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Effects of chlortetracycline and copper supplementation on antimicrobial resistance of fecal *Escherichia coli* from weaned pigs

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ABSTRACT

Feed-grade chlortetracycline (CTC) and copper are both widely utilized in U.S. pig production. Cluster randomized experiment was conducted to evaluate the effects of CTC and copper supplementation in weaned pigs on antimicrobial resistance (AMR) among fecal *Escherichia coli*. Four treatment groups: control, copper, CTC, or copper plus CTC were randomly allocated to 32 pens with five pigs per pen. Fecal samples were collected weekly from three pigs per pen for six weeks. Two *E. coli* isolates per fecal sample were tested for phenotypic and genotypic resistance against antibiotics and copper. Data were analyzed with multilevel mixed effects logistic regression, multivariate probit analysis and discrete time survival analysis. CTC-supplementation was significantly (99% [95% CI = 98–100%]) associated with increased tetracycline resistance compared to the control group (95% [95% CI = 94–97%]). Copper supplementation was associated with decreased resistance to most of the antibiotics tested, including cephalosporins, over the treatment period. Overall, 91% of the *E. coli* isolates were multidrug resistant (MDR) (resistant to ≥3 antimicrobial classes). *tet*A and bla_{CMY-2} genes were positively associated ($P < 0.05$) with MDR categorization, while *tet*B and *pco*D were negatively associated with MDR. *tet*A and bla_{CMY-2} were positively associated with each other and in turn, these were negatively associated with both *tet*B and *pco*D genes; which were also positively associated with one another. Copper minimum inhibitory concentration was not affected by copper supplementation or by *pco*D gene carriage. CTC supplementation was significantly associated with increased susceptibilities of *E. coli* to copper (HR = 7 [95% CI = 2.5–19.5]) during treatment period. In conclusion, *E. coli* isolates from the nursery pigs exhibited high levels of antibiotic resistance, with diverse multi-resistant phenotypic profiles. The roles of copper supplementation in pig production, and *pco*-mediated copper resistance among *E. coli* in particular, need to be further explored since a strong negative association of *pco* with both *tet*A and bla_{CMY-2} points to opportunities for selecting a more innocuous resistance profile.

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1. Introduction

Chlortetracycline (CTC) and copper are commonly used in U.S. pig production (Dewey et al., 1999; Jacela et al., 2009, 2010; Apley et al., 2012). Widely employed for prevention, control and treatment of bacterial diseases, the use of antibiotics as growth promoters in animal production has been much more hotly debated as a cause of antimicrobial resistance (AMR) problems in human pathogens (Phillips et al., 2004; Marshall and Levy, 2011). Multiple previous studies have shown that the use of in-feed antibiotics on pig farms is associated with increased AMR (Thakur and Gebreyes, 2005; Akwar et al., 2008a, 2008b; Lutz et al., 2011; Keelara et al., 2013). Chlortetracycline is used either alone or in combination with other drugs such as sulfamethazine, sulfathiazole, or penicillin G (Dewey et al., 1999; Apley et al., 2012). Tetracycline resistance is the most commonly reported phenotype among bacteria isolated from pigs (Scott et al., 2005; Funk et al., 2006; Alali et al., 2008; Tadesse et al., 2012). Tetracycline resistance is inducible and occurs primarily due to acquisition of *tet* or *otr* genes that are involved in the active efflux of the molecule or else ribosomal protection from its action (Roberts, 2011). Co-selection of other arguably more important resistance types is of great concern. In a field trial to evaluate the effect of subtherapeutic use of CTC in grower pigs, Funk et al., 2006 reported that CTC supplementation was significantly associated with increased levels of tetracycline, ampicillin and ceftriaxone resistance among Gram negative aerobic fecal flora.

Copper is an essential micro-mineral that is included in the feed of pigs at National Research Council (NRC) recommended levels (5–6 ppm) (NRC, 2012); however, to compensate for dietary factors that could reduce its absorption, copper is usually supplemented at a higher dose than is typically recommended at 16.5 ppm in U.S. (Hasman et al., 2006; NSNG, 2010). In the U.S. at higher dietary levels (typically 100–250 ppm), it is also used for growth promotion purposes in pig production (Jacela et al., 2010; NRC, 2012). Among *Escherichia coli*, in addition to elaborate chromosomally mediated copper handling mechanisms, copper resistance is mediated through a plasmid-borne copper (*pco*) resistance cluster consisting of seven genes: *pco*ABCDRSE (Rouch and Brown, 1997).

There is growing concern that heavy metals, such as copper, help to maintain and expand the pool of antibiotic-resistant bacteria through co-selection (co- and cross-resistance mechanisms) (Baker-Austin et al., 2006). Transferable copper resistance (*tcr*B) in enterococci has previously been found to be associated with tetracycline (*tet*M), macrolide (*erm*B) and glycopeptide (*van*A) resistance genes (Aarestrup et al., 2002; Hasman and Aarestrup, 2002; Amachawadi et al., 2010, 2011a, 2013). Although the effects of metals on Gram-positive enteric bacteria have been extensively studied in several farm animal species (Hasman and Aarestrup, 2002; Amachawadi et al., 2011a, 2013), associations between copper supplementation and antimicrobial resistance (and tetracycline resistance in particular) among *E. coli* have not yet been examined in properly controlled experiments reflecting typical agricultural field conditions.

It has been reported that administration of ceftiofur in feedlot cattle is associated with increased tetracycline resistance in *E. coli* (Lowrance et al., 2007) and, conversely, administration of in-feed CTC in feedlot cattle is also associated with ceftiofur-resistant *E. coli* (Platt et al., 2008). However, experimental studies evaluating the effects of feed-grade use of CTC and elevated levels of copper supplementation in weaned pigs on AMR in their fecal flora are lacking. Therefore, we conducted an experimental study to investigate the effects of CTC and copper (and their combination) as feed supplements in pigs on phenotypic susceptibilities of *E. coli* to antibiotics and copper; and on the prevalence of genotypic elements coding for tetracycline (*tet*), copper (*pco*D) and ceftiofur (bla_{CMY-2}) resistance among fecal *E. coli*.

2. Materials and methods

2.1. Experimental design and E. coli isolation

A total of 160 weaned pigs (PIC 1050 barrows; PIC North America, Hendersonville, TN) of three weeks old obtained from a commercial breeder farm were randomly distributed by block (initial body weight) to pens (5 per pen). The average arrival body weight of the pigs was 7.8 kg. The study was performed at Kansas State University Segregated Early Weaning (SEW) research facility. The SEW facility receives about 400 weanling pigs every nine weeks from a commercial swine facility and after eight weeks (~27 kg) they are then moved to a different commercial facility for finishing. The facility has two barns each housing 40 pens (1.5 m × 1.2 m), each of which has an ad libitum feeder and nipple waterer. The pens have metal tri-bar flooring and typically hold five pigs (0.3 m^2/pig). Waste products were shallow pit emptied and the pens were washed and disinfected between batches of pigs. Antibiotics were not routinely included into feed except for experimental purposes. Animal handling and experimental designs were approved by the university Institutional Animal Care and Use Committee (IACUC# 2773). The treatment groups consisted of control (16.5 ppm of copper sulfate ($CuSO_4$)), elevated copper (125 mg/kg of feed), CTC (CTC 50; Alpharma, Fort Lee, NJ; at 550 mg/kg of feed), or copper plus CTC (elevated copper and CTC as above). Full dietary formulations are reported elsewhere (Amachawadi et al., 2011b). Copper in the basal diet was from the trace mineral premix which was analyzed at the Ward Labs, Kearney, NE to verify copper concentration. Premix batch sheets were routinely audited to verify copper sulfate inclusion rate. Trace mineral premix was analyzed to further verify copper inclusion. The same basal premix was included at the same concentration in all experimental diets. The treatment groups were randomly assigned to pens (8 pens/treatment), blocked by barn ($n = 2$) and accounting for the geographical distribution of pens within barn to avoid imbalance in potential fecal cross-contamination between pens. After two weeks of adaptation, pigs were fed experimental diets continuously for 21 days followed by a final washout period of two weeks (Amachawadi et al., 2011b).

In total, 576 fecal samples (144 samples/treatment group) were expected to be collected weekly from three

pigs per pen over 6 weeks. Fecal samples were collected *per* rectum, placed into individual WhirlPak bags (Nasco, Ft. Atkinson, WI), and transported on ice to the laboratory. *E. coli* were isolated from 0.1 ml of fecal suspension prepared from approximately 1 g of feces mixed in 9 ml of buffered peptone water by spread-plating on MacConkey agar (Difco™, Becton Dickinson Co., Sparks, MD) and incubated at 37 °C for 24 h. Two distinct lactose-fermenting colonies per sample were individually re-streaked onto blood agar plates (Remel Inc., Lenexa, KS) and incubated at 37 °C for 24 h. *E. coli* isolates were presumptively identified by indole test and stored at −70 °C on protect beads (Cryocare®, Key Scientific Products, Round Rock, TX) for later analysis. In total, 1152 isolates (288 per treatment group) were tested for their phenotypic antimicrobial susceptibilities and for the detection of *tet*, *pco*D and bla_{CMY-2} genes.

2.2. Antimicrobial and copper susceptibility testing

Minimum inhibitory concentrations (MIC) for 15 antibiotics were determined by the broth microdilution method using the Sensititre system (Trek Diagnostic Systems, Cleveland, OH) and following the Clinical Laboratory Standards Institute (CLSI) veterinary antimicrobial susceptibility testing protocol (CLSI, 2008). The antimicrobial concentration ranges and clinical breakpoints of the panels are depicted in Table 1. Antimicrobial susceptibility testing was performed with the National Antimicrobial Resistance Monitoring System (NARMS) custom-made plates for Gram-negative bacteria (CMV1AGNF and CMV2AGNF). Because CMV1AGNF was no longer commercially available after we began our laboratory analyses, we used CMV2AGNF for the last 350 isolates. The sole difference between the plates was that on the newer CMV2AGNF NARMS plates, amikacin was replaced by azithromycin. Based on MIC values, the *E. coli* isolates were categorized into susceptible (included intermediates) or resistant according to CLSI MIC interpretative criteria for *Enterobacteriaceae* (CLSI, 2008), except for streptomycin, for which NARMS consensus breakpoints were used. *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. coli* strains ATCC 25922 and 35218, and *Pseudomonas aeruginosa* ATCC 27853 strains from American Type Culture Collections (ATCC) (Manassas, VA) were used as quality controls for susceptibility testing.

Copper susceptibility was determined using broth microdilution in Mueller-Hinton II broth (Beckton Dickinson and Co.) from bacteria grown on Mueller-Hinton II agar (Beckton, Dickinson and Co.) for 24 h at 37 °C according to the CLSI procedures described above. $CuSO_4$ concentrations used were 0, 12, 16, 20, 24, 28, and 32 mM. Because the addition of $CuSO_4$ to the medium caused a significant drop in pH (Tetaz and Luke, 1983), the medium was adjusted to a pH of 7.0 by adding NaOH prior to bacterial inoculation. To ensure legitimate direct comparison of our results with those from other published studies relying on an agar dilution method (Aarestrup and Hasman, 2004), the agar dilution method was performed on 10 isolates selected from each MIC dilution to compare with the broth microdilution method we used.

2.3. PCR detection of resistance genes

E. coli isolates were tested for *tet* (A, B, C, E), bla_{CMY-2} and *pco*D genes from DNA extracted by heating each bacterial suspension in 500 μl of nuclease-free water at 95 °C for 10 min. After centrifugation at 14,000 rpm for 2 min, the lysate was separated and used as template for PCR. Primers and positive controls (and their sources) are shown in Table 2. All PCR reactions were performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany), and PCR products were analyzed by capillary gel electrophoresis in the QIAxcel Fast Analyzer system (Qiagen, Valencia, CA).

For the detection of *tet* (A, B, C, E) genes, a multiplex PCR assay (Ng et al., 2001) was adapted using a pre-optimized commercially available multiplex PCR master mix kit (Qiagen) according to the manufacturer's instructions. The PCR mixture of 50 μl consisted of 17 μl of nuclease-free water, 25 μl of master mix, 5 μl of primer mix, and 3 μl of DNA template. PCR conditions were initial activation at 95 °C for 15 min followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. For detection of the *pco*D gene, a total of 25 μl of the PCR reaction mixture consisted of 12.5 μl HotStarTaq master mix (Qiagen), 2 μl of each of the primers, 2 μl of DNA template, and 6.5 μl of nuclease-free water. The thermal profile consisted of initial activation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. For bla_{CMY-2} (Alali et al., 2009), the final 25 μl PCR reaction mixture consisted of 8.5 μl of nuclease-free water, 12.5 μl of master mix (Promega Corporation, Madison, WI), 1 μl of each of the primers, and 2 μl of DNA template. PCR conditions were initial denaturation for 2 min at 95 °C followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 30 s, with final extension at 72 °C for 5 min.

2.4. Statistical analysis

Data were analyzed using STATA version 12.1 (Stata Corp LP, College Station, TX), and $P < 0.05$ was considered significant. Our unit of concern was the individual *E. coli* isolate, with sampling procedures designed to reflect the population of these bacteria in the gut of weaned pigs in each of the various treatment arms and periods of the study. A full-factorial model evaluating the effects of copper, CTC, treatment period and their interactions was used throughout the analyses. Trial days were categorized into treatment periods as follows: pretreatment period (day 0), treatment period (days 7, 14, and 21) and post-treatment period (days 28 and 35). For descriptive statistics, binary outcomes were expressed as proportions with exact 95% binomial confidence intervals. A likelihood ratio chi-square test was used to compare the unadjusted effects of treatment and treatment period.

A three-level (i.e., isolates nested within fecal samples, fecal samples nested within pens) mixed effects logistic regression model was used to model the fixed effects of two treatments and three treatment periods (and their

Table 1
Antibiotic concentration ranges and resistance break points used for susceptibility testing of *E. coli* ($n = 1152$) isolated from fecal samples of weaned pigs experimentally fed chlortetracycline, copper, both or neither.

Antimicrobial class (FDA, 2012)	Antimicrobial agent	Abbreviations	WHO classification (WHO, 2012)	Concentrations used (μg/mL)	Resistance break point (μg/mL)[a]
Aminoglycosides	Amikacin	AMI	Critically important	0.5–64	≥64
	Gentamicin	GEN	Critically important	0.25–16	≥16
	Kanamycin	KAN	Critically important	8–64	≥64
	Streptomycin[b]	STR	Critically important	32–64	≥64
β-lactam/β-lactamase inhibitor combinations	Amoxicillin/clavulanic acid	AUG	Critically important	1/0.5–32/16	≥32/16
Cephems	Cefoxitin	FOX	Highly important	0.5–32	≥32
	Ceftiofur	TIO	Critically important	0.12–8	≥8
	Ceftriaxone	AXO	Critically important	0.25–64	≥4
Folate pathway inhibitors	Sulfisoxazole	FIS	Highly important	16–256	≥512
	Trimethoprim/sulfamethoxazole	SXT	Highly important	0.12/2.38–4/76	≥4/76
Macrolides	Azithromycin	AZI	Critically important	0.12–16	≥8
Penicillins	Ampicillin	AMP	Critically important	1–32	≥32
Phenicols	Chloramphenicol	CHL	Highly important	2–32	≥32
Quinolones	Ciprofloxacin	CIP	Critically important	0.015–4	≥4
	Nalidixic acid	NAL	Critically important	0.5–32	≥32
Tetracyclines	Tetracycline	TET	Highly important	4–32	≥16

[a] Breakpoints were based on Clinical Laboratory Standards Institute guidelines (CLSI, 2008).
[b] National Antimicrobial Resistance Monitoring System breakpoint was used (FDA, 2012).

interactions) accounting for random effects of pen and fecal sample on: (1) both individual binary phenotypic and genotypic resistance outcomes, and (2) MDR – binary classified as resistance to ≥3 antibiotic classes (FDA, 2012). This approach was also used to model the fixed effects of resistance genes (and their interactions) on MDR. Multivariate probit analysis (Cappellari and Jenkins, 2003) was used to simultaneously model multiple binary AMR outcomes. Copper MIC data were analyzed using discrete time survival analysis (Stegeman et al., 2006; Rabe-Hesketh and Skrondal, 2012). Time-to-event of a typical survival analysis was replaced by concentration-to-event, with the event being bacterial growth inhibition. The log-rank test was employed to compare the survival experiences between the treatment groups and treatment periods. The Bonferroni correction was used to adjust for multiple comparisons throughout the data analysis.

3. Results

3.1. Antimicrobial susceptibilities

The overall MIC distribution and resistance prevalence of *E. coli* isolates for the 15 antibiotics tested are illustrated as a 'squashtogram' in Table 3. The MIC values for ampicillin, streptomycin, sulfisoxazole, and tetracycline were right-censored beyond the highest concentration tested for greater than 60% of the isolates. All isolates were susceptible to amikacin ($n = 802$) and the quinolones (ciprofloxacin and nalidixic acid; $n = 1152$); as a result, these three antimicrobials were excluded from subsequent multivariable analyses. The prevalence of azithromycin resistance did not significantly ($P > 0.05$) differ by treatment. Because not all isolates were tested for azithromycin ($n = 350$ isolates), it was also excluded from multivariable analysis. Resistance to the beta-lactams significantly ($P < 0.05$) decreased over time in all three of the treated groups, with no change observed in the control group (Table 4). Multivariable multilevel mixed effects logistic regression modeling also illustrated that resistance to most of the antimicrobials tested decreased over time in all groups, including the CTC and copper-supplemented groups.

In total, 101 unique resistance phenotypes were observed. Only three isolates (0.35%) were pan-susceptible to all 15 antibiotics tested; in contrast, 7 isolates (0.6%) were susceptible to an observed maximum of 12 antibiotics. The predominant phenotype (22.5%; $n = 1152$) was of multidrug resistance to 10 drugs, followed by a phenotype exhibiting resistance to 8 different antibiotics (12%; $n = 1152$). In contrast to these highly MDR phenotypes, isolates exhibiting resistance solely to tetracycline were limited to just under 6% of the total isolates ($n = 1152$). A resistance profile consisting of ampicillin, amoxicillin, 3rd generation cephalosporins, sulfisoxazole, streptomycin and tetracycline was frequently observed when CTC was supplemented alone, or in combination with copper (Table 5). Pairwise correlations (associations) among all possible sets involving the nine antibiotics that were included in the multivariate analysis (Table 6) showed that, as expected, beta-lactam resistances were highly correlated (90–98%); with the cephalosporin resistances being especially highly correlated with one another (94–98%).

3.2. Multidrug resistance

According to the NARMS definition of MDR as resistance to ≥3 classes of antimicrobials (thereafter, treated as a binary classification), 91% (95% CI = 89–92%) of all the isolates ($n = 1152$) were MDR. CTC supplementation increased the likelihood of *E. coli* isolates being classified as MDR ($P = 0.033$) when compared with the control group (94%

Table 2
Primer sequences, amplicon size and positive control strains used for PCR detection of antimicrobial resistance genes from fecal *E. coli* ($n = 1152$) obtained from weaned pigs that received chlortetracycline, copper, both or neither.

Primers used					Positive control		
Resistance gene	Primer	Primer sequence (5′–3′)	Amplicon size (bp)	References	*E. coli* strains	Gene bank accession no.	Source
*tet*A	*tet*A-F	GCT ACA TCC TGC TTG CCT TC	210	Ng et al., 2001	D20-15	X61367	Marilyn Roberts[a]
	*tet*A-R	CAT AGA TCG CCG TGA AGA GG					
*tet*B	*tet*B-F	TTG GTT AGG GGC AAG TTT TG	659	Ng et al., 2001	D20-16	J01830	» »
	*tet*B-R	GTA ATG GGC CAA TAA CAC CG					
*tet*C	*tet*B-F	CTT GAG AGC CTT CAA CCC AG	418	Ng et al., 2001	D20-6	J01749	» »
	*tet*B-R	ATG GTC GTC ATC TAC CTG CC					
*tet*E	*tet*B-F	AAA CCA CAT CCT CCA TAC GC	278	Ng et al., 2001	D22-14	L06940	» »
	*tet*B-R	AAA TAG GCC ACA ACC GTC AG					
bla_{CMY-2}	585F	CAG ACG CGT CCT GCA ACC ATT AAA	454	Alali et al., 2009	M1		University of Illinois, Chicago
	1038R	TAC GTA GCT GCC AAA TCC ACC AGT					
*pco*D	*pco*D-F	CAGGAACGGTGATTGTTGTA	700	This study	*E. coli* (pRJ1004)	X83541.1	Henrik Hasman[b]
	*pco*D-R	CCGTAAAATCAAAGGGCTTA					

[a] University of Washington, Seattle, WA.
[b] National Food Institute, Lyngby, Denmark.

Table 3
Minimum inhibitory concentration (MIC) distribution (squashtogram) of *E. coli* ($n = 1152$) isolated from fecal samples of weaned pigs supplemented with chlortetracycline, copper, both or neither.

Antimicrobial[a]	% Resistant	95% CI[b]	Distribution of MICs in ug/ml (%)[c]															
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
AMI	0.0	0.0, 0.5						0.3	19.2	60.5	19.6	0.5						
AZI	6.3	4.0, 9.4							5.7	49.4	38.6	3.7	2.6					
AMP	74.6	71.9, 77.1							1.2	10.1	13.5	0.1	0.6	1.4	73.2			
AUG	70.2	67.5, 72.9							0.4	3.4	20.7	4.2	1.2	46.7	23.4			
AXO	68.9	66.0, 71.4					28.7	1.2	1.2	0.2	5.6	19.7	33.0	9.9	0.5	0.1		
CHL	38.5	35.6, 41.3								3.9	36.3	17.5	3.9	3.7	34.8			
CIP	0.0	0.0, 0.3	92.0	6.1	1.7	0.1				0.2								
SXT	4.1	3.0, 5.4				35.7	47.1	11.7	1.2	0.2	0.2	3.9						
FOX	66.9	64.1, 69.6							0.4	9.8	17.6	0.9	4.4	22.4	44.5			
GEN	42.3	39.4, 45.2					4.3	36.6	13.5	1.4	0.9	1.1	9.2	33.1				
KAN	23.5	21.1, 26.1										66.2	10.1	0.3	0.3	23.3		
NAL	0.0	0.0, 0.3						0.1	6.2	63.0	29.1	1.5	0.2					
FIS	82.3	80.0, 84.5											13.3	2.7	1.5	0.1	0.2	82.3
STR	71.0	68.3, 73.6												29.0	10.7	60.3		
TET	97.1	96.0, 98.0									2.8	0.1	0.5	24.8	71.8			
TIO	64.8	61.9, 67.5				0.4	11.5	16.1	1.5	1.6	4.3	28.6	36.2					

[a] For abbreviations refer to Table 1. AMI ($n = 802$) and AZI ($n = 350$) were tested with CMV1AGNF and CMV2AGNF panels respectively.

[b] 95% confidence interval for proportion of isolates resistant was calculated by using the exact binomial method.

[c] The unshaded areas indicate the range of dilutions tested for each antimicrobial. The vertical bars indicate CLSI breakpoints for resistance. Numbers in the gray-shaded areas indicate the percentages of isolates with MICs greater than the highest concentration tested. Numbers listed for the lowest tested concentration represent the percentages of isolates with MICs ≤ the lowest tested concentration.

Table 4
Antimicrobial resistance prevalence (95% CI) of *E. coli* isolated from fecal samples of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither cross tabulated by treatment group and period.

Antimicrobial agent[a]	Treatment group	Treatment period[b]			
		Before ($n=48$)[c]	During ($n=144$)[c]	After ($n=96$)[c]	P-value[d]
AMP	Control	79.2 (65.0, 89.5)	74.3 (66.4, 81.2)	76.0 (66.3, 84.2)	0.786
	CTC	77.1 (62.7, 88.0)	79.2 (71.6, 85.5)	60.4 (49.9, 70.3)	0.005
	Copper	91.7 (80.0, 97.7)	78.5 (70.9, 84.9)	53.1 (42.7, 63.4)	<0.001
	Copper + CTC	89.6 (77.3, 96.5)	81.3 (73.9, 87.3)	66.7 (56.3, 76)	0.003
	P-value[e]	0.126	0.549	0.007	
AZI[f]	Control	–	11.7 (4.8, 22.6); $n=60$	13.3 (3.8, 30.7); $n=30$	1.00
	CTC	–	3.5 (0.4, 12.1); $n=57$	6.7 (0.8, 22.1); $n=30$	0.606
	Copper	–	0.0 (0.0, 6.1); $n=59$	3.7 (0.09, 1.9); $n=27$	0.314
	Copper + CTC	–	1.8 (0.04, 9.4); $n=57$	16.7 (5.6, 34.7); $n=30$	0.017
	P-value[e]		0.011	0.379	
AUG	Control	72.9 (58.2, 84.7)	68.1 (59.8, 75.6)	72.9 (62.9, 81.5)	0.664
	CTC	77.1 (62.7, 88.0)	70.8 (62.7, 78.1)	56.3 (45.7, 66.4)	0.017
	Copper	91.7 (80.0, 97.7)	75 (67.1, 81.9)	52.1 (41.6, 62.4)	<0.001
	Copper + CTC	79.2 (65.0, 89.5)	78.5 (70.9, 84.9)	62.5 (52, 72.2)	0.016
	P-value[e]	0.085	0.199	0.017	
AXO	Control	66.7 (51.6, 79.6)	66.7 (58.3, 74.3)	71.9 (61.8, 80.6)	0.939
	CTC	72.9 (58.2, 84.7)	72.2 (64.2, 79.4)	56.3 (45.7, 66.4)	0.552
	Copper	75.0 (60.4, 86.4)	75.7 (67.9, 82.4)	52.1 (41.6, 62.4)	0.027
	Copper + CTC	77.1 (62.7, 88.0)	77.1 (69.3, 83.7)	61.5 (51.0, 71.2)	0.356
	P-value[e]	0.693	0.199	0.030	
CHL	Control	54.2 (39.2, 68.6)	41.0 (32.9, 49.5)	40.6 (30.7, 51.1)	0.237
	CTC	60.4 (45.3, 74.2)	21.5 (15.1, 29.1)	36.5 (26.9, 46.9)	<0.001
	Copper	54.2 (39.2, 68.6)	29.2 (21.9, 37.3)	34.4 (25, 44.8)	0.008
	Copper + CTC	58.3 (43.2, 72.4)	40.3 (32.2, 48.8)	38.5 (28.8, 49)	0.056
	P-value[e]	0.902	0.001	0.828	
FIS	Control	79.2 (65.0, 89.5)	78.5 (70.9, 84.9)	89.6 (81.7, 94.9)	0.059
	CTC	93.8 (82.8, 98.7)	81.3 (73.9, 87.3)	85.4 (76.7, 91.8)	0.102
	Copper	83.3 (69.8, 92.5)	71.5 (63.4, 78.7)	88.5 (80.4, 94.1)	0.004
	Copper + CTC	81.3 (67.4, 91.1)	81.9 (74.7, 87.9)	85.4 (76.7, 91.8)	0.731
	P-value[e]	0.153	0.134	0.752	
FOX	Control	70.8 (55.9, 83.0)	67.4 (59.1, 74.9)	62.5 (52.0, 72.2)	0.567
	CTC	75.0 (60.4, 86.4)	70.8 (62.7, 78.1)	45.8 (35.6, 56.3)	<0.001
	Copper	91.7 (80.0, 97.7)	75 (67.1, 81.8)	42.7 (32.7, 53.2)	<0.001
	Copper + CTC	72.9 (58.2, 84.7)	77.8 (70.1, 84.3)	60.4 (49.9, 70.3)	0.015
	P-value[e]	0.034	0.205	0.008	
GEN	Control	62.5 (47.4, 76.0)	40.3 (32.2, 48.8)	37.5 (27.8, 48)	0.011
	CTC	60.4 (45.3, 74.2)	29.2 (21.9, 37.3)	33.3 (24, 43.7)	0.093
	Copper	56.3 (41.2, 70.5)	39.6 (31.5, 48.1)	38.5 (28.8, 49.0)	0.001
	Copper + CTC	62.5 (47.4, 76.0)	47.9 (39.5, 56.4)	41.7 (31.7, 52.2)	0.060
	P-value[e]	0.914	0.012	0.692	
KAN	Control	8.3 (2.3, 20.0)	11.8 (7.0, 18.2)	34.4 (25, 44.8)	<0.001
	CTC	43.8 (29.5, 58.8)	24.3 (17.6, 32.1)	42.7 (32.7, 53.2)	0.003
	Copper	27.1 (15.3, 41.8)	13.2 (8.1, 19.8)	38.5 (28.8, 49.0)	<0.001
	Copper + CTC	31.3 (18.7, 46.3)	11.1 (6.5, 17.4)	20.8 (13.2, 30.3)	0.005
	P-value[e]	0.001	0.008	0.007	
STR	Control	58.3 (43.2, 72.4)	70.8 (62.7, 78.1)	71.9 (61.8, 80.6)	0.220
	CTC	75.0 (60.4, 86.4)	79.2 (71.6, 85.5)	63.5 (53.1, 73.1)	0.028
	Copper	66.7 (51.6, 79.6)	67.4 (59.1, 74.9)	71.9 (61.8, 80.6)	0.716
	Copper + CTC	87.5 (74.8, 95.3)	72.2 (64.2, 79.4)	66.7 (56.3, 76)	0.019
	P-value[e]	0.009	0.142	0.522	
SXT	Control	12.5 (4.7, 25.2)	0.7 (0.0, 3.8)	3.1 (0.6, 8.9)	0.002
	CTC	14.6 (6.1, 27.8)	2.1 (0.4, 6.0)	12.5 (6.6, 20.8)	0.001
	Copper	18.8 (8.9, 32.6)	0 .0 (0.0, 2.5)	0.0 (0.0, 3.8)	<0.001
	Copper + CTC	12.5 (4.7, 25.2)	0.0 (0.0, 2.5)	0.0 (0.0, 3.8)	<0.001
	P-value[e]	0.870	0.201	<0.001	
TET	Control	91.7 (80.0, 97.7)	94.4 (89.3, 97.6)	92.7 (85.6, 97.0)	0.694
	CTC	97.9 (88.9, 99.9)	100 (97.5, 100)	100 (96.2, 100)	0.167
	Copper	91.7 (80.0, 97.7)	98.6 (95.1, 99.8)	97.9 (92.7, 99.7)	0.058
	Copper + CTC	97.9 (88.9, 99.9)	100 (97.5, 100)	95.8 (89.7, 98.9)	0.022
	P-value[e]	0.322	0.001	0.027	

Table 4 (*Continued*)

Antimicrobial agent[a]	Treatment group	Treatment period[b]			
		Before (n = 48)[c]	During (n = 144)[c]	After (n = 96)[c]	P-value[d]
TIO	Control	66.7 (51.6, 79.6)	65.3 (56.9, 73.0)	61.5 (51, 71)	0.774
	CTC	68.8 (53.7, 81.3)	70.1 (62.0, 77.5)	49 (38.6, 59.4)	0.003
	Copper	75.0 (60.4, 86.4)	72.9 (64.9, 78.0)	41.7 (31.7, 52.2)	<0.001
	Copper + CTC	68.8 (53.7, 81.3)	75.7 (67.9, 82.4)	59.4 (48.9, 69.3)	0.028
	P-value[e]	0.824	0.249	0.019	

[a] AMP = ampicillin, AZI = azithromycin, AUG = amoxicillin/clavulanic acid, AXO = ceftriaxone, CHL = chloramphenicol, FIS = sulfisoxazole, FOX = cefoxitin, GEN = gentamicin, KAN = kanamycin, STR = streptomycin, SXT = trimethoprim/sulfamethoxazole, TET = tetracycline, TIO = ceftiofur. Results were not shown for amikacin, ciprofloxacin and nalidixic acid since all isolates were susceptible by binary classification.
[b] Sampling days were categorized into treatment periods as pretreatment (day 0); treatment (days 7, 14, and 21) and post-treatment (days 28 and 35).
[c] Number of *E. coli* isolates per treatment group per treatment period.
[d] Likelihood ratio chi-square (LR χ^2) P-value with 2 df comparing treatment periods by treatment group.
[e] LR χ^2 P-value with 3 df comparing treatment groups by treatment period.
[f] Azithromycin was tested only on 350 isolates using NARMS CMV2AGNF panel. Amikacin was tested on 802 isolates by using CMV1AGNF.

[95% CI = 91–97%] vs. 88% [95% CI = 84–91%], respectively) (Fig. 1A).

3.3. Determination of copper susceptibility

The results from both agar dilution and broth microdilution methods we used on selected isolates from each copper MIC distribution were comparable with respect to dilution. The median MIC was 20 mM, ranging from 12 to 32 mM (essentially, the limits of our assay) regardless of the treatment groups. Fig. 1B shows copper MIC frequency distribution subgraphed by treatment group. The MIC_{50} and MIC_{90} of the *E. coli* were 20 mM and 28 mM across all the isolates, respectively. Unexpectedly, using the log rank test *E. coli* from the control group exhibited survival at higher concentrations (thus, were more resistant to copper) (P = 0.027, 3 df) when compared with the treatment groups across all periods. As expected, isolates obtained during the treatment period exhibited higher survival concentrations (i.e., were less susceptible) (P < 0.0001). *E. coli* isolates in the CTC-only supplemented group obtained during treatment period had reduced survival in copper (P = 0.0018) compared with *E. coli* obtained from the no-CTC group. Using discrete time survival analysis, the CTC by period interaction showed a significant association with the survival of the *E. coli* isolates; that is, *E. coli* isolates from the CTC-supplemented group obtained during the treatment period (HR = 7 [95% CI = 2.5–19.5]; P < 0.001) and post-treatment period (HR = 4.1; 95% CI = 1.2–14.2; P = 0.025) exhibited increased susceptibility to copper when compared with isolates from the control group.

3.4. Prevalence of resistance genes

Across all treatments, unadjusted prevalence of the resistance genes in order of magnitude was bla_{CMY-2} (72% [95% CI = 69–75%]), *tet*A (66% [95% CI = 64–69%]), *tet*B (48% [95% CI = 45–51%]), *pco*D (16% [95% CI = 14–18%]), *tet*C 2.1% (95% CI = 1.3–3.1%) and *tet*E 1.6% (95% CI = 0.9–2.5%). In unadjusted analyses, prevalence of *tet*A was significantly higher among the *E. coli* isolates obtained from copper plus CTC – supplemented pigs during the treatment period (76% vs. 59%; P = 0.019) compared with isolates obtained from the copper only group. *tet*A was also significantly (72% vs. 51%; P = 0.024) higher in the isolates obtained from the copper plus CTC – supplemented group in the post-treatment period compared with isolates obtained from CTC-only – supplemented pigs during this period. The prevalence of *tet*B was higher among *E. coli* isolates obtained from pigs supplemented with CTC (50%) or copper (50%) during the treatment period (P = 0.004) compared with the control (35%) and copper plus CTC (35%) groups. The prevalence of *tet*C decreased during treatment and post treatment periods compared to the baseline in the copper (P = 0.004) and copper plus CTC (P < 0.001) supplemented groups. *tet*E was detected more frequently from the copper supplemented group (Table 7).

Prevalence of *tet*A and bla_{CMY-2} exhibited a decreasing trend through subsequent treatment periods, regardless of treatment group; in contrast, the prevalence of *tet*B greatly increased across the same treatment periods. The prevalence of *pco*D decreased significantly (P = 0.007) through subsequent treatment periods only in the control pigs (Table 7). *tet*A and bla_{CMY-2} were positively and strongly associated with each other; in turn, these were negatively associated with both *tet*B and *pco*D. The latter two genes were also positively associated with each another (Table 8).

Model-adjusted prevalence from multilevel mixed-effects logistic regression analysis for each of the resistance genes is plotted across treatment periods in Fig. 2. Prevalence of *tet*A decreased through subsequent treatment periods; that is, it was significantly lower during the treatment (P = 0.001; odds ratio (OR) 0.15 [95% CI = 0.05–0.46]) and post-treatment periods (P < 0.001; OR = 0.08 [95% CI = 0.02–0.26]) when compared with day 0 isolates with no treatment effect. The isolates obtained from the copper-supplemented group were 2.6 (95% CI = 1.0–7.0; P = 0.05) times more likely to have *tet*B than isolates from the control group using OR as the measure of association. Similarly, CTC supplementation was significantly associated with an increased prevalence of *tet*B compared to the control group (OR = 3.3 [95% CI = 1.2–8.7]; P = 0.017). Paradoxically, copper plus CTC supplementation (i.e., treatment interaction) was significantly associated with decreased prevalence of *tet*B (OR = 0.14 [95% CI = 0.04–0.57], P = 0.006) when compared with the control groups. *E. coli* isolates in the post-treatment period exhibited a higher prevalence of *tet*B (P = 0.024), which was 2.5 (95% CI = 1.13–5.5)

Table 5
Prevalence (%) across all treatment periods of major antimicrobial resistance phenotypes of *E. coli*, isolated from weaned pigs fed diets supplemented with chlortetracycline (CTC), copper, both or neither.

No. of antimicrobials	Phenotype[a]	Treatment group				Overall (n = 1152)
		Control (n = 288)	Copper (n = 288)	CTC (n = 288)	Copper + CTC (n = 288)	
0	Pansusceptible	0.35	0.00	0.00	0.69	0.26
1	tet	7.64	5.56	2.78	6.60	5.64
	Other phenotypes (amp, fis, str)	0.69	0.69	0.00	0.35	0.43
2	fis_tet	0.69	2.08	1.39	1.74	1.48
	Other phenotypes (amp_fis, fis_fox, kan_tet, str_tet)	2.43	1.04	1.39	1.39	1.56
3	fis_kan_tet	1.74	4.86	9.72	3.47	4.95
	chl_fis_tet	0.35	0.69	0.69	0.35	0.52
	fis_gen_tet	2.08	0.69	0.69	1.74	1.30
	Other phenotypes (too numerous to list)	2.78	1.04	0.00	0.35	1.04
4	fis_kan_str_tet	4.86	10.42	8.33	5.21	7.20
	amp_fis_str_tet	1.39	1.39	2.43	1.74	1.74
	fis_gen_str_tet	1.04	1.74	0.00	1.74	1.13
	Other phenotypes (too numerous to list)	2.43	0.00	1.39	0.69	1.13
5	amp_chl_fis_str_tet	0.35	0.00	1.04	0.00	0.35
	chl_fis_kan_str_tet	0.35	0.00	1.04	0.00	0.35
	Other phenotypes (too numerous to list)	1.04	0.00	1.39	0.35	0.69
6	amp_aug_axo_fox_tet_tio	4.17	7.29	5.56	4.17	5.30
	Others phenotypes (too numerous to list)	1.04	1.39	1.39	1.39	1.30
7	amp_aug_axo_fis_fox_tet_tio	4.86	5.90	0.69	4.17	3.91
	amp_axo_fis_fox_str_tet_tio	0.69	0.00	0.69	0.69	0.52
	Other phenotypes (too numerous to list)	3.47	2.08	1.04	1.74	2.08
8	amp_aug_axo_fis_fox_str_tet_tio	7.99	9.38	15.28	14.24	11.72
	amp_aug_axo_chl_fis_gen_str_tet	0.35	1.74	1.04	1.04	1.04
	amp_aug_axo_chl_fis_str_tet_tio	0.69	0.69	0.69	0.35	0.61
	amp_aug_axo_fox_gen_str_tet_tio	2.78	5.21	5.21	2.78	3.99
	Other phenotypes (too numerous to list)	2.08	3.13	1.74	1.04	2.00
9	amp_aug_axo_fis_fox_kan_str_tet_tio	1.74	1.04	3.47	1.74	2.00
	amp_aug_axo_chl_fis_fox_gen_str_tet	1.74	1.74	1.04	0.69	1.30
	amp_aug_axo_chl_fis_fox_str_tet_tio	4.51	0.69	1.74	0.00	1.74
	amp_aug_axo_chl_fis_gen_str_tet_tio	1.39	0.35	1.04	0.35	0.78
	amp_aug_axo_fis_fox_gen_str_tet_tio	0.69	0.35	0.69	0.35	0.52
	Other phenotypes (too numerous to list)	1.04	0.00	1.04	0.00	0.52
10	amp_aug_axo_chl_fis_fox_gen_str_tet_tio	22.57	22.57	12.85	31.94	22.48
	amp_aug_axo_chl_fis_fox_str_sxt_tet_tio	0.00	0.00	2.43	0.35	0.69
	amp_aug_axo_fis_fox_gen_kan_str_tet_tio	2.78	1.39	3.13	1.04	2.08
	Other phenotypes (too numerous to list)	1.39	0.00	0.00	0.00	0.35
11	amp_aug_axo_chl_fis_fox_gen_str_sxt_tet-tio	2.78	1.74	3.47	1.39	2.34
	amp_aug_axo_chl_fis_fox_gen_kan_str_tet_tio	0.35	2.08	1.74	3.82	2.00
	Other phenotypes (too numerous to list)	0.35	0.00	1.04	0.00	0.35
12	amp_aug_axo_chl_fis_fox_gen_kan_str_sxt_tet_tio	0.35	1.04	0.69	0.35	0.61
	Total	100	100	100	100	100

[a] amp = ampicillin, aug = amoxicillin/clavulanic acid, axo = ceftriaxone, chl = chloramphenicol, fis = sulfisoxazole, fox = cefoxitin, gen = gentamicin, kan = kanamycin, str = streptomycin, sxt = trimethoprim/sulfamethoxazole, tet = tetracycline, tio = ceftiofur.

times higher than for baseline samples. The prevalence of *pco*D was significantly lower among isolates during treatment (OR = 0.38 [95% CI = 0.14–0.99]; P = 0.047) and post-treatment periods (OR = 0.34 [95% CI = 0.12–0.95]; P = 0.040) compared with pretreatment levels. Similar to *tet*A, the prevalence of bla_{CMY-2} was significantly lower in the isolates from the post-treatment period compared with baseline isolates (OR = 0.2 [95% CI = 0.07–0.55]; P = 0.002).

Genotypic profiles for four of the resistance genes (excluding the low prevalent *tet*C and *tet*E genes) detected among *E. coli* (n = 1152) are shown in Fig. 3. The most common singly carried gene was *tet*B (11%); on the other hand, *tet*A was the gene most commonly (38%) found in association with the bla_{CMY-2} gene. The most common three-gene profile observed was *tet*A-*tet*B-bla_{CMY-2}, accounting for 14% of the isolates tested. Only about 4% of the isolates contained all the four genes together, and 2% of the isolates carried none of the four genes.

At least one of the four *tet* (A, B, C, and E) genes was detected in 97% (1117 of 1152) of the *E. coli* isolates.

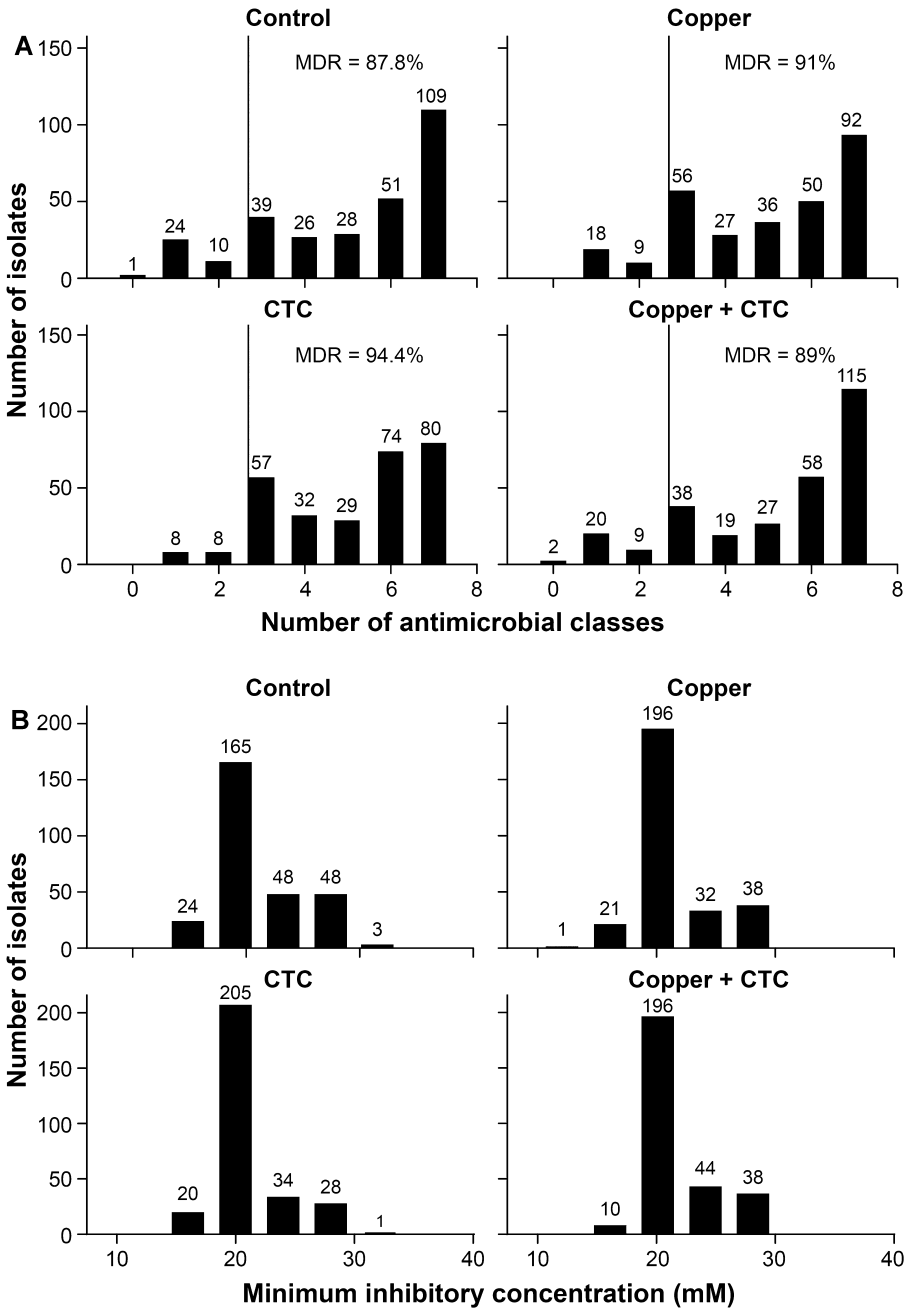

Fig. 1. Multidrug resistance (MDR) and copper minimum inhibitory concentration (MIC) distributions of *E. coli* isolates ($n = 1152$) obtained from feces of pigs by treatment group ($n = 288$/group). (A) Frequency distribution of MDR *E. coli*. Vertical bars at ≥3 represent cutoff value for MDR. (B) Frequency distribution of copper MIC of *E. coli* isolates.

Among these ($n = 1117$), about half of the tetracycline resistance was conferred by *tet*A (49%), followed closely by *tet*B (29.5%) and 19% of the isolates carried both *tet*A and *tet*B genes together. The presence of *tet*A and *tet*B, singly or in combination, explained the majority of the observed phenotypic resistance to tetracycline, accounting for 99% of the isolates ($n = 1117$) with at least one *tet* gene and 96% of all the isolates tested ($n = 1152$). The *tet*E gene was carried alone in 12 (67%) of the 18 isolates that were positive for *tet*E. The *tet*C gene, however, was always found along with *tet*A or *tet*B but never with *tet*E. Except for *tet*C, these genes were mostly found individually in the *E. coli* isolates. Out of 33 isolates that were phenotypically susceptible to tetracycline, 22 (67%) of them did not carry any of the four *tet* genes. Out of 35 isolates in which none of the *tet* genes were detected, 13 (37%) were phenotypically resistant to tetracycline. Among phenotypically tetracycline-resistant *E. coli* ($n = 1119$), 99% were positive for one or more of the

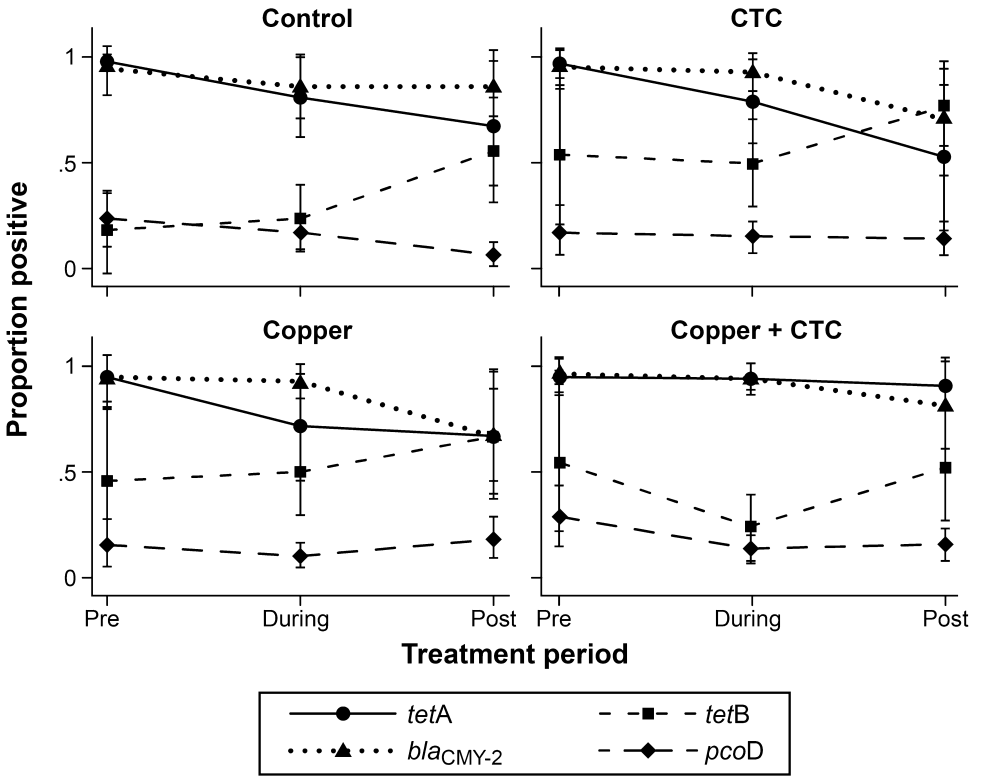

Fig. 2. Adjusted predictions (with 95% CI) of the resistance genes detected from *E. coli* ($n = 1152$) obtained from fecal samples of pigs supplemented with chlortetracycline (CTC) or copper. *tet*A (solid lines), *tet*B (dashed lines), bla_{CMY-2} (dotted lines) and *pco*D (long dashes with dots).

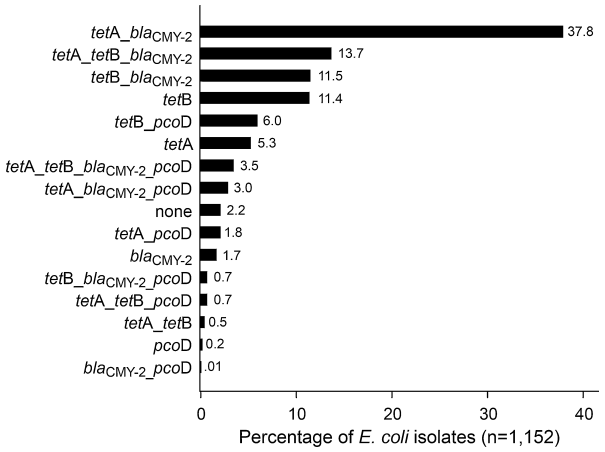

Fig. 3. Distribution of genotypic profiles of 4 resistance genes detected from *E. coli* isolates ($n = 1152$) obtained from feces of weaned pigs supplemented with chlortetracycline, copper both.

four *tet* genes tested. A duplex PCR targeting only *tet*A and *tet*B would explain 99% of the phenotypic tetracycline resistance ($n = 1119$) in this study.

3.5. Associations between resistance genotypes and MDR

Among *E. coli* isolates ($n = 1043$) that were resistant to ≥3 antimicrobial classes, 41% of them had *tet*A and bla_{CMY-2} genes together. The presence of *tet*A (96% [95% CI = 94–97%] vs. 81% [95% CI = 76–85%]) and bla_{CMY-2} (98% [95% CI = 97–99%] vs. 72% [95% CI = 66–76%]) were significantly ($P < 0.001$) and positively associated with isolates being resistant to three or more antimicrobial classes. On the other hand, the presence (vs. absence) of *tet*B (87% [95% CI = 84–89%] vs. 94% [95% CI = 92–96%]) and *pcoD* (69% [95% CI = 62–76%] vs. 95% [95% CI = 93–96%]) were significantly and negatively associated with MDR. The presence of *tet*B and *pco*D genes together was significantly ($P < 0.001$) associated with a reduced prevalence of MDR classification among the isolates. On the basis of the number of antimicrobials (theoretically, from 0 to 15) to which isolates were resistant, the median MDR count was the highest when the bla_{CMY-2} gene was found in association with either *tet*A or *tet*B (Fig. 4).

4. Discussion

We evaluated the impact of CTC and copper supplementation in weaned pigs on antimicrobial and copper resistance in fecal *E. coli*. The treatment period (i.e., 3 weeks vs. two weeks) was deemed necessary to ensure sufficient time for both antimicrobial treatments (especially copper) to have a meaningful impact on the bacterial ecosystem of the gut. Importantly, extending all treatments to three weeks maintained balance among the trial arms, reflecting comparable selection pressure periods. In our experimental design there could be cross contamination between

Table 6
Model-defined pairwise correlations (with 95% CI) between phenotypic AMR of *E. coli* ($n = 1152$), obtained from fecal samples of weaned pigs fed diets supplemented with chlortetracycline (CTC), copper, both or neither against different antimicrobials included in the multivariate probit analysis and adjusted for clustering by pen.

	AMP[a,b]	AUG	AXO	CHL	FIS	FOX	GEN	STR	TIO
AMP	1								
AUG	0.94(0.87, 0.97)	1							
AXO	0.95(0.9, 0.97)	0.97(0.95, 0.99)	1						
CHL	0.54(0.45, 0.62)	0.56(0.46, 0.64)	0.55(0.46, 0.63)	1					
FIS	0.28(0.10, 0.43)	0.28(0.11, 0.44)	0.30(0.12, 0.47)	0.61(0.51, 0.70)	1				
FOX	0.90(0.83, 0.94)	0.94(0.92, 0.96)	0.94(0.91, 0.96)	0.49(0.37, 0.59)	0.26 (0.08, 0.44)	1			
GEN	0.57(0.47, 0.66)	0.59(0.49, 0.68)	0.61(0.51, 0.69)	0.75(0.66, 0.81)	0.41 (0.24, 0.56)	0.5 (0.38, 0.61)	1		
STR	0.59(0.51, 0.66)	0.59(0.52, 0.65)	0.59(0.51, 0.67)	0.69(0.61, 0.76)	0.66 (0.54, 0.76)	0.52 (0.42, 0.62)	0.72 (0.64, 0.79)	1	
TIO	0.94(0.89, 0.97)	0.96(0.92, 0.98)	0.98(0.97, 0.99)	0.49(0.38, 0.59)	0.28 (0.09, 0.44)	0.95 (0.93, 0.97)	0.52 (0.39, 0.63)	0.53 (0.43, 0.63)	1

[a] All pairwise correlations were significant ($P \leq 0.004$). AMP = ampicillin, AUG = amoxicillin/clavulanic acid, AXO = ceftriaxone, CHL = chloramphenicol, FIS = sulfisoxazole, FOX = cefoxitin, GEN = gentamicin, STR = streptomycin, TIO = ceftiofur.

[b] Amikacin (AMI), kanamycin (KAN), trimethoprim/sulfamethoxazole (SXT), azithromycin (AZI), ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline (TET) were excluded from the model because of sparse cell counts.

Fig. 4. Median MDR count of *E. coli* ($n = 1152$) on the basis of the number of antimicrobials. (A) Box plot depicting genotypic profiles (*tet*A, *tet*B, and bla_{CMY-2}) vs. number of antimicrobial agents. A = *tet*A, B = *tet*B, and CMY2 = bla_{CMY-2}; *n* represents the number of isolates with the unique genotypic combination. (B) Bar graph of median number of antimicrobials phenotypically resistant vs. profiles of four resistance genes (*tet*A, *tet*B, and bla_{CMY-2} and *pco*D). Numbers on the bar graph represent the number of isolates with unique genotypic profiles.

pens that might have affected the results by reducing the power of observed effects of treatments. To make the effect of cross contamination uniform across pens we blocked the experiments by geographical location within each barn. Secondly the experiments were randomly allocated to the pens-not in a systematic way. This set up reduces the bias associated with cross contamination; that is to say, any effect of cross contamination is similar in all the experimental groups and likely to be biased toward the null if present.

Greater amounts of in-feed antibiotics and copper are commonly applied in nursery pigs, as compared to later phases of pig production, in order to prevent, control, and treat disease, or to enhance growth (Dewey et al., 1999; Cromwell, 2002; Shryock and Page, 2006; Apley et al., 2012). Resistance to most antibiotics tested decreased over time when CTC and copper were supplemented – either alone or in combination – compared with the control group. Similar results have been reported across agricultural production species and systems, pointing to strong and generalized associations between isolates derived from neonatal animals and higher MDR phenotypes (Langlois et al., 1986, 1988; Dewulf et al., 2007; Berge et al., 2010).

Despite high background resistance observed at baseline and in control pigs, CTC supplementation did significantly ($P < 0.05$) increase both phenotypic and genotypic tetracycline resistance as was also previously reported (Dewulf et al., 2007). In one reported on-farm trial (Funk et al., 2006), subtherapeutic use of CTC in grower pigs was significantly associated with increased levels of tetracycline resistance in Gram negative aerobic fecal flora.

Copper supplementation was significantly associated with reduced resistance to most of the antibiotics tested, including cephalosporins. Thus copper may prove to be a promising replacement for antimicrobial growth promoters (AGPs) in pigs (Verstegen and Williams, 2002). In this nursery pig population involved in our study both elevated level of copper ($P < 0.07$) and dietary CTC ($P < 0.01$) supplementations were associated with improved average daily gain and average daily feed intake during the treatment period (d 0–21) compared with the control pigs provided only with basal copper (Amachawadi et al., 2011b). The fact that copper supplementation, or the presence of *pco*D, was not associated with a shift in copper MIC distribution of the *E. coli* isolates remains a paradoxical finding. The absence of significant treatment effects on the level of *pco*D detection suggest that the levels of copper supplementation used in our trial did not result in acquired copper resistance mediated through the *pco* gene. This result contrasts sharply with those seen for Gram-positive enterococci in pigs and cattle. When copper was supplemented at high levels, this resulted in expansion of those isolates that harbored the plasmid-borne *tcr*B gene (Amachawadi et al., 2010, 2011a, 2013). A combination of both plasmid and chromosomal genes are required for copper resistance in *E. coli*, suggesting that resistance to high copper concentrations can be achieved while maintaining the intracellular copper concentrations required for normal physiological functions (Williams et al., 1993).

The high doses of copper (100–250 ppm) used in U.S. pig production (Hasman et al., 2006) for over six decades (Williams et al., 1993), and exposure to excreted copper in the ambient environment, may have led enteric Gram-negative bacteria to become intrinsically adapted to higher copper concentrations (Aarestrup and Hasman, 2004). Gram negative enteric bacteria (*E. coli* and *Salmonella*) have been found by others to tolerate higher concentrations of copper, likely through chromosomally mediated mechanisms, than the Gram positive enteric bacteria (enterococci) (Aarestrup and Hasman, 2004). Experimental studies on copper susceptibilities in *E. coli* are lacking; most studies are based on isolate banks, often from diagnostic laboratory submissions. Nevertheless, the MIC distribution of the *E. coli* isolates that we studied is almost identical to that reported from livestock-associated *E. coli* in Denmark derived from diagnostic laboratory submissions (Aarestrup and Hasman, 2004). Standardized breakpoints for copper susceptibility testing are somewhat lacking. Based on a previous study (Williams et al., 1993) that identified copper resistant *E. coli* isolates as those able to grow at 18 mM of $CuSO_4$, 93% of our isolates with an MIC of $\geq$20 mM would have been categorized as resistant. Despite the fact that elevated copper supplementation in our study did not appear to favor *pco*D-borne isolates, the possibility remains, based on observed genotypic associations, to select against resistance to critically important antibiotics such as 3rd generation cephalosporins.

As mentioned earlier, *E. coli* isolates generally were highly multidrug resistant. Greater than 88% of the isolates ($n = 288$) from each of the treatment groups (Fig. 1A) were resistant to at least 3 antimicrobial classes and, surprisingly, 38%, 28%, 32% and 40% of the isolates ($n = 288$) from the control, CTC, copper and copper plus CTC groups, respectively, were resistant to 7 (out of 8) antimicrobial classes. Ceftiofur and tetracycline resistances both were associated with higher MDR counts. Ceftiofur-resistant isolates had a median MDR of nine antimicrobial agents, compared with the median of four for a typical ceftiofur-susceptible isolate. Similarly, median MDR was 8 and 3 among tetracycline-resistant and susceptible *E. coli*, respectively. The median number of antibiotics to which both tetracycline- and ceftiofur-resistant isolates were resistant was also nine, compared with a median of two antimicrobials for isolates susceptible to both drugs. The percentage (36%; $n = 1152$) of *E. coli* isolates that specifically included penta-resistant ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline) in their resistance phenotype was higher than that reported for *E. coli* isolates from feedlot cattle (Platt et al., 2008). This observation was corroborated by high resistance observed (Table 3) to these older antimicrobials, all of which have been used extensively in animals and which could have led to their co-selection. About 89% of the isolates with ACSSuT were also co-resistant to ceftiofur, similar to reports from cattle *E. coli* (Lowrance et al., 2007; Platt et al., 2008). Because CTC supplementation significantly increased tetracycline resistance (both phenotypically and genotypically), ceftiofur and tetracycline resistances were strongly associated ($P < 0.001$; OR = 5.1[95% CI = 2.4–11.2]), and *tet*A and bla_{CMY-2} were also positively associated, it can be inferred that direct selection for tetracycline resistance also co-selects for ceftiofur-resistant *E. coli* in this swine population (Funk et al., 2006). Logically, ceftiofur use should also select for tetracycline resistance (Lowrance et al., 2007; Akwar et al., 2008a); however, it was not used in this facility during the trial period and thus cannot be evaluated here.

The highest prevalent gene observed throughout the present study (compared with other genes) was that of bla_{CMY-2}. The significant association of bla_{CMY-2} with higher level of MDR, especially in the absence of selective pressure exerted by ceftiofur use in our study population, suggests its expansion and maintenance is achieved through genetic linkage with other antimicrobial resistance genes (particularly with *tet*A). However, factors that contribute to long-term maintenance of the bla_{CMY-2} gene in the absence of ceftiofur use remain unclear, especially when considering issues of relative strain fitness (Lutz et al., 2011; Schmidt et al., 2013). The bla_{CMY-2} gene is commonly associated with large plasmids that can also carry other antimicrobial resistance genes (Rankin et al., 2002) such as *tet*A (Call et al., 2010); thus, selection pressure exerted by other antimicrobials such as tetracyclines could result in the expansion, dissemination, and persistence of the bla_{CMY-2} gene (Lutz et al., 2011).

Table 7
Prevalence (%) of resistance genes among fecal *E. coli* isolates ($n = 1152$) from weaned pigs fed diets supplemented with chlortetracycline (CTC), copper, both or neither cross-tabulated by treatment group and period.

Resistance gene	Treatment group	Treatment period[a,*]			
		Before ($n = 48$)[d]	During ($n = 144$)[d]	After ($n = 96$)[d]	*P*-value[b]
*tet*A	Control	89.6 (77.3, 96.5)	64.6 (56.2, 72.4)	58.3 (47.8, 68.3)	<0.001
	CTC	81.3 (67. 4, 91.1)	63.2 (54.8, 71.1)	51.0 (40.6, 61.4)	0.001
	Copper	77.1 (62.7, 88.0)	59.0 (50.5, 67.1)	57.3 (46.8, 67.3)	0.041
	Copper + CTC	79.2 (65, 89.5)	75.7 (67.9, 82.4)	71.9 (61.8, 80.6)	0.610
	P-value[c]	0.371	0.019	0.024	
*tet*B	Control	29.2 (17.0, 44.1)	35.4 (27.6, 43.8)	53.1 (42.7, 63.4)	0.005
	CTC	52.1 (37.2, 66.7)	50.0 (41.6, 58.4)	65.6 (55.2, 75.0)	0.048
	Copper	47.9 (33.3, 62.8)	50.0 (41.6, 58.4)	59.4 (48.9, 69.3)	0.274
	Copper + CTC	52.1 (37.2, 66.7)	34.7 (27.0, 43.1)	51.0 (40.6, 61.4)	0.016
	P- value[c]	0.067	0.004	0.161	
*tet*C	Control	4.2 (0.5, 14.3)	1.4 (0.2, 4.9)	0.0 (0.0, 3.8)	0.111
	CTC	4.2 (0.5, 14.3)	0.7 (0.0, 3.8)	1.0 (0.0, 5.7)	0.167
	Copper	6.3 (1.3, 17.2)	0.0 (0.0, 2.5)	0.0 (0.0, 3.8)	0.004
	Copper + CTC	18.8 (8.9, 32.6)	2.8 (0.8, 7.0)	0.0 (0.0, 3.8)	<0.001
	P- value[c]	0.044	0.226	1.000	
*tet*E	Control	0.0 (0.0, 7.4)	2.8 (0.8, 7.0)	0.0 (0.0, 3.8)	0.228
	CTC	0.0 (0.0, 7.4)	0.7 (0.0, 3.8)	2.1 (0.3, 7.3)	0.581
	Copper	4.2 (0.5, 14.3)	4.2 (1.5, 8.8)	1.0 (0.0, 5.7)	0.350
	Copper + CTC	0.0 (0.0, 7.4)	1.4 (0.2, 4.9)	0.0 (0.0, 3.8)	0.666
	P-value[c]	0.132	0.231	0.622	
bla_{CMY-2}	Control	77.1 (62.7, 88.0)	69.4 (61.2, 76.8)	70.8 (60.7, 79.7)	0.586
	CTC	79.2 (65.0, 89.5)	75.0 (67.1, 81.8)	60.4 (49.9, 70.3)	0.007
	Copper	77.1 (62.7, 88.0)	76.4 (68.6, 83.1)	58.3 (47.8, 68.3)	0.021
	Copper + CTC	83.3 (69.8, 92.3)	79.2 (71.6, 85.5)	65.6 (55.2, 75.0)	0.023
	P-value[c]	0.854	0.283	0.267	
*pco*D	Control	25.0 (13.6, 39.6)	18.8 (12.7, 26.1)	7.3 (3.0, 14.4)	0.007
	CTC	18.8 (8.9, 32.6)	15.3 (9.8, 22.2)	14.6 (8.2, 23.3)	0.170
	Copper	16.7 (7.5, 30.2)	11.1 (6.5, 17.4)	19.8 (12.4, 29.2)	0.781
	Copper + CTC	29.2 (17.0, 44.1)	13.9 (8.7, 20.6)	15.6 (9.0, 24.5)	0.057
	P-value[c]	0.458	0.338	0.083	

[*] Mean proportions of isolates and exact binomial 95% confidence interval.
[a] Treatment period (before = day 0), during (days 7, 14, and 21), and after (days 28 and 35).
[b] LR χ^2 *P*-value with 2 df comparing treatment periods by treatment group.
[c] LR χ^2 *P*-value with 3 df comparing treatment group by treatment period.
[d] Number of *E. coli* isolates per treatment group per treatment period.

Previous studies also reported that the use of CTC in the diet of pigs was positively associated with the occurrence of MDR Gram-negative bacteria (Dawson et al., 1983; Funk et al., 2006). This is explained by the observation that *tet*A and bla_{CMY-2} were positively associated with both phenotypic and genotypic ceftiofur resistance, further supporting the premise that these two genes are usually co-localized on a single mobile genetic element such as a plasmid, a transposon, or an integron (Boerlin et al., 2005). Previous reports have typically found both genes harbored on the same IncA/C plasmid in cattle (Call et al., 2010). In contrast, *tet*B and *pco*D were instead associated with ceftiofur-susceptible isolates, both phenotypically and genotypically. One of the most remarkable features we observed was that MDR was typically associated with either bla_{CMY-2} or *tet*A; on the other hand, the presence of either of *tet*B or *pco*D was associated with lower MDR.

We observed that individual supplementation of CTC or copper significantly expanded *tet*B, with no significant effect on *tet*A. Furthermore, copper plus CTC supplementation increased the prevalence of *tet*A, while decreasing that of *tet*B. Importantly, when examining multiple genes that exhibit negative correlations with one another, and when each gene explains a similar phenotype among isolates, gene substitution is to be expected. In other words,

Table 8
Model-defined pairwise correlations (with 95% CI) based on a multivariate probit model, among four resistance genes in fecal *E. coli* ($n = 1152$) from weaned pigs fed diets supplemented with chlortetracycline, copper, both or neither.

	tetA	tetB	bla_{CMY-2}	pcoD
tetA	1			
tetB	−75.3 (−81.0, −67.8)	1		
pcoD	−20.9 (−38.0, −2.2)	33.4 (18.9, 46.4)	−39.7 (−53.5, −23.9)	1
bla_{CMY-2}	63.5 (50.4, 73.8)	−38.5 (−51.7, −23.4)	1	

when prevalence (expressed as a proportion) of one *tet* gene goes up, in the absence of true statistical independence, the other must go down. As a result, explaining which gene was selected for and which was selected against remains difficult.

Another salient feature of the two *tet* genes is that whereas the prevalence of *tet*A seemed to decrease through time – irrespective of treatment groups – the prevalence of *tet*B increased. This longer-term secular trend also suggests a form of gene substitution at the population – though not at the individual isolate – level among isolates. Put another way, this substitution does not occur within the isolates themselves; rather, it implies that isolates with *tet*B tended to outcompete those with *tet*A over time. The features that result in a preferred ecological niche for the former may well have nothing to do with the specific *tet* genes themselves; however, as has been observed elsewhere (Langlois et al., 1988; Berge et al., 2010), MDR is most common in neonatal farm animals and typically trends downward through time, and in our study *tet*A was significantly associated with higher MDR than *tet*B.

The presence of *tet*B conferred a higher level of tetracycline resistance in that the prevalence of *tet*B (61% [95% CI = 58–64%]) was significantly higher among *E. coli* isolates ($n = 827$) with MIC values >32 μg/ml than in isolates ($n = 325$) with MIC values of ≤32 μg/ml (15% [95% CI = 11–19%]). This was in contrast to *tet*A (90% [95% CI = 86–93%] vs. 57% [95% CI = 53–60%]) and bla_{CMY-2} (85% [95% CI = 81–89%] vs. 67% [95% CI = 64–70%]) which were significantly more likely in isolates with tetracycline MIC values ≤32 μg/ml than in isolates with MIC values >32 μg/ml, respectively. This, perhaps paradoxically, suggests that use of higher sustained levels of tetracycline could favor lower MDR isolates harboring *tet*B vs. higher MDR isolates with *tet*A and bla_{CMY-2}. On the other hand, using lower levels of tetracycline, such as by feeding tetracyclines for growth promotion, could result in a greater expansion of the high MDR fraction, or the fraction of *E. coli* harboring a gene coding for resistance to a critical antimicrobial such as 3rd generation cephalosporins (e.g., ceftiofur and ceftriaxone).

5. Conclusion

The *E. coli* derived from weaned pigs exhibited a very high prevalence of resistance to most of the antimicrobials studied, including cephalosporins, and were highly multi-drug resistant, exhibiting a diverse range of resistance profiles. As expected, CTC supplementation increased already high levels of tetracycline resistance, possibly through expansion of the *E. coli* population harboring *tet*B over *tet*A; gene substitution at the bacterial population level, as weaned pigs grow older, likely explains the relative increase in *tet*B prevalence. The roles of copper supplementation in swine production, and *pco*-mediated copper resistance in *E. coli* in particular, need to be further explored; this, because strong negative associations with *tet*A and bla_{CMY-2} genes point to potential opportunities to select for a more innocuous tetracycline resistance profile among the gut bacteria.

Conflict of interest

The authors declare no conflict of interest.

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