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In-Feed Use of Heavy Metal Micronutrients in U.S. Swine Production Systems and Its Role in Persistence of Multidrug-Resistant Salmonellae

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The study aimed to characterize the role of heavy metal micronutrients in swine feed in emergence of heavy-metal-tolerant and multidrug-resistant *Salmonella* organisms. We conducted a longitudinal study in 36 swine barns over a 2-year period. The feed and fecal levels of Cu^{2+} and Zn^{2+} were measured. *Salmonella* was isolated at early and late finishing. MICs of copper sulfate and zinc chloride were measured using agar dilution. Antimicrobial susceptibility was tested using the Kirby-Bauer method, and 283 isolates were serotyped. We amplified *pcoA* and *czcD* genes that encode Cu^{2+} and Zn^{2+} tolerance, respectively. Of the 283 isolates, 113 (48%) showed Cu^{2+} tolerance at 24 mM and 164 (58%) showed Zn^{2+} tolerance at 8 mM. In multivariate analysis, serotype and source of isolates were significantly associated with Cu^{2+} tolerance ($P < 0.001$). Fecal isolates were more likely to be Cu^{2+} tolerant than those of feed origin (odds ratio [OR], 27.0; 95% confidence interval [CI], 2.8 to 250; $P = 0.0042$) or environmental origin (OR, 5.8), implying the significance of gastrointestinal selective pressure. *Salmonella enterica* serotypes Typhimurium and Heidelberg, highly significant for public health, had higher odds of having >20 mM MICs of Cu^{2+} than did "other" serotypes. More than 60% of *Salmonella* isolates with resistance type (R-type) AmStTeKm (32 of 53) carried *pcoA*; only 5% with R-type AmClStSuTe carried this gene. *czcD* gene carriage was significantly associated with a higher Zn^{2+} MIC ($P < 0.05$). The odds of having a high Zn^{2+} MIC (≥8 mM) were 14.66 times higher in isolates with R-type AmClStSuTe than in those with R-type AmStTeKm ($P < 0.05$). The findings demonstrate strong association between heavy metal tolerance and antimicrobial resistance, particularly among *Salmonella* serotypes important in public health.

Nontyphoidal *Salmonella enterica* serotypes are among the most important food-borne bacterial pathogens, with a broad host range, including food animals and humans. *Salmonella enterica* remains one of the leading causes of food-borne illness (11%), hospitalization (35%), and death (28%) in the United States (1). In addition, most strains of the commonly occurring serovars, such as Typhimurium, have been shown to exhibit multidrug resistance, resistance to two or more antimicrobials (2–4). Previous studies on antimicrobial resistance have shown the emergence of multidrug-resistant (MDR) *Salmonella* in swine production systems even when there was no history of using antimicrobials, either as therapeutics or as growth promoters (5–7). The emergence and persistence of MDR *Salmonella* serovars in a swine production environment where there is no history of antimicrobial use suggest the presence of other risk factors such as selective pressure, including the use of heavy metal micronutrients in intensive swine production units.

Micronutrients such as copper and zinc, among many others, are included in swine feed and other livestock to achieve growth promotion and increase feed efficiency (8, 9). Zinc and copper are essential trace elements for prokaryotic and eukaryotic cellular metabolic functions. Zinc is a cofactor of more than 300 metalloenzymes, including alkaline phosphatases, whereas copper is needed for activation of several oxidative enzymes required for normal cellular metabolism (10, 11). Due to the proven and anticipated beneficial effects of zinc and copper in swine production, in-feed supplementation of zinc and copper in commercial production systems has been very common (12, 13).

On the other hand, tolerance to various chemicals among bacterial pathogens, mediated by different mechanisms, has also been on the rise. Multidrug efflux systems have been shown to be important mechanisms of resistance against antimicrobial agents and other structurally unrelated compounds, including heavy metals and biocides. The mechanisms of heavy metal resistance to copper in *Enterococcus faecium* isolates from pigs have been associated with the carriage of a conjugative plasmid carrying copper resistance determinants such as *tcrB* (14–16). Another efflux system that has also been associated with copper tolerance reported in Gram-negative organisms is the PCO operon, which mediates resistance to Cu^{2+}.

Resistance to Zn^{2+} and other metals such as Co^{2+} and Cd^{2+} is conferred by genetic determinants often carried by a plasmid such as pMOL30. In the CZC operon system, the products of the *czc* gene clusters function as a main component of an efflux protein

Received 27 December 2013 **Accepted** 28 January 2014
Published ahead of print 31 January 2014
Editor: H. L. Drake
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doi:10.1128/AEM.04283-13

(CzcA), a cation funnel (CzcB), a modulator of substrate specificity (CzcC) (17–20), and a protein involved in regulation of the operon (CzcD). The CzcD gene is involved in the regulation of a zinc, cobalt, and cadmium efflux system, the Czc system, which mediates resistance to these heavy metal cations (19, 21).

Coselection of heavy-metal-tolerant and antimicrobial-resistant phenotypes often occurs when the genes are colocated on genetic elements such as a plasmid, transposon, or integron (22–24). The efflux pump in bacteria plays a major role in development of resistance to several classes of antimicrobial agents. The presence and significance of resistance factors against micronutrients among *Salmonella* strains in the United States are poorly understood, and their association with antimicrobial resistance in the production environment has not been investigated. The present study was conducted to characterize the role of heavy metal micronutrient interventions (such as those by copper and zinc) in the emergence of heavy-metal-tolerant *Salmonella* and also its coselective association with multidrug-resistant *Salmonella*. Further, we investigated the association with carriage of specific efflux gene markers.

MATERIALS AND METHODS

Study design and sample collections. This study was part of a large longitudinal group-randomized controlled study designed to investigate the association of heavy metal micronutrients in swine feed with the occurrence and persistence of multidrug-resistant (MDR) *Salmonella*. Briefly, three vertically integrated commercial swine production systems (systems 1, 2, and 3) selected based on their history of *Salmonella* occurrence were included. From each system, three farms were selected (total of 9 farms). At each farm, four barns were randomly selected for further follow-up in this study, and all barns used standardized disinfection systems to limit introduction of additional potential confounding effects. We visited each farm at two stages (early finishing and late finishing) in four replicates (repeated visits to the same barns during the study period of October 2007 to November 2009). Each replicate visit consisted of sampling assigned barn floors before and after disinfection, pigs at early and late finishing stages, and pooled feed samples (25). Sampling was done from all the 36 barns for a period of more than 2 years. A total of 48 fresh fecal samples (25 g) were aseptically collected from each barn in four replicates at the early finishing stage (6 to 9 weeks of age) ($n = 6,842$) and at late finishing stages (26 to 28 weeks of age) of production ($n = 6,093$) from individual pigs. Some samples were lost/missed at different stages of the study (48 samples/barn × 36 barns × 4 replicates = 6,912 samples). Approximately 100 g of pooled feed samples (1 sample per barn collected from all 36 barns at 2 stages and 4 replicates with 13 losses to follow-up) was aseptically collected from 36 barns ($n = 275$) over a period of 2 years. Each pooled feed sample per barn was aseptically collected from the feeder bin in sterile Whirl-Pak bags and shipped to the laboratory on the same day as collection. For each farm, a survey assessment including questions about basic production, herd health management, biosecurity, and in-feed use of heavy metals (copper and zinc) was done.

***Salmonella* isolation and identification.** Salmonellae were isolated and identified according to conventional methods as described previously (26, 27). Briefly, a 10-g portion of each fecal and feed sample was preenriched in 90 ml of buffered peptone water (BPW; Becton, Dickinson, Sparks, MD), and 90 ml of BPW was added to each Whirl-Pak bag containing individual drag swabs and incubated at 37°C overnight. The remaining portions of fecal and feed samples were stored at −20°C. After overnight incubation, 100 μl of the preenriched suspension was added into 9.9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Becton, Dickinson, Sparks, MD) and incubated at 42°C for 24 h. A 10-μl portion of the suspension was inoculated onto xylose-lactose-Tergitol 4 (XLT-4) agar (Becton, Dickinson, Sparks, MD) plates and incubated at 37°C for 24 h, and incubation was extended to 48 h in cases where colonies were doubtful. Three presumptive *Salmonella* colonies were selected from each positive plate for biochemical testing. Each selected presumptive *Salmonella* colony was then inoculated onto triple sugar iron (TSI) agar slants (Becton, Dickinson, Sparks, MD), lysine iron agar (LIA) slants (Becton, Dickinson, Sparks, MD), and urea broth (Becton, Dickinson, Sparks, MD) and incubated at 37°C for 24 h. All biochemically confirmed *Salmonella* isolates were then stored at −80°C until further testing.

Phenotyping. *Salmonella* isolates recovered from swine feed ($n = 30$), swine barn floors ($n = 1,628$), and swine feces ($n = 4,504$) were serogrouped using commercially available polyvalent O and group-specific antisera (Mira Vista, Copenhagen, Denmark) according to the recommendations of the manufacturer. Of all *Salmonella* isolates biochemically confirmed ($n = 6,162$), 283 *Salmonella* isolates were systematically selected based on origin and phenotypic characteristics (serogrouping and antimicrobial resistance profiles) and submitted to the National Veterinary Services Laboratories (USDA-NVSL, Ames, IA) for serotyping. *Salmonella* isolates were tested for antimicrobial susceptibility to a panel of 12 antimicrobials using the Kirby-Bauer disc diffusion method according to the guidelines of the CLSI (28). The antimicrobials used and their respective disc potencies were as follows: ampicillin (Am; 10 μg/ml), amoxicillin-clavulanic acid (Ax; 30 μg/ml), amikacin (An; 30 μg/ml), ceftriaxone (Ce; 30 μg/ml), cephalothin (Ch; 30 μg/ml), chloramphenicol (Cl; 30 μg/ml), ciprofloxacin (CIP; 5 μg/ml), gentamicin (Gm; 10 μg/ml), kanamycin (Km; 30 μg/ml), streptomycin (St; 10 μg/ml), sulfisoxazole (Su; 250 μg/ml), and tetracycline (Te; 30 μg/ml). We used *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 as control strains. *Salmonella* isolates showing resistance to three or more antimicrobials were classified as multidrug resistant (MDR), and those isolates showing intermediate resistance profiles were considered susceptible.

Copper and zinc level determinations in swine feed and fecal samples. Pooled feed and fecal samples were shipped to the Ohio State University soil analytic laboratory for copper and zinc level determinations. The concentrations of copper and zinc in pooled feed and fecal samples were measured by inductively coupled plasma emission spectroscopy (PerkinElmer Optima 2000) using a method adapted from the work of Luo et al. (29). Quality control samples included a negative control (blank), positive control (two matrix spikes), and one standard reference material per batch of 20 samples. The methods have been modified from those of Lindsey et al. (30), Ollers et al. (31), Zhu et al. (32), and USEPA 3051b (33).

MIC of copper sulfate and zinc chloride. Three hundred forty-nine *Salmonella* isolates, from 283 samples, recovered from floor swabs ($n = 179$ samples), feces ($n = 94$ samples), and feed samples ($n = 10$ samples) with different antimicrobial resistance patterns were systematically selected for tests of tolerance to different concentrations of zinc chloride ($ZnCl_2$) and copper sulfate ($CuSO_4$). The agar plate-dilution method was used to determine the MIC against *Salmonella* as described before (34). The susceptibilities were determined on Mueller-Hinton II (MH-II) agar plates with a dilution range for zinc chloride of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 mM, with the pH of the medium adjusted to 5.5. Copper sulfate solutions contained the dilution range of 0, 1, 2, 4, 8, 16, 20, 24, 28, and 32 mM with the pH of the medium adjusted to 7.2. Briefly, 25 ml of MH agar was aseptically dispensed and allowed to solidify. Bacterial suspensions were adjusted to 10^7 CFU/ml (100 μl of each inoculum at a 0.5 McFarland standard plus 900 μl of sterile 0.85% NaCl solutions). Each of the 400-μl suspension was aseptically aliquoted to a corresponding well of the replicator inoculum block. All test *Salmonella* isolates and control strains were tested in triplicate. The inoculated plates were incubated at 37°C for 16 to 20 h. Plates were assessed for growth, and the MIC was determined. The MIC was defined as the lowest concentration that inhibits the visible growth of *Salmonella*. *Enterococcus faecium* A17 sv 1 HHA 210, *S. aureus* C10682, *S. aureus* ATCC 29213, and *S. aureus* SO385 were used as reference strains. The reference strains were generously provided by Henrik Hasman (Technical University of Denmark).

Identification of heavy metal micronutrient (Cu^{2+} and Zn^{2+}) tolerance genes. The 283 *Salmonella* isolates were tested for the carriage of selected tolerance genes (*pcoA* for copper tolerance and *czcD* for zinc tolerance) using PCR. Briefly, *Salmonella* isolates were inoculated onto tryptic soy agar (TSA) plates and incubated at 37°C overnight. The genomic DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's instructions (Qiagen Ambion, Austin, TX, USA). Primers used for amplification of the *pcoA* gene included Forward (5′-CGTCTCGACGAACTTTCCTG-3′) and Reverse (5′-GGACTTCACGAAACATTCCC-3′). The thermocycling conditions included Hot Start *Taq* activation at 95°C for 5 min, denaturation at 95°C for 1.5 min, annealing at 57°C for 1.5 min, and extension at 72°C for 2 min, and amplification was done in 34 cycles (35). Primers used for amplification of the *czcD* gene included Forward (5′-TTTAGATCTTTTACCACCATGGGCGC-3′) and Reverse (5′-TTTCAGCTGAACATCATACCCTAGTTT-3′) (36). The PCR amplification conditions were initial denaturation at 94°C for 2.5 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and then the amplification cycle was repeated for a further 28 cycles and final extension was done at 72°C for 5 min. Ten microliters of the PCR product of each isolate tested was electrophoresed on a 1% agarose gel stained with 5 μl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5× Tris-borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

Data analysis. Data were first summarized descriptively: levels of *Salmonella* prevalence in different samples, proportions of isolates that were resistant or were carrying heavy metal tolerance genes, and copper and zinc MICs were calculated. To explore the role of heavy metals and heavy metal tolerance in *Salmonella* in swine production, the data were analyzed with SAS v.9.3 (SAS Institute Inc., Cary, NC) using a generalized linear mixed model approach (PROC GLIMMIX), with binary distribution and logit link. The clustered data structure was accounted for by testing and including production company, farm, and barn as random effects in the models. Only one isolate per sample was included in the statistical modeling. Isolates were categorized based on their heavy metal (Zn and Cu) MIC values into high- or low-MIC groups (Zn MIC equal to 8 = high; Cu MIC equal to 24 mM = high), as well as on carriage of the zinc (*czcD*) or copper (*pcoA*) tolerance gene (yes/no). These characteristics were used as outcomes in the statistical analysis, and separate models were run for Cu and Zn.

Isolates were categorized into three groups based on their serotype: Typhimurium, Heidelberg, and "other." Based on their resistance patterns (R-type), isolates were also categorized into four groups: AmClStSuTe, AmStTeKm, resistant to any other combination of drugs, or pansusceptible. Associations between the above-mentioned outcomes and feed and fecal levels of the metals, source of the isolates (fecal, feed, or floor swab), different serotypes, and resistance patterns were assessed. Initially, all factors were individually modeled against the outcome in a univariate screening, and all that were associated with the outcome with P of <0.2 were included in a full model. Nonsignificant ($P > 0.05$) variables were dropped one at a time until all variables remaining in the model were significant. Of the 283 isolates selected for further characterization, 100% of serovar Heidelberg and Typhimurium isolates were multidrug resistant; thus, no further statistical modeling was performed on multidrug resistance.

RESULTS

Antimicrobial resistance of *Salmonella*. *Salmonella* isolates were detected in 17.2% (1,180/6,842) and 7.1% (431/6,093) of the pigs at early and late finishing stages, respectively. Interestingly, in each of the three production companies different *Salmonella* serovars appeared to dominate: Typhimurium was found most frequently in production system 1 (61.8% of all isolates), Heidelberg was found most often in production system 2 (50.3%), and "other" serovars were isolated most commonly in production system 3 (56.5%). Antimicrobial resistance was very common among the isolates, with 90% (27 of 30) of *Salmonella* isolates that originated from feed samples, 92.3% (1,503 of 1,628) of isolates from drag swabs, and 98.02% (4,415 of 4,504) of isolates from swine fecal samples showing resistance to one or more of the antimicrobials tested. Almost two-thirds, 60% (18 of 30), of the *Salmonella* isolates recovered from feed samples, 51.4% (836 of 1,628) of the isolates from barn floor drag swabs, and 76.1% (3,428 of 4,504) of the isolates from swine fecal samples were multidrug resistant (MDR). Table 1 summarizes the phenotypic properties of *Salmonella* serotypes ($n = 283$) further characterized using phenotypic and genotypic approaches.

Fecal and feed levels of heavy metal micronutrients (Zn^{2+} and Cu^{2+}). The level of zinc in the pooled feed samples varied between 77 mg/kg and 2,000 mg/kg of feed with a median of 139.8 mg/kg. The levels of Zn^{2+} in fecal samples were found to be significantly higher than the in-feed levels, ranging between 536.5 mg/kg and 12,557.2 mg/kg with a median of 941.1 mg/kg. Copper levels were found to range between 3.2 mg/kg and 365.2 mg/kg in pooled feed samples, with a median of 31.5 mg/kg. Copper levels in fecal samples were also higher, ranging from 71.2 mg/kg to 2,397 mg/kg with a median of 137.6 mg/kg. Table 2 depicts the levels of copper and zinc in feed and fecal samples. Overall, the Zn^{2+} levels in feed or fecal samples were not associated with the occurrence of high tolerance of Zn^{2+} (≥8 mM). On the other hand, copper tolerance was significantly associated with the Cu^{2+} levels found in fecal matter but not in feed.

Analysis of copper (Cu^{2+}) tolerance. Source of the isolates (fecal, feed, or floor swab sample), serotype, the resistance pattern that the isolates exhibited, and the copper level in fecal matter were associated with Cu^{2+} MICs in the univariate screening ($P < 0.2$). Forty-seven percent (134 of 283) of the isolates showed tolerance to Cu^{2+} at 24 mM, while the remaining 53% showed tolerance ranging from 4 mM to 20 mM. Carriage of the *pcoA* gene ($P = 0.8473$), copper level in the swine feed ($P = 0.6501$), or stage of sampling was not significantly associated with copper tolerance of *Salmonella* isolates. The carriage of the *pcoA* gene versus Cu MIC in *Salmonella* isolates recovered from fecal matter, feed, and barn floors is shown in Tables 1 and 3. When using multivariate analysis, two variables, serotype ($P = 0.0006$) and source of the isolates ($P < 0.0001$), remained significant in the model (Table 4). Resistance pattern became nonsignificant, and its effect appeared to be explained by the serotype when the two were included in the model simultaneously.

The odds of *Salmonella* isolates having high Cu^{2+} MICs (≥20 mM) were 5.8 times higher if the isolates originated from fecal samples rather than from floor swabs (95% confidence interval [CI] for an odds ratio [OR] of 3.1 to 11.1, $P = 0.0002$), indicating the significance of selective pressure in the gut ecosystem. Isolates of fecal origin were also significantly more likely to be tolerant to Cu^{2+} (MIC, ≥20 mM) than were those originating from feed samples (OR, 27.0; 95% CI, 2.8 to 250; $P = 0.0042$) (Table 4).

We found that serotype Heidelberg had 5.6-times-higher odds (95% CI for OR, 2.3 to 13.5; $P = 0.0002$) of having a MIC higher than 20 mM MIC than did "other" serotypes. Similarly, Typhimurium isolates were 1.3 times as likely to have high Cu^{2+} MICs (>20 mM) as were the "other" serotypes, but the difference was not statistically significant (95% CI for OR, 0.7 to 2.4; $P = 0.4741$). Also, serotype Heidelberg was 4.4 (95% CI for OR, 1.8 to 10.5; $P = 0.0009$) times as likely to have a MIC higher than 20 mM as was serovar Typhimurium (results not shown).

TABLE 1 Summary of phenotypic properties of *Salmonella* isolates ($n = 283$)

Serotype (no. of strains)	Resistance pattern (R-type), no. of strains	Source	Zn				Cu			
			MIC (mM)	Presence of *czcD* (no. of strains)[a]	Level in sample (mg/kg)		MIC	Presence of *pcoA* (no. of strains)[a]	Level in sample (mg/kg)	
					Feed	Fecal			Feed	Fecal
Schwarzengrund (3)	AmStTeAxChKm (1)	Swab	8	0	196	1,436	24	0	14	260
	Pansusceptible (2)	Swab	8	0	196	1,436	24	0	14	260
Mbandaka (7)	AmClStSuTeAx (1); AmSuChCe (1); StSuTe (2)	Swab	8	0	196–527.3	1,436–4,167.2	20–24	0	14–194.7	260–1,209.8
	AmStTe (1); AmClStSuTeAxCh (1)	Fecal	4–8	0	109.0–144	674–9,027.2	20–24	0 (1), + (1)	157.7–173	1,142–1,704.2
	SuTe (1)	Feed	8	0	1,668	10,530	16	1	268	1,852
Anatum (9)	AmTeAxChCeXNL (1); AmTeAxChXNL (1); AmStTeAxCh/XNL (3)	Swab	8	0	140.1–171.3	774–1,383.1	20–24	0	11.9–97	137.8–742
	Pansusceptible (2)	Swab	4–8	0	171.3–201	1,017–1,691	20	0	34	406
	AmStTeAxCh (2)	Fecal	8	0	96	787	24	0	146	1,496
Derby (17)	StSuTe (4)	Swab	4–8	0	132.7–201	939.9–11,631	20	0	9.4–34	112.9–406
	AmStSuTeAxChCe/XNL (6); AmClStSuTe (1); AmStSuTe/Km (2)	Fecal	4–8	0	108.8–2,000	865.9–11,185	20–24	0	8.3–316	110.6–1,911
	AmStSuTe (1); AmStSuTeAxCh (1)	Swab	4–8	0	1,389.1–1,393.2	8,714.8–10,146.2	20	1	249.3–268.3	1,645.5–1,875.3
	Pansusceptible (1)	Fecal	4	0	91.4–166.3	639.2–1,229.4	20–24	0 (6), + (1)	6.2–173	95–143.9
	Te (1)	Feed	8	0	147.6	719.7	16	0	146.2	919.2
Heidelberg (72)	AmStTeKm (1)	Feed	4	0	127.2	736.1	20	1	20.9	86.7
	AmStTeKm (12); AmStTeKmGm (2); AmStSuTeKmGm/Ax (2)	Swab	4–8	0	93.4–7,383.7	710.8–8,494.7	20–24	0 (7), + (9)	18.7–1,384.3	106.6–1,564.3
	AmStTeKm/AxChkmXNL (31); AmStSuTeKm/Gm (10); AmClStSuTeKm (1)	Fecal	4–8	0	79–2,000	675.3–11,631	20–24	0 (17), + (25)	135.4–308	137.3–2,130.7
	AmStTeAxChKmXNL (5); AmStTeAxChKmGm (4)	Fecal	4–8	0	92.9–1,549.1	675.3–8,952	20–24	0 (4), + (5)	203.2–291.1	12,176–1,839.1
	AmStTeAxChKmAnXNL (1); AmStTeAxChKm (2)	Fecal	8	0	178–191	1,059–1,197	24	0	14–87	160–172
	AmStTeKm (1)	Feed	4	0	1,330.3	948.6	20	0	229.5	1,412.7
Typhimurium (122)	AmClStSuTe (28); AmStSuTeKm/Ax (4); AmClStSuTeAx/Ch (3)	Swab	4–8	0 (2), + (12)	109.5–183.1	541–970	16–24	0 (10), + (4)	7.8–173	81.9–1,142
	AmClStSuTe/Ch (2); AmClStSuTeKm/Gm (2)	Swab	4–8	0 (5), + (14)	97.5–1,685	541–11,365	16–24	0 (18), + (1)	7.3–271	89.3.1–2,001
	AmClStSuTe (39); AmClStSuTeAx (9); AmStSuTeKm (4)	Fecal	4–8	0 (7), + (45)	81.3–1,762.2	787–11,365	16–24	0 (47), + (5)	9.9–262	90.7–2,001
	AmStTeKm (8); AmClStSuTeKm (3)	Fecal	4–8	0 (11)	89.1–1,728.2	692.6–7,852.4	20–24	0 (2), + (9)	7.5–365.2	152.3–1,703
	AmClStSuTeAxCh (6); AmClStSuTeAxChKmXNL (2)	Fecal	4–8	0 (1), + (7)	140–161.1	674–1,383.1	20–24	0 (7), + (1)	7.6–173	119.1–1,142
	AmClStSuTeAxChXNL (4); AmClStSuTeAxChKmGm (1); AmClStSuTeCh (1)	Fecal	4–8	0 (4), + (1)	144–160.9	674–1,063.6	20–24	0	14.4–173	123.8–1,142
	AmClStSuTeAxKmGm (1); ClStSuTe (2); AmClStSuTeChKmGm (2)	Fecal	4–8	0 (3), + (2)	99.1–1,347.5	541–8,952.0	4–24	0 (3), + (2)	3.2–203.2	101.8–1,543.4
	AmClStSuTe (1)	Feed	8	1	116.2	761.3	20	0	19.4	87.8
Worthington (8)	AmTeAxChCeXNL (2)	Fecal	8	0	115.2–134.4	1,066.7–1,463.5	24	2	9.3–31.4	126.8–177
	AmStTeAxChKmGm (1)	Swab	8	0	81.9	732.1	24	1	133	935.7
	AmStTeAxChKmXNL (1)	Fecal	8	0	1,645	12,325	24	1	302	2,397
	AmStTe (1)	Feed	8	0	1,685	11,365	24	1	271	2,001
	AmTeAxChCe (1); AmTeAxChCeXNL (1)	Swab	8	0	119.6	734.6	20	1	7.3	89.3
	AmStTeAxChKmGmXNL (1)	Swab	8	0	81.9	732.1	24	0	133	935.7
Rissen (1)	AmStTeKm (1)	Swab	8	0	129.6	1,254.7	20	1	15.7	138.7
Enteritidis (1)	AmClStSuTe (1)	Swab	8	0	140	774	16	0	97	742

Infantis (9)	AmClStSuTeAxChKmXNL (2); AmClStSuTe (1)	Fecal	4–8	0 (2), + (1)	86.4–146.9	1,383.1	16–20	0	11.9–16.2	135.6
	AmClStSuTeAxChKmGm (2); AmClStSuTeAxChKmGmXNL (1); AmAxChXNL (1)	Fecal	4–8	0	111–169.1	674–1,266	16–20	0	17–173	149.2–1,142
	AmClStSuTeChKmGmXNL (1); AmAxChXNL (1)	Swab	8	0	97.5–160.9	990.5–1,063.6	20	0	11.7–15.2	123.8–144.4
Agona (5)	StSuTeKmGm (2); AmStSuTeAxChKmGmXNL (1)	Fecal	4	0	105.1–177.3	991-.9–1,007.7	16–20	0	8.5–14.9	133.9–140.5
	AmStSuTeAxChKmGmXNL (1)	Swab	8	0	173.6	883.2	20	1	10.1	133.9
	Te (1)	Feed	8	0	1,375	6,895.0	16	0	279.2	1,391.4
Senftenberg (7)	AmClStSuTeAxChGmKmXNL (4); AmClStSuTeGm (1); StSuTeKmGm (1)	Fecal	4–8	0	178–1,636.2	657.8–8,364.9	20–24	0 (1), + (4)	14–291.1	172–1,643.1
	Pansusceptible (1)	Swab	8	0	191	1,059	24	1	87	160
Amsterdam (1)	Pansusceptible (1)	Swab	8	0	198	1,048	20	0	21	161
London (1)	Te (1)	Feed	4	0	1,558	12,120	20	1	262	2,144
Ohio (2)	Pansusceptible (1)	Swab	8	0	191	1,059	24	0	87	160
	AmClStSuTeKmGm (1)	Fecal	4	0	155.2	1,043.7	24	1	10.9	107.8
Inverness (1)	Pansusceptible (1)	Feed	8	0	131	924	20	0	4.8	99
Muenchen (3)	AmStTeKm (1)	Fecal	8	0	147	1,691	24	0	138	406
	Pansusceptible (2); AmStTeKm (1)	Fecal	8	0	192	1,183	20	0	15	120
Rough_Ob:e,n,x (1)	Pansusceptible (1)	Fecal	8	0	192	1,183	20	0	15	120
Serogroup E (5)	AmStSuTeChCeGm (1); AmStTeKmGm (1); AmClStSuTeChKmGm (1); ClStSuTe (1)	Fecal	4–8	0	139.5–1,393.2	855.2–1,215.9	16–24	0 (2), + (2)	23.1–249.3	85.5–1,875.3
	Te (1)	Feed	8	0	119.9	731.4	16	0	14.1	102.0
Serogroup A-I+ (3)	AmStTeKmGm (1); AmStTe (1)	Swab	8	0	81.9–1,552.1	732.1–8,629.2	16	+ (2)	133–229.2	935.7–1,498.1
	AmStSuTeKmGm (1)	Fecal	8	0	111	675.4	16	1	17	78.2
Serogroup B (5)	AmClStSuTeChKmGm (2); AmClStSuTe (1); AmStTeKmGm (1)	Fecal	4–8	0	116.2–1,393.2	761.3–10,146.2	16	+ (4)	8.8–249.3	87.8–1,875.3
	StSuTe (1)	Swab	8	0	1,389.1	8,714.8	20	1	268.3	1,645.5
Serogroup A-S+ (2)	AmClStSuTe (1); AmStTeKmGm (1)	Fecal	8	0	119.6–155.2	734.6–1,142.1	16	+ (2)	7.3–10.3	89.3–127.8

[a] 0, negative; +, positive.

TABLE 2 Zinc and copper levels in swine feed and fecal samples[a]

Heavy metal	Type of sample	Level (mg/kg)				Dietary Zn and Cu requirement (mg/kg)
		Median	Minimum	Maximum	95% CI of $\bar{x}$	
Zinc	Feed	139.8	79	7,383.7	563.14 ± 79.57	50–100
	Feces	941.1	541	12,325	3,070.17 ± 393.47	
Copper	Feed	31.5	3.2	1,384.3	123.33 ± 13.64	3–6
	Feces	137.6	71.2	2,397	806 ± 73.89	

[a] Dietary requirements are based on the guidelines of the National Research Council, 2012 (8).

The copper extrusion efflux gene, *pcoA*, was detected in 35% (99/283) of the *Salmonella* isolates (Table 3). More than 60% of *Salmonella* isolates with R-type AmStTeKm (32 of 53) carried the *pcoA* gene, while 5% of those with R-type AmClStSuTe (4 of 84) carried *pcoA*. The *pcoA* gene was detected in all three categories of serotypes regardless of their MDR status (Table 3). Of the detected *pcoA* genes, 40% were found in serovar Heidelberg and 22% were found in serovar Typhimurium. A lower proportion of these genes was also detected in the following serotypes: Senftenberg, Worthington, Derby, Ohio, Mbandaka, London, Agona, and Rissen. This finding implies the common occurrence of the gene in *Salmonella* (Table 3).

Analysis of zinc (Zn^{2+}) tolerance. In the univariate screening, carriage of the *czcD* gene, serotype, resistance pattern, and source of the isolates were all significantly associated with high Zn^{2+} MICs. However, neither Zn^{2+} levels in the feed ($P = 0.9613$) nor those in the feces ($P = 0.8043$) were significantly associated with high Zn^{2+} MIC levels. Sixty percent (171 of 283) of the isolates showed zinc tolerance at 8 mM, and the remaining 40% (112 of 283) showed tolerance at 4 mM. The carriage of the *czcD* gene versus Zn MIC in *Salmonella* isolates recovered from fecal samples, feed, and barn floors is shown in Tables 1 and 3. In the final model, carriage of the *czcD* gene, serotype, and source of the isolates remained significantly associated with high Zn^{2+} tolerance (Table 5). Isolates carrying the *czcD* gene had 10.6-times-higher odds of having a Zn^{2+} MIC of 8 mM or more than did those not carrying the gene (95% CI for OR, 4.0 to 27.8; $P < 0.0001$), adjusting for the serotype. "Other" serotypes had 4.5 (95% CI for OR, 2.2 to 9.5; $P < 0.0001$)- and 2.4 (95% CI for OR, 1.0 to 5.6; $P = 0.0504$)-times-higher odds than serotype Typhimurium of having a high Zn^{2+} MIC, whereas Heidelberg isolates were only approximately half as likely to have a high Zn^{2+} MIC as were Typhimurium isolates (OR = 0.52; 95% CI for OR, 0.211 to 1.289), but the difference was not statistically significant ($P = 0.1578$) (Table 5). In the model with serotypes, resistance pattern became nonsignificant and its effect appeared to be explained by the serotype. In contrast to the findings with copper tolerance, isolates from floor swabs had 6.5-times-higher odds (95% CI for OR, 3.2 to 12.9; $P < 0.0001$) of having high tolerance for Zn^{2+} than did fecal isolates. Also, isolates from feed samples had 3.0-times-higher odds of having a Zn^{2+} MIC of >8 mM than did fecal isolates, even though the difference was not significant (95% CI for OR, 0.7 to 13.5; $P = 0.1546$) (Table 5).

Assessment of association between tolerance and gene carriage showed that isolates with a high Zn^{2+} MIC also were more likely to carry the *czcD* gene (Table 3). The odds of *czcD* gene carriage were 5.2 times higher for isolates with high Zn^{2+} MICs than for those with low Zn^{2+} MICs (OR = 5.2; 95% CI, 2.4 to 11.5; $P < 0.0001$). However, 9.8% of the *Salmonella* isolates (11/112) with low Zn^{2+} MICs also carried the *czcD* gene, suggesting that carriage of this gene is not always associated with a Zn^{2+}-tolerant phenotype. Of the high-zinc-tolerant isolates, 52% belonged to serovar Typhimurium and 12% belonged to serovar Heidelberg. No *Salmonella* serovar Heidelberg carried the *czcD* gene, indicating a different mechanism for tolerance, whereas 69% of the Typhimurium isolates carried the *czcD* gene. Interestingly, none of the pansusceptible *Salmonella* isolates (12 of 283, 4.2%) were found to carry the *czcD* gene even though 66.7% (8/12) were tolerant to Zn^{2+} at 8 mM (Table 3). This indicates the presence of other mechanisms apart from the *czcD* gene. While the finding is just in contrast to the carriage of the *pcoA* gene encoding copper tolerance, it shows the occurrence of a strong association between distinct heavy metal tolerance and antimicrobial resistance (R-types).

The Zn^{2+} tolerance gene, *czcD*, was detected in 30% (85 of 283) of the *Salmonella* isolates. None of the *Salmonella* isolates with R-type AmStTeKm carried the *czcD* gene, while 84% of those with R-type AmClStSuTe (71 of 84) carried *czcD*. The *czcD* gene was almost exclusively detected in serotype Typhimurium (84 of 85); none of the Heidelberg isolates carried the gene and only one of the isolates belonging to the "other" serotypes carried the gene.

DISCUSSION

The magnitude of multidrug resistance in *Salmonella* and other pathogens at the human-animal and ecosystem interface has been a major concern globally. As we previously reported (25), in addition to isolation of *Salmonella* from feces and barn floor swabs, in the current study, *Salmonella* was also detected in 3.6% (10/275) of the commercially processed swine feed samples. Besides the direct selective pressure of antimicrobial resistance, coselection due to other structurally related or unrelated chemical agents has also been a concern for the rising trend in multidrug resistance. However, there have been very limited studies conducted in this area. The current study attempts to fill the knowledge gap, mainly focusing on the use of heavy metal micronutrients. Previous studies have identified genetic elements among *Salmonella* strains that render some strains resistant to heavy metal micronutrients, including copper (37–39) and zinc (39, 40). Such resistant strains were shown to carry genes associated with multiple antimicrobial resistance factors (34, 38).

Heavy metal micronutrients such as Zn^{2+} at relatively low concentrations are essential for microorganisms since they provide vital cofactors for metalloproteins and enzymes (2, 41). The use of copper in swine feed has also been repeatedly shown to have a positive effect in production performance (42, 43), especially when dietary Cu^{2+} is supplemented above the National Research Council (NRC) (8) requirement of 5 mg Cu^{2+}/kg (44). Copper

TABLE 4 Multivariable model with copper tolerance (Cu MIC = 24 mM) as outcome

Serotype/sample	Estimate	SE	OR (95% confidence interval)	*P* value
Serotype				0.0006
Typhimurium	0.2373	0.3311	1.27 (0.66–2.43)	0.4741
Heidelberg	1.7212	0.4486	5.59 (2.31–13.52)	0.0002
Other	Reference			
Sample type				<0.0001
Fecal	1.7633	0.3266	5.83 (3.07–11.09)	<0.0001
Feed	−1.5257	1.1477	0.22 (0.02–2.08)	0.1849
Floor swab	Reference			

and zinc are considered two of the most widely researched alternatives to growth promotion antibiotics to enhance swine performance and maintain health (45). In the current study, we found a wide range in the levels of copper and zinc used in swine feed and concentrations often much higher than the NRC recommendations, as depicted in Table 2. In addition, it is worth noting that the concentrations of both micronutrients were higher in the fecal samples than in the feed. This is expected, and it was previously reported that as the various feed ingredients are absorbed within the gastrointestinal tract, a few elements such as heavy metal micronutrients tend to be more concentrated in the feces, and as a result, a large percentage of the consumed dietary copper and zinc ends up in feces (8), indicating a relatively low retention in the intestine and significant excretion of these minerals in feces. Previous reports (46) also showed that this phenomenon is particularly more significant in swine and poultry feces and reported that the concentration of zinc in swine feces is 10 to 100 times higher than that in dairy manure and that liquid swine manure had six times as much copper as did liquid dairy manure. In addition, the recent NRC report (8) indicated that even though high levels of dietary copper and zinc have been shown to improve animal performance, a large percentage of consumed minerals such as copper and zinc (approximately 90 to 95%) is excreted in the feces and ends up as environmental contaminants. In this study, we observed that the level of micronutrients, particularly zinc, in the

TABLE 5 Multivariable model with zinc tolerance (Zn MIC = 8 mM) as outcome

Serotype, sample type, or carriage	Estimate	SE	OR (95% confidence interval)	*P* value
Serotype				0.0003
Heidelberg	−0.6507	0.4594	0.52 (0.211–1.289)	0.1578
Others	0.8609	0.4379	2.37 (0.999–5.602)	0.0504
Typhimurium	Reference			
Sample type				<0.0001
Feed	1.095	0.7672	2.99 (0.66–13.54)	0.1546
Floor swabs	1.8727	0.3493	6.51 (3.27–12.94)	<0.0001
Fecal	Reference			
czcD carriage				<0.0001
Yes	2.3576	0.4912	10.57 (4.02–27.79)	<0.0001
No	Reference			

TABLE 3 Heavy metal micronutrient (Zn^{2+} and Cu^{2+}) phenotypes, genotypes (*czcD* and *pcoA*), and association with resistance types (R-types)

Heavy metal micronutrient	MIC (mM)	Heavy metal tolerance gene[a]	R-type, *n* (%)									
			AmClStSuTe/Ax/Ch	AmStTeKm/Gm	AmStSuTeKm/Gm	AmStTeAxChKm/Gm	AmStTeAxChKmXNL	StSuTe	Te	Pansusceptible	Others	Total
Zinc	4	*czcD* (+)	11 (9.1)						1 (0.9)	4 (3.5)		11 (3.8)
		czcD (−)	4 (3.5)	42 (37.5)	4 (3.5)	3 (2.7)	5 (27)				38 (34)	101 (35.6)
	8	*czcD* (+)	67 (39.2)								7 (4.1)	74 (31.1)
		czcD (−)	10 (8.3)	18 (14.9)	11 (6.4)	2 (1.2)	1 (0.6)	7 (4.1)	3 (1.8)	8 (4.7)	37 (21.6)	97 (34.2)
Copper	1	*pcoA* (+)										
		pcoA (−)										
	2	*pcoA* (+)										
		pcoA (−)										
	4	*pcoA* (+)									1 (0.5)	1 (0.3)
		pcoA (−)										
	8	*pcoA* (+)										
		pcoA (−)										
	16	*pcoA* (+)	1 (0.5)	3 (1.5)					3 (3.0)		94 (47.5)	98 (35)
		pcoA (−)	3 (3.0)				1 (1.4)	2 (2.8)	1 (1.4)		94 (47.5)	100 (35.3)
	20	*pcoA* (+)		10 (13.9)	2 (2.6)						12 (16.7)	28 (9.9)
		pcoA (−)	5 (6.9)	6 (8.3)	1 (1.3)			5 (6.9)		7 (9.7)	20 (2.8)	44 (15.5)
	24	*pcoA* (+)	2 (1.9)	25 (24.8)	11 (10.9)	4 (3.9)	4 (3.9)			1 (1.0)	9 (8.9)	56 (19.8)
		pcoA (−)	3 (2.9)	16 (15.8)	19 (18.8)	2 (1.9)	1 (1.0)			4 (3.9)		45 (15.9)

[a] +, detected; −, not detected.

swine feed is much higher than the NRC daily requirements. While we cannot confirm it, the high level of use could potentially ameliorate the coselective pressure and its association with antimicrobial resistance. This area may need further investigation.

A few studies have reported different tolerance levels of important food-borne pathogens such as *Salmonella* and *E. coli* to different concentrations of Zn^{2+} and Cu^{2+} (34, 47). This study has reported up to 8 mM as the highest level of tolerance of *Salmonella* isolates to Zn^{2+}, which is higher than the level reported by Aarestrup and Hasman (39). The highest level of tolerance of *Salmonella* isolates to Cu^{2+} reported in our study was 24 mM. This is smaller than the maximum tolerance reported by the same study (39). The increased tolerance level of *Salmonella* isolates to Zn^{2+} and Cu^{2+} is attributable to the use of the respective micronutrients in swine feed. Zinc is known to inhibit some of the bacterial populations in the intestinal tract and thereby improve the health or feed conversion ratio of the food animals (39, 48, 49).

Carriage of the *pcoA* gene or level of copper in swine feed was not significantly associated with copper tolerance of *Salmonella* isolates. This may have different implications. One clear reason might be the presence of various other mechanisms that may result in copper tolerance besides *pcoA* and associated operon systems. Other genes, including *cuiD* and *scsC*, and other mechanisms have also been reported to encode copper tolerance in *Salmonella* (50, 51).

The PCR results on the level of *czcD* gene carriage by *Salmonella* isolates were consistent with the increase in the level of the Zn^{2+} MIC of *Salmonella* isolates. The PCR results on the level of *pcoA* gene carriage by *Salmonella* isolates were also consistent with the increase in the level of the Cu^{2+} MIC of *Salmonella* isolates. The two multidrug-resistant R-types AmClStSuTe and AmStTeKm were observed to be the highest-occurrence resistance types in the *Salmonella* isolates in this study; however, their occurrence varied by the type of heavy metal tolerance gene carriage. It should be noted that *Salmonella enterica* serovar Typhimurium strains of phage type DT104, often containing the R-type AmClStSuTe, are some of the major strains reported worldwide and are commonly isolated from humans and food animals (52, 53). While there have been very limited studies conducted to date investigating the association between heavy metal tolerance and specific antimicrobial resistance patterns, a study in 1984 in drinking water reported the overall association between copper and zinc tolerance and antimicrobial resistance (54). Heavy metal tolerance was more common among isolates from the fecal samples than among those of environmental origin (drag swabs).

In summary, the findings in this study clearly demonstrated the presence of a strong association between decreased susceptibility to heavy metals and antimicrobial resistance among *Salmonella* serovars isolated from swine, swine feed, and barn floors. The detection of decreased susceptibility to heavy metal micronutrients (copper and zinc) and associated genetic determinants among various *Salmonella* strains has implications for the control of multidrug-resistant *Salmonella* strains, which are of public health and veterinary medicine significance. However, further studies investigating the role of coselection and mechanisms of genetic linkage could shed further light on the relationship and its significance.

ACKNOWLEDGMENTS

This work was funded by the USDA-NRI, Epidemiological Approaches for Food Safety (grant no. 2007-01778). J.J.M. was financially supported through a fellowship from the Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University.

We thank members of the Infectious Diseases Molecular Epidemiology Laboratory (IDMEL) for technical assistance.

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