RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Alternatives to antibiotics? Effects on MRSA prevalence in swine production systems – **NPB #13-021** **revised**

Investigator: Dr. H. Morgan Scott

Institution: Kansas State University

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Industry Summary:

Antimicrobial resistant bacteria in animal products are of major public health concern. The concerns about prudent use of antimicrobials have largely focused on feed grade antibiotic products used both for growth promotion and for routine prevention. Resistance to antibiotics among the normal swine commensal bacterium, *Staphylococcus aureus*, is of public health significance because of its zoonotic potential as an opportunistic pathogen of humans. Methicillin-resistant *Staphylococcus aureus* (MRSA) is widely recognized as hospital acquired, community associated zoonotic pathogen, which is of major concern in both humans and animals. The majority of nosocomial infections in intensive settings occur largely due to hospital acquired and community associated MRSA strains; very little evidence exists of common livestock-associated strains causing clinical illness in humans. Studies from the European Union have hypothesized that the emergence and dissemination of MRSA among swine production system is influenced by the use of in-feed antimicrobials, particularly tetracycline. The primary objective of our research was to evaluate the potential association of high levels of in-feed zinc supplementation (as an alternative to tetracycline), versus in-feed subtherapeutic or therapeutic levels of chlortetracycline (CTC), on the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in post-weaned pigs.

A field experiment was conducted using weaned piglets, which were randomized to pens by weight and allowed to acclimatize to their surroundings and diet. All piglets received normal dietary rations as per NRC recommendations. The pens were assigned randomly to six treatment groups (incomplete factorial design with low CTC, high CTC, high Zn being the factors; low and high CTC could not be interacted per FDA regulations). Swab samples (nasal, skin, and tonsils) were collected on Days 0, 21 and 42. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were recovered after streaking swabs onto a selective medium, CHROM™ MRSA agar

(Hardy Diagnostics) at 37°C for 24 h. Bacterial isolation was performed by picking three distinct colonies from each plate and re-streaking onto blood agar plates. We isolated 720 bacterial colonies for each sampling site. All isolates were subjected to species confirmation by PCR followed by detection of *mecA* (methicillin resistance) and *czrC* (zinc resistance) genes. All MRSA isolates were also subjected for *spa* typing, sequencing the *spa* gene repeat region, to further study the molecular epidemiology (this added step resulted in a request for a one-year no-cost extension to the original grant).

Overall, the prevalence of *mecA*-positive MRSA was 42.8% (308/720), 37.2% (268/720), and 42.6% (307/720) among nasal, skin, and tonsillar samples, respectively. The prevalence of *mecA*- and *czrC*-positive MRSA was affected by Zn ($P < 0.05$) but not CTC supplementation ($P > 0.05$). The prevalence of *czrC*-positive MRSA was 20% (144/720), 21.1% (152/720), and 14.3% (103/720) in nasal, skin, and tonsillar swabs, respectively. The occurrence of the *czrC* gene was strongly associated with *mecA*-positive MRSA isolates ($P < 0.0001$). The median minimum inhibitory concentrations (MICs) of Zn for zinc resistant and susceptible isolates were 8 and 4 mM, respectively ($P < 0.001$). We conducted *spa* gene repeat sequence analysis on all MRSA isolates originating from the three anatomic pig collection sites. The preliminary results from several isolates are presented here as the sequence analysis is still in progress (July 2015). Among the nasal isolates (n=121), the majority of them belong to t034 (50; 41.3%), unknown *spa* type (42; 34.7%), and t007 (29; 23.9%) *spa* types. In case of tonsillar MRSA isolates (n=109), the major *spa* types were t034 (41; 37.6%), t007 (37; 33.9%), and 28.4% (31) of unknown *spa* types. In the case of skin isolates (n=105), the major *spa* types were t034 (89; 84.7%), t127 (8; 7.6%), t007 (7; 6.6%), with only a single isolate not belonging to any known *spa* types.

There was a clear epidemic spread in the first 21 days of the feeding trial which provided most of the increase in the prevalence of MRSA in nasal, tonsillar, and skin regions of piglets and across all treatment groups. This suggests that either: 1) the MRSA strains were endemic in the barn environments before pigs were introduced, or 2) the MRSA strains were present in a very few piglets upon arrival and the stress of transport, mixing, and new environs triggered epidemic spread of the organism. Since our study was longitudinal in nature with repeated sampling it helped us to identify infectious disease dynamics and a clearly discernable epidemic spread of MRSA. In contrast, most previous studies of MRSA in swine in North America have been cross-sectional in design; or, with spacing of visits and animal sampling such that infectious disease dynamics could not be observed.

The genetic link between *czrC* and *mecA* genes points to the potential importance of elevated Zn supplementation in co-selection and propagation of antibiotic resistance; most notably, under non-epidemic situations. Importantly, Zn is a required micro-nutrient in swine diets and so a truly negative Zn diet is impractical, at best, and obviously dangerous to swine health and well-being at worst. As well, it is very unlikely that Zn supplemented at baseline nutrient requirement levels will select for those strains of *Staphylococcus aureus* harboring the czr gene. Based on our *spa* typing results, *spa* type t034 is the most predominant from all three collection sites. This *spa* type belongs to the clonal complex398 (CC398) and these are the most predominant livestock associated-MRSA clonal lineage, primarily in pigs, that have been reported from many countries, including the United States. The

reason for the emergence of CC398 in pigs is not well understood and might involve a complex interplay between management and dietary factors. Both t007 and t127 also form a part of LA-MRSA complex and also have been reported from many countries including the United States. Although these clones or sequence types are reported from pigs, farmers, and veterinarians there are rarely cases of human infection (as opposed to colonization) reported. Importantly, if this situation changes and these strains become associated with human illness, they may pose a serious therapeutic challenge in the case of invasive infections since most of these isolates possess several virulence factors and toxins beside antimicrobial resistance determinants.

The data generated from our study will help to clarify the link between zinc and chlortetracycline use in swine production to other antibiotic resistance in a major pathogen of public health significance. It is imperative that the speculated associations between the use of these antibiotic and non-antibiotic antimicrobials and MRSA be examined in realistic field settings so as to more quantitatively evaluate the potential risks to public health. These data are essential for conducting meaningful quantitative risks assessments (QRA) and to draw effective intervention strategies with the help of more epidemiological surveillance. More importantly, our research findings will help swine producers to make informed decisions in optimizing existing management practices (such as mineral supplementation and the use of feed grade antimicrobials) in order to best manage public health risks in their settings.

For more information please contact:

H. Morgan Scott DVM, PhD
Department of Veterinary Pathobiology
Mail Stop 4467
Texas A&M University
College Station, TX, USA 77843-4467
Email: hmscott@cvm.tamu.edu

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Scientific Abstract:

Zinc (Zn) is sometimes supplemented at elevated concentrations (2,000-3,000 ppm) in swine diets, ostensibly to prevent enteric infections and promote growth. Studies from Denmark have suggested a genetic linkage and a phenotypic association among Zn resistance, encoded by the *czrC* gene, methicillin-resistance, encoded by the *mecA* gene, and tetracycline resistance (encoded by the *tetM* gene in *S. aureus*. We have previously shown that nasal carriage of *mecA*-positive MRSA exhibits a dose-response to zinc supplementation in pigs. A longitudinal study was performed to evaluate the association of in-feed Zn and chlortetracycline (CTC) supplementation (at two levels) with the prevalence of MRSA in pigs. The study consisted of 240 weaned piglets, housed in 48 pens (5 piglets/pen), randomly assigned to six treatments in a 2×3 complete factorial design. Treatment factors included diets with normal (30 mg/kg) or high (2,500 mg/kg of feed) concentrations of Zn, with or without CTC at low (5 mg/kg) or high (22 mg/kg BW) levels, and the interaction terms for Zn with low or high CTC. Nasal, skin, and tonsillar swabs were collected from all piglets on Days 0, 21, and 42.

The samples were inoculated onto MRSA CHROMagar and presumptive MRSA colonies were confirmed by genus (*Staph*) and species (*nuc*) specific PCR. The presumptive MRSA isolates were tested for *mecA* and *czrC* by PCR. Zinc susceptibility was determined by the agar gel dilution method. Statistical analyses were carried out using STATA (v.12.1). Overall treatments and days, the prevalence of *mecA*-positive MRSA was 42.8% (308/720), 37.2% (268/720), and 42.6% (307/720) in nasal, skin, and tonsillar samples, respectively. The prevalence of *czrC*-positive MRSA was 20% (144/720), 21.1% (152/720), and 14.3% (103/720) in nasal, skin, and tonsillar swabs, respectively. The Zn, sampling day (period) and treatment interactions had a significant effect on the prevalence of *mecA* and *czrC*-positive MRSA in all three collection sites ($P < 0.001$). The occurrence of *czrC* gene was strongly associated with *mecA*-positive MRSA isolates ($P < 0.0001$). The median MICs of Zn for zinc resistant and susceptible isolates were 8 and 4 mM, respectively ($P < 0.001$). The prevalence of *mecA*- and *czrC*-positive MRSA was affected by Zn ($P < 0.05$) but not CTC supplementation ($P > 0.05$). The observed association between *czrC* and *mecA* genes points to the importance of elevated Zn supplementation in selection and propagation of antibiotic resistant bacteria such as MRSA in swine production.

Introduction:

A number of heavy metals are used in trace amounts in food animal production to maintain the normal physiology and the healthy status of animals. Two of these elements, copper (Cu) and zinc (Zn), are supplemented in animal feeds at levels higher than their physiological requirement (Hasman et al., 2006). Cu and Zn are more often employed during the first week post-weaning in piglets to control diarrhea and to improve the performance of piglets. Resistance to antimicrobials, including heavy metals, is important for bacterial survival in competitive environments such as the gut lumen. Often, heavy metal and antibiotic resistance genes are co-located on the same mobile genetic elements such as plasmids, transposons, and integrons (Aminov and Mackie, 2007). Therefore, there is a distinct possibility that natural selection pressures imposed by heavy metals may indirectly select for resistance to antimicrobials. Our previous studies, including those funded through the Pork Checkoff, have shown that copper supplementation in weaner diets confers resistance to copper in *Enterococcus faecium* and *E. faecalis* isolates and co-selects for resistance to macrolides and tetracyclines (Amachawadi et al., 2010 & 2011) but not to vancomycin (in the U.S.).

Zinc (Zn) is a micronutrient essential for growth, development, and differentiation of all living organisms. Zn has numerous functions in biological systems, including both structural and catalytic roles in a number of enzymes (O'Halloran, 1993). It has been shown that supplementing pharmacological levels of Zn (2,000-3,000 ppm) can also stimulate growth in young pigs. However, Zn in excess concentration is highly reactive and toxic to cells because of its ability to generate intracellular superoxide or reactive oxygen radicals. Homeostasis is necessary to maintain intracellular concentrations of Zn and to avoid either its deprivation or toxicity. Responses by bacteria to Zn and antibiotics seem to be additive. Acquired resistance to Zn is common, and this has been reported both in Gram-negative and Gram-positive bacteria. The Zn resistance mechanism has been very well documented in *Ralstonia eutrophus*, a Gram-negative soil bacterium, in which the resistance is mediated by the *czc* system that confers resistance to cadmium (Cd), Zn, and cobalt by effluxing

cations from the cells (Nies, et al., 1999). A gene cluster, *czrCBA*, cation-antiporter efflux system is involved in Zn and Cd resistance in *Pseudomonas aeruginosa*. Resistance to heavy metals, arsenic, mercury, and cadmium also has been described among several *Staphylococcus aureus* isolates (Aarestrup and Hasman, 2004).

It has been hypothesized that the emergence and dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) among pigs is impacted by the use of feed grade antimicrobials, including tetracycline (Tet) and Zn, in the diet (de Neeling et al., 2007; Price et al., 2012). MRSA has emerged as an important opportunistic zoonotic pathogen in food animal production around the world (Kluytmans, 2010; Cavaco et al., 2011). Previous studies from Denmark have demonstrated a genetic linkage and a strong phenotypic association between Zn and methicillin among *S. aureus* CC398 isolates of pigs (Aarestrup et al., 2010). Isolates belonging to the clonal complex398 (CC398) are the most predominant LA-MRSA clonal lineage, primarily in pigs, and have been reported from many countries (Leedom Larson et al., 2011). This clonal lineage is of public health significance and constitutes a large reservoir for transmission to humans working on the swine farm or working/living with those who have direct contact with animals (Aarestrup et al., 2010; Leedom Larson et al., 2012). The emergence and success of MRSA CC398 in animal reservoirs is attributed to its ability to persist in the farm environment, propensity for dissemination, and resistance to routinely used antimicrobials. The presence of the *mecA* gene defines MRSA, and this gene is found on a large mobile genetic element called the Staphylococcal Chromosomal Casette mec (SCCmec). Another gene, *czrC*, has been found to confer resistance both to Zn and Cd in *S. aureus* and it is genetically located within the type V SCC*mec* elements, which are prevalent among MRSA CC398 isolates (Cavaco et al., 2010). However, *in vivo* studies relating the use of Tet and Zn, alone or in combination, on the prevalence of MRSA in U.S. swine production are completely lacking. One study in Denmark looked at injectable Tet and Zn supplementation on MRSA prevalence (Moodley et al., 2011); however, no studies have examined feed grade Tet and Zn in North America. Therefore, there is a pressing need to better understand the complexities involving emergence, dissemination, propagation, and maintenance of MRSA in *S. aureus* of pigs and their relation to approved antibiotic formulations at different doses and high-level supplementation with Zn oxide.

Objectives:

The primary objective of the proposed research was to evaluate the potential association of high levels of in-feed zinc supplementation, and in-feed subtherapeutic or therapeutic levels of chlortetracycline (CTC), on the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in pigs. Specifically, we proposed to achieve the following objectives:

1. Investigate the effects of supplementing swine diets with high levels of zinc, alone and in combination with high or low doses of chlortetracycline, on the prevalence of MRSA, of zinc resistance (*czrC* gene), and of several antibiotic resistances among *Staphylococcus aureus* isolates in pigs,
2. Determine the phenotypic susceptibilities of MRSA isolates to zinc and chlortetracycline and their genotypic properties and quantify these resistance traits among all *Staphylococcus aureus* in the samples,

3. Genetically characterize the MRSA isolates via *spa* typing (a protein A gene variable repeat region), to assess their relationship to global MRSA epidemiology, and
4. Investigate the molecular ecology, epidemiology, and public health significance of high-level zinc supplementation versus in-feed antibiotics and their potential for co-selection among populations of MRSA.

Materials & Methods:

Animal Trial and Experiments

The animal trial was conducted from 10/28/2013 to 12/10/2013 at the segregated early weaning (SEW) facility of Kansas State University. The SEW facility has two metal buildings (south and north barns) with 40 pens each. We received weaned piglets on 10/21/2013 and randomized them by weight and allowed them to acclimatize to their surroundings and diet. All piglets received normal diet as per NRC recommendations. Following the brief period of acclimatization, we started the pigs on their medicated treatment diets (per random allocation). The piglets were assigned randomly to six treatment groups by dividing each barn into 4 different quadrants; with main effects of added zinc oxide (0 vs. 2,500 ppm of Zn) and chlortetracycline (0, 5, 22 mg/kg body weight). In total, we used 8 quadrants or replicates of treatment distributed equally among the barns. The swab samples (nasal, skin, and tonsils) were collected on days 0, 21 and 42. Piglets were removed from the high CTC test diets for one day in the middle of the d 0 to 21 test period to meet the FDA approved label guidelines of feeding CTC for less than 14 d continuously. This allowed us to balance on periods (pre-treatment, treatment, and post-treatment) and also keep us on label for chlortetracycline high dosage, and to balance time on treatments across groups.

Processing of swabs and bacterial isolation

The collected swab samples were brought on ice to the laboratory for processing. Direct plating of swab samples was performed by streaking onto CHROM™ MRSA agar plates (Hardy Diagnostics) for selective isolation and identification of MRSA and incubating the plates at 37°C for 24 h. The bacterial isolation was done by picking three distinct colonies from each plate (for both the bacteria) and re-streaking them onto blood agar plates. We isolated 720 bacterial colonies for each sampling site. After isolation and preliminary identification, we preserved these bacterial isolates on cryogenic beads at -80°C for further use.

Identification of MRSA

The genus (*staph* for genus-specific 16S rRNA) and species (*nuc*) confirmation of MRSA were carried out by PCR as per the procedure described by Zhang *et al.* (2004). *S. aureus* strain 9B (Dr. Henrik Hasman, National Food Institute, Technical University of Denmark) served as positive control for *staph* and *nuc* genes. The amplified PCR products were 756 bp and 279 bp for *staph* and *nuc* genes, respectively.

PCR detection of *mecA* and *czrC* genes

DNA from the MRSA isolates were extracted by boiling a suspension of the bacterial colonies in sterile nuclease free water for 10 min. The suspension was centrifuged at 20,000 g for 1 min and the supernatant was treated with lysostaphin (Sigma-Aldrich, Saint Louis, MO) at 1µg/ml for 1 h at 37°C. DNA from each of the isolates was tested for methicillin and zinc resistance determinants, *mecA* and *czrC* genes, respectively. The primers, PCR conditions, and electrophoresis conditions were carried out as per the procedures described by Amachawadi et al. (2015).

Zinc and tetracycline susceptibility determinations

Zinc susceptibilities of *S. aureus* isolates were determined by the agar dilution method (Aarestrup and Hasman, 2004). Mueller Hinton agar plates containing 0, 1, 2, 4, 8, 12, and 16 mM of zinc chloride (Sigma-Aldrich, St. Louis, MO) were used, with pH adjusted to 5.5 using 50% HCl. Bacterial inocula were prepared by growing the isolates in Mueller Hinton II broth for 5-6 h and turbidity adjusted to a McFarland turbidity standard of 0.5. The plates, in duplicates, were spot inoculated with 15-20 µL of bacterial growth and incubated for 48 h at 37°C to determine growth or no growth. The minimum inhibitory concentration (MIC) of tetracycline was determined by microbroth dilution (adapted from CLSI, 2008 with variations described below). Tetracycline susceptibilities were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, and 0.098 µg/ml of by broth microdilution as per Amachawadi et al. (2011).

***spa* typing and sequencing**

All the MRSA confirmed isolates were subjected to molecular typing by *spa* gene PCR and sequence analyses. The primers and PCR conditions for *spa* typing were as described by Shopsin et al. (1999). The PCR products were purified and subjected for sequencing using both forward and reverse *spa* primers at Genewiz (South Plainfield, NJ). The resultant sequences were analyzed using Ridom Staph Type software (Ridom® GmbH, Munster, Germany) to detect the *spa* repeat and assigning them to *spa* types and the sequence types.

Statistical Analysis

Data were analyzed using STATA SE (v. 12). The data were considered to be multilevel and longitudinal in nature since pens were clustered within each treatment, animals were clustered within pens, and within-animal dependency existed because of repeated sampling of the same animals during the study period. The unbalanced factorial design with repeated measures and subsequent mixed model analysis allowed us to compare responses at specific times, over time, and the available interactions thereof. In addition, the main effects and interaction of the treatments were explored using random effects multi-level logistic regression model. Zinc and tetracycline MIC data were analyzed using non-parametric survival analyses. Results were considered significant at $P < 0.05$.

Results:

A total of 720 *Staphylococcus aureus* were tested for the presence of *mecA* gene in each of the three sampling sites; nasal, tonsil, and skin. The overall prevalence of *mecA* was 42.8 % (308/720), 42.6% (307/720), and 37.2% (268/720) among nasal, tonsil and skin *S. aureus* isolates (Table 1). The overall prevalence of *czrC* was 20% (144/720), 14.3% (103/720), and 21.1% (152/720) among nasal, tonsil and skin *S. aureus* isolates (Table 2). The Zn, sampling day (period) and treatment interactions had a significant effect on the prevalence of *mecA* and *czrC*-positive MRSA in all three collection sites ($P < 0.001$). The prevalence of *mecA*- and *czrC*-positive MRSA was affected by Zn ($P < 0.05$) but not CTC supplementation ($P > 0.05$). There was a clear epidemic spread in the first 21 days of our feeding trial that provided most of the observed increase in the prevalence of MRSA in nasal, tonsillar, and skin regions of piglets (Fig. 1). The occurrence of *czrC* gene was strongly associated with *mecA*-positive MRSA isolates ($P < 0.0001$; Table 3). The median MICs of Zn for zinc resistant and susceptible isolates were 8 and 4 mM, respectively ($P < 0.001$; Table 4). Both, the *czrC*-positive and –negative isolates were resistant to tetracycline. Use of non-parametric analyses (i.e., MIC50 and MIC90; see Fig 2, 3 and 4) revealed similar findings for MICs at median and 90th percentile, respectively for isolates from nasal, tonsil and skin. Among the nasal isolates (n = 121), majority of them belong to t034 (50; 41.3%), unknown (42; 34.7%), and t007 (29; 23.9%) *spa* types. In case of tonsil isolates (n=109), major *spa* types were t034 (41; 37.6%), t007 (37; 33.9%), and 28.4% (31) does not belong to any known *spa* types. Whereas, in case of skin isolates (n=105), the major *spa* types were t034 (89; 84.7%), t127 (8; 7.6%), t007 (7; 6.6%), and one isolate did not belong to any known *spa* types. The complete *spa* sequence analyses are in progress and this will allow us to understand the global epidemiology of predominant MRSA sequence types originating from food animal agriculture.

Discussion:

From our study, it is evident that Zn, if fed at an elevated level, is capable of increasing the prevalence of *czrC* and thus *mecA* positive *S. aureus* in post-weaned pigs. Since mecA was the primary gene of importance in conferring methicillin resistance to the bacteria, Zn cannot be ignored as a risk factor for MRSA. There was a clear epidemic spread in the first 21 days of our feeding trial which provided most of the observed increase in the prevalence of MRSA in nasal, tonsillar, and skin regions of piglets. Later, the prevalence declined as the epidemic waned. The genetic link between *czrC* and *mecA* genes suggests the importance of elevated Zn supplementation in co-selection and propagation of antibiotic resistance, more importantly, under non-epidemic situations. Importantly, Zn is a required micro-nutrient in swine diets and so a truly negative Zn diet is impractical, and potentially dangerous to swine health and well-being. A bias towards the null hypothesis of no effect is the most likely statistical impact of the $ZnSO_4$ included in the diet at basal levels; despite this, we detected a significant association with the ZnO levels added beyond the basal levels provided by the $ZnSO_4$. Based on our *spa* typing results, *spa* type t034, is the most predominant from all three anatomic collection sites. This *spa* type, t034, belongs to the clonal complex398 (CC398) which is the most predominant

livestock associated-MRSA clonal lineage, primarily in pigs, and has been reported from many countries including the United States. The reason for the emergence of CC398 in pigs is not understood and might involve a complex interplay between management and dietary factors. Both t007 and t127 *spa* types also form a part of LA-MRSA complex and have been reported from many countries, including the United States. Although these clones or sequence types are reported from pigs, farmers, and veterinarians there have rarely been cases of infection reported in the United States. That said, these clones could pose a serious therapeutic challenge in case of invasive infections as these lineages possesses several virulence factors and toxins beside antimicrobial resistance determinants. The presence of MRSA has been confirmed in this cohort of pigs and in this facility. The complete *spa* typing and sequencing results will shed further light on the possible effect of zinc and or CTC on the prevalence of *spa* and sequence types.

Publications / Presentations / Abstracts

1. Amachawadi, R. G., H. M. Scott, J. Vinasco, J. Feldpausch, M. D. Tokach, S.S. Dritz, J. L. Nelssen, R. D. Goodband, and T. G. Nagaraja. 2014. Nasal, skin, and tonsillar carriage of methicillin-resistant and zinc-resistant *Staphylococcus aureus* in piglets fed diets supplemented with zinc and chlortetracycline. 95th Proceedings of Conference on Research Workers in Animal Diseases, December 7-9, Chicago, Illinois: Poster presentation.

2. Amachawadi, R. G., H. M. Scott, J. Vinasco, J. Feldpausch, M. D. Tokach, S.S. Dritz, J. L. Nelssen, R. D. Goodband, and T. G. Nagaraja. 2015. Effects of chlortetracycline and zinc supplementation on nasal, skin, and tonsillar carriage of methicillin-resistant and zinc-resistant *Staphylococcus aureus* in piglets. 4th ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens. May 8-11, 2015. Washington, DC: Oral presentation.

List of Tables

Table 1: Prevalence of *mecA* positive methicillin-resistant Staphylococcus aureus isolated from nasal, tonsil, and skin region of piglets

Treatments	Number tested	Nasal		Tonsil		Skin	
		No. positive	Positive (%)	No. positive	Positive (%)	No. positive	Positive (%)
Control	120	42	35.0	48	40.0	43	35.8
Zinc	120	56	46.6	48	40.0	44	36.6
High CTC	120	51	42.5	53	44.2	46	38.3
Low CTC	120	57	47.5	55	45.8	43	35.8
Zinc + High CTC	120	48	40.0	50	41.6	44	36.6
Zinc + Low CTC	120	54	45.0	53	44.2	48	40.0
TOTAL	**720**	**308**	**42.8**	**307**	**42.6**	**268**	**37.2**

Table 2: Prevalence of *czrC* positive methicillin-resistant Staphylococcus aureus isolated from nasal, tonsil, and skin region of piglets

Treatments	Number tested	Nasal		Tonsil		Skin	
		No. positive	Positive (%)	No. positive	Positive (%)	No. positive	Positive (%)
Control	120	18	15.0	18	15.0	25	20.8
Zinc	120	31	25.8	18	15.0	24	20.0
High CTC	120	21	17.5	14	11.7	26	21.7
Low CTC	120	26	21.7	17	14.2	19	15.8
Zinc + High CTC	120	25	20.8	17	14.2	30	25.0
Zinc + Low CTC	120	23	19.2	19	15.8	28	23.3
TOTAL	**720**	**144**	**20.0**	**103**	**14.3**	**152**	**21.1**

Table 3: Association between *mecA* and *czrC*-positive methicillin-resistant *Staphylococcus aureus* isolated from nasal, tonsil, and skin region of piglets

Sampling site	Odds Ratio (OR)	95% Confidence Interval	*P*-value
Nasal	3.33	[2.92-3.79]	< 0.0001
Tonsil	2.89	[2.57-3.25]	< 0.0001
Skin	4.82	[4.10-5.67]	< 0.0001

Table 4: Minimum inhibitory concentrations of zinc and tetracycline against methicillin-resistant *Staphylococcus aureus* isolated from nasal, tonsil, and skin region of piglets

	czrC-negative isolates		*czrC*-positive isolates	
Sampling site	**Zinc (mM)**	**Tetracycline (µg/ml)**	**Zinc (mM)**	**Tetracycline (µg/ml)**
Nasal	1.5	> 100	4.5	> 100
Tonsil	1	> 100	2	> 100
Skin	1.3	> 100	3.6	> 100

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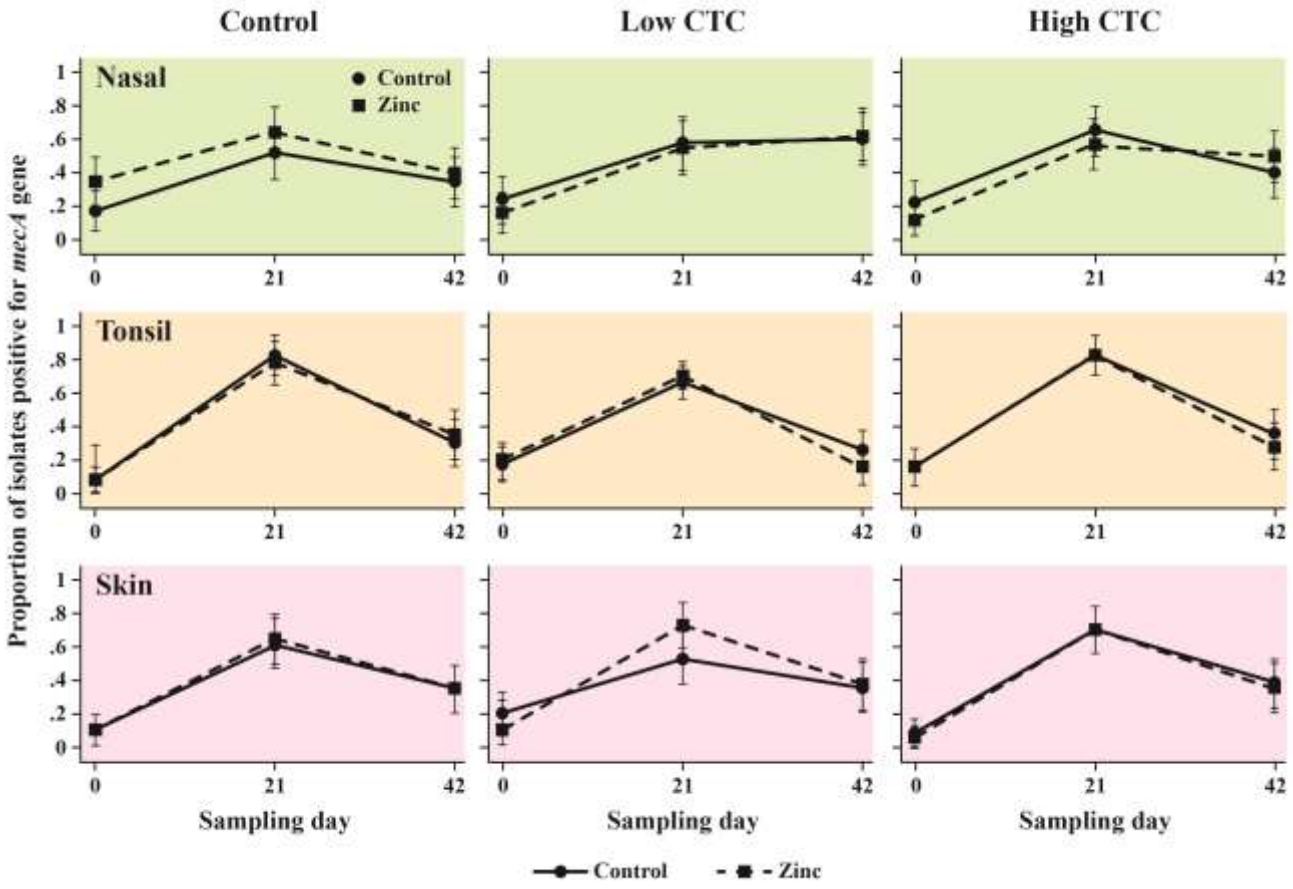

Figure 1: Proportion of *mecA* positive isolates by sampling day illustrating epidemic spread during the first 21 days of feedling period among nasal, skin, and tonsillar isolates

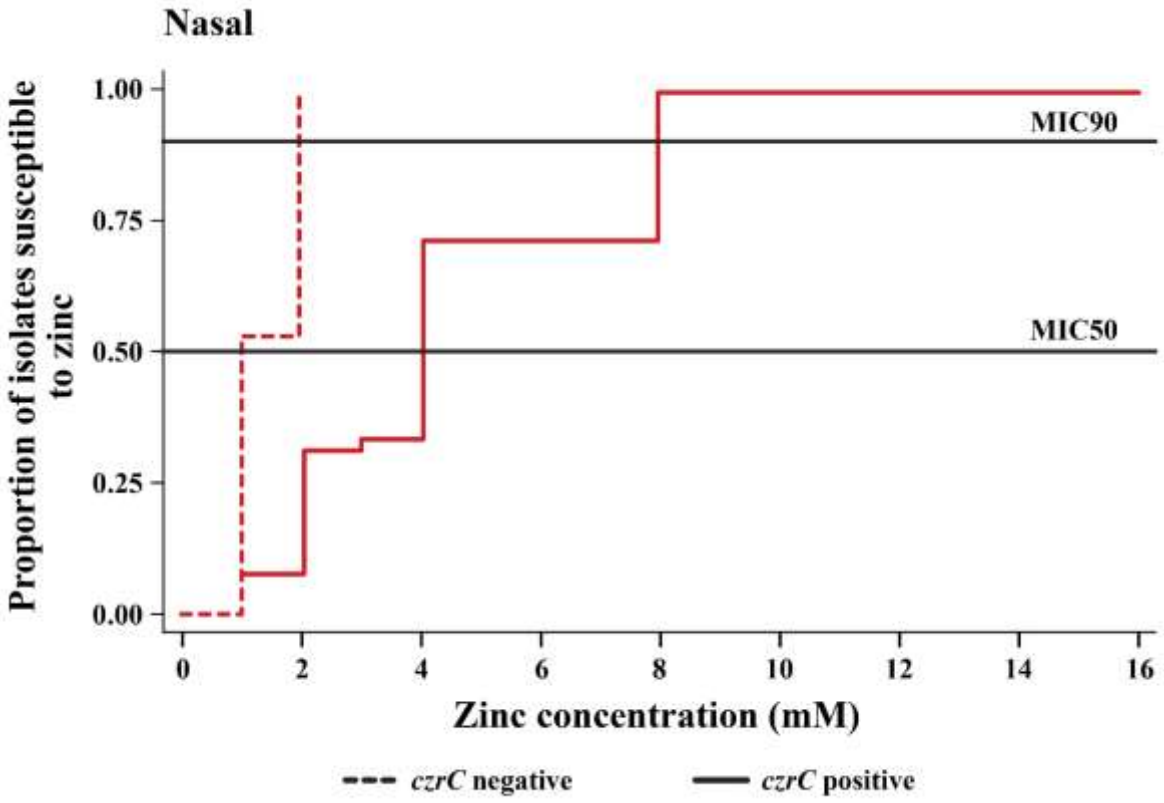

Figure 2: Failure function graph showing cumulative susceptibilities of *czrC*-positive and *czrC*-negative nasal isolates to increasing zinc concentrations

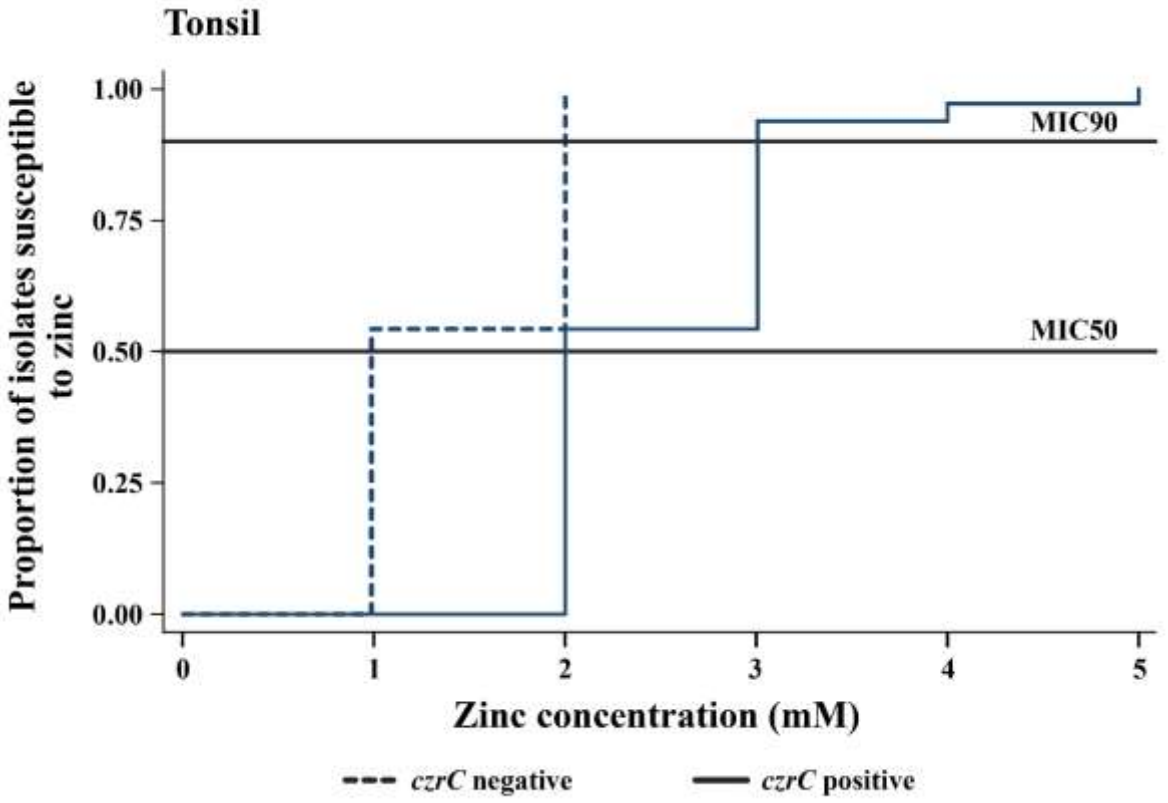

Figure 3: Failure function graph showing cumulative susceptibilities of *czrC*-positive and *czrC*-negative tonsil isolates to increasing zinc concentrations

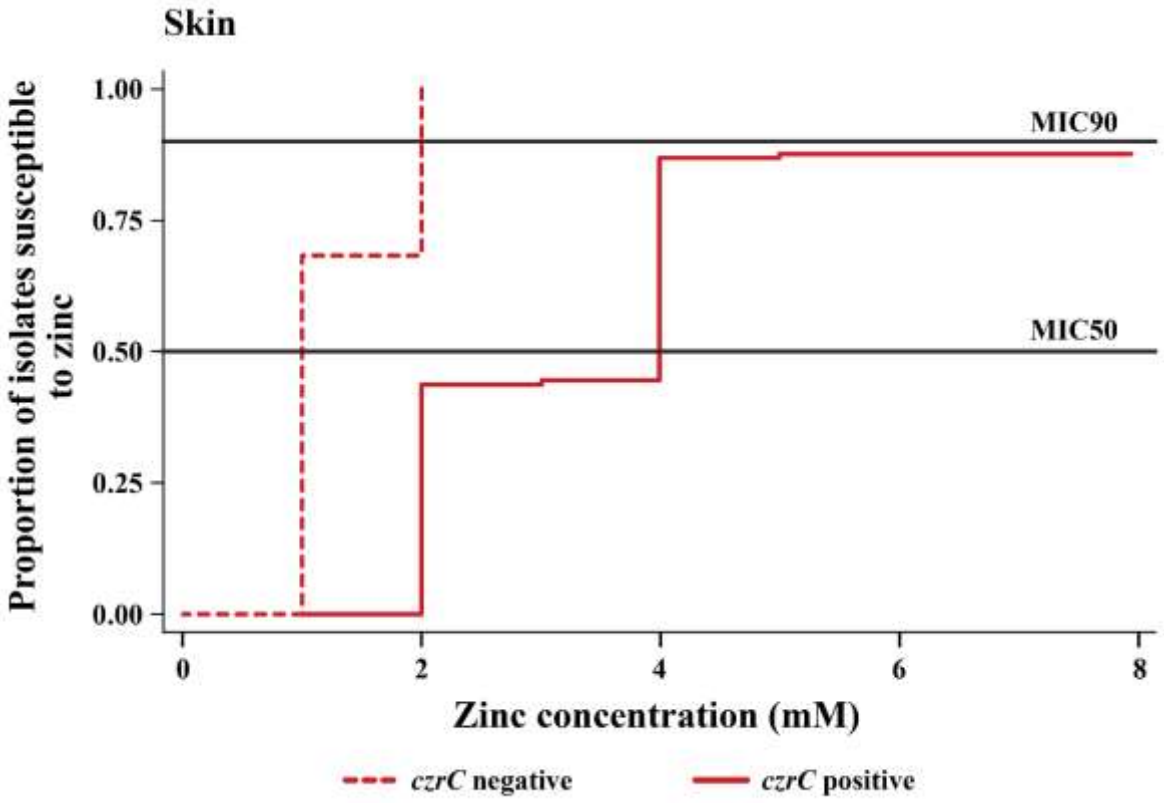

Figure 4: Failure function graph showing cumulative susceptibilities of *czrC*-positive and *czrC*-negative skin isolates to increasing zinc concentrations