 isolates obtained from humans with no swine contact was significantly higher than that among swine associated ST5 isolates ($p < 0.0001$) (Table 2).

Zinc Chloride Susceptibility Testing

Susceptibility testing revealed no (0/82) swine associated MRSA ST5 isolates were resistant to zinc chloride, while phenotypic resistance was seen in all (14/14) LA-MRSA ST398 isolates. Prevalence of phenotypic resistance to zinc among MRSA ST5 isolates obtained from humans with no swine contact was intermediate (25%, 18/73), but more prevalent than among swine associated MRSA ST5 isolates ($p < 0.0001$) (Table 2). Two MRSA ST5 isolates obtained from humans with no swine contact exhibited phenotypic resistance despite not harboring the *czrC* gene. Phenotypic zinc chloride resistance in the absence of *czrC* has been previously reported for MRSA ST398 and non-ST398 isolates by Cavaco et al. and indicates an alternative mechanism for zinc resistance is present in MRSA ST5 isolates, as well (21).

SCC*mec* Typing

The swine associated LA-MRSA ST5 isolates carried SCC*mec* type III (17/82, 21%), type IV (42/82, 51%), or were unable to be typed using the primer sets previously published (23/82, 28%) (Table 3). Of the 23 untypable isolates, 20 (24.4% of LA-MRSA ST5 isolates) carried a Class D *mec* gene complex, which has not been assigned to a *mec* type, and 3 of the isolates carried a Class A *mec* gene complex without the traditional *ccrAB* gene combination. All LA-MRSA ST398 isolates tested harbored SCC*mec* type V (14/14). The MRSA ST5 isolates from humans with no known swine contact carried SCC*mec* type II element (69/73, 95%). A small portion were type IV (4/73, 5.5%).

Discussion:

The recent emergence of MRSA in livestock worldwide has become a focal point in discussions regarding the role of antibiotic use in food animal production and the development of antibiotic resistant clinical infections in humans. However, the mechanisms and factors responsible for this emergence, as well as the factors contributing to the geographical variation in genotypes of swine associated MRSA found globally are poorly understood. Although some causal role of antibiotic use in the emergence of LA-MRSA is hypothesized and may seem obvious, epidemiological evidence of such relationships has not been readily demonstrated (33). It is clear that other factors, including disinfectants and metals, may play selective roles in the emergence of particular MRSA clones in humans and animals (20, 34, 35).

In this study, zinc resistance mediated by the *czrC* gene was examined as a potential contributor to the prevalence of LA-MRSA ST5 on swine farms in the USA. A documented association exists between the presence of *czrC* and the *mecA* gene in LA-MRSA ST398 isolates obtained from swine farms, with swine associated isolates having a higher prevalence of *czrC* than LA-MRSA ST398 isolates obtained from veal calves or humans (19, 21). The high correlation (99%) reported between isolates harboring the *czrC* gene and phenotypic zinc resistance in LA-MRSA ST398 indicates this gene orchestrates the predominant mechanism mediating zinc resistance in these isolates (21). The specific importance of the *czrC* gene and the physical link between *mecA* and *czrC* within the SCC*mec* element provides a mechanism by which dietary supplementation of zinc in swine rations could contribute to the persistence of methicillin resistance through co-selection (19, 20, 22, 23). Evidence of the practical relevance of this mechanism comes from Denmark, where widespread use of zinc in weaned pig diets as an alternative to antibiotic therapy for controlling enteric disease followed the banning of antibiotics for growth promotion in 2000, approximately a decade before LA-MRSA ST398 became highly prevalent in these pigs (22).

Sequencing studies have demonstrated the *czrC* gene is located within the type V and type VIII SCC*mec* elements (19, 36). The majority of European LA-MRSA ST398 isolates investigated have carried the type V SCC*mec* element containing the *czrC* gene, but none of the LA-MRSA ST5 isolates we examined carried the SCC*mec* type V element. All of the MRSA ST5 isolates from humans with no swine contact carrying the *czrC* gene contained the SCC*mec* type II (Table 3), which has not previously been reported to carry the *czrC* gene. In these isolates, the *czrC* gene is located within the SCC*mec* element similar to the type V elements previously reported in LA-MRSA ST398 isolates. The fact that none of the swine associated LA-MRSA ST5 isolates harbored the SCC*mec* types seen in ST5 MRSA isolates from clinical infections is further evidence that the animal and human reservoirs of ST5 MRSA appear to be phylogenetically distinct (37).

Previous reports examining *czrC* in LA-MRSA isolates indicate a higher prevalence of this gene in MRSA clonal complex (CC) 398 isolates (72.5%) as compared to all non-CC398 isolates evaluated (25.5%) (21). An absence of *czrC* in LA-MRSA of the CC5 and CC9 lineage in both European and Asian isolates has been reported, which is consistent with our results evaluating LA-MRSA ST5 isolates from the USA. Collectively, our results considered with previously published data indicate the *czrC* gene has a lineage association and is prevalent in the ST398 lineage, while absent from the ST5 and ST9 lineages (21). An alternate explanation for the elevated prevalence of the *czrC* gene in the ST398 lineage is the selection pressure incurred with the use of elevated levels of zinc in feed. However, the prevalence of the *czrC* gene in non-ST398 LA-MRSA isolates from European swine was reported to be 30% of tested isolates, while the phenotypic zinc resistance was reported to be 60% for the same isolates (21), arguing against selection pressure incurred with the use of elevated levels of zinc in feed as the sole factor controlling MRSA prevalence in swine. Although no national data concerning the use of zinc in swine rations exists, the practice is thought to be widespread in the United States (Dr. Mike Tokach, personal communication). This appears not to have played the same role in propagating methicillin resistance in livestock isolates of *S. aureus* in the US, as the majority of herds tested in recent reports are MRSA negative (14, 38).

Our results reported here, combined with previously reported results (21), open new avenues of research to be explored. First, the *czrC* gene has been identified in two methicillin susceptible *S. aureus* ST398 isolates (18). The presence of this gene without the SCC*mec* element should be evaluated to determine if *czrC* is a remnant from a previously methicillin resistant isolate or if the *czrC* gene has been integrated through a different mechanism. There have also been both LA-MRSA isolates (21) and swine associated methicillin susceptible *S. aureus* isolates (Sun et al., in preparation) identified that show phenotypic zinc resistance without carrying the *czrC* gene. Such isolates should be screened for other mechanisms of zinc resistance to determine the impact alternative genes have on conferring a resistant phenotype. Evaluation of the impact of *czrC* in non-ST398 LA-MRSA also bears further investigation, specifically the ability of LA-MRSA ST5 isolates to acquire and harbor *czrC* and the impact of in feed zinc on the capability of LA-MRSA ST398 isolates to outcompete other lineages in swine. Ultimately, zinc resistance in LA-MRSA is more complex than the presence or absence of *czrC* or the use of zinc in feed as an antimicrobial agent to combat disease in livestock. Further investigation

is needed to determine the mechanisms leading to zinc resistance as well as illuminating the impact of selective pressure on the emergence of particular MRSA clones in humans and animals.

Overall, the data reported here indicate that zinc co-selection associated with in feed zinc supplementation has not contributed to the persistence or prevalence of LA-MRSA ST5 in the US swine population. This conclusion is contrary to theories surrounding the dissemination of LA-MRSA ST398 in Europe and, considering the presence of *czrC* in LA-MRSA ST398 isolates in the US, also show a potential link between genetic lineage and carriage of resistance markers, as seen with *qacA* resistance in CC22 in the hospital setting (35). Further, the data reported here indicate that multiple mechanisms contribute to fitness and the ability of LA-MRSA ST5 and other lineages to compete and persist in the nasal microbiota of pigs.

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Table 1: Primer sets used to SCC*mec* type isolates.

	Nucleotide Sequence	Expected Product (Forward Primer)	Source
ccrB	ATTGCCTTGATAATAGCCITCT		Ito et al. 2001 (15)
ccrA1	AACCTATATCATCAATCAGTACGT	694 (ccrB)	Ito et al. 2001 (15)
ccrA2	TAAAGGCATCAATGCACAAACACT	937 (ccrB)	Ito et al. 2001 (15)
ccrA3	AGTCAAAAGCAAGCAATAGAAT	1791 (ccrB)	Ito et al. 2001 (15)
ccrA4	GTATCAATGCACCAGAACTT	1287 (ccrB)	Kondo et al. 2007 (16)
ccrC-F	CGTCTATTACAAGATGTTAAGGATAAT		Kondo et al. 2007 (16)
ccrC-R	CCTTTATAGACTGGATTATTCAAAATAT	518 (ccrC-F)	Kondo et al. 2007 (16)
mecI-F	CAAGTGAATTGAAACCGCCT		Okuma et al. 2002 (17)
mecI-R	CAAAAGGACTGGACTGGAGTCCAAA	187 (mecI-F)	Okuma et al. 2002 (17)
mecR1-R	GTCTCCACGTTAATTCCATT	1920 (mecI-F)	Kobayashi et al. 1996 (18)
Class B	TATACCAAACCCGACAAC		Katayama et al. 2001 (19)
IS1272	AACGCCACTCATAACATATGGAA	1996 (ClassB)	Okuma et al. 2002 (17)
Class C	AACGTTGTAACCACCCAAGA		Hiramatsu et al. 1992 (20)
IS431	TGAGGTTATTCAGATATTTTCGATGT	2072 (Class C)	Katayama et al. 2001 (19)

Table 2: The prevalence of phenotypic zinc chloride resistance and *czrC* presence in isolates from human with no swine contact and swine-associated isolates.

	MRSA ST5 from Humans with no Swine Contact	LA-MRSA ST5	LA-MRSA ST398
<i>czrC</i> PCR Prevalence	16/73 (21.9%)	0/82 (0%)	14/14 (100%)
Phenotypic Zinc Chloride Resistance	18/73 (24.7%)	0/82 (0%)	14/14 (100%)

Table 3: SCC*mec* type and *czrC* gene

prevalence in swine associated LA-MRSA ST5 and ST398 and MRSA ST5 isolates from humans with no swine contact.

	SCC <i>mec</i> Type	<i>czrC</i> prevalence
LA-MRSA ST5	III	0/17
	IV	0/42
	Untypable ^a	0/23
MRSA ST5 from Humans with no Swine Contact	II	16/69
	IV	0/4
LA-MRSA ST398	V	14/14

^a – denotes those isolates that are unable to be classified into a SCC*mec* type due to the presence of *ccr* gene or *mec* complex unable to be determined using available primer sets or the presence of a *ccr* and *mec* complex combination not currently assigned a SCC*mec* type.