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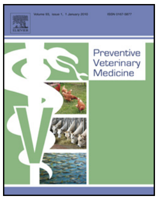

Effects of chlortetracycline and copper supplementation on antimicrobial resistance of fecal *Escherichia coli* from weaned pigs

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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*$_{\text{CMY-2}}$ 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*$_{\text{CMY-2}}$ 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*$_{\text{CMY-2}}$ 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 ($bla_{\text{CMY-2}}$) 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