EFFECTS OF CHLORTETRACYCLINE AND COPPER SUPPLEMENTATION ON LEVELS OF ANTIMICROBIAL RESISTANCE IN THE FECES OF WEANED PIGS

by

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DVM, Addis Ababa University, 2003
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Department of Diagnostic Medicine/Pathobiology
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Abstract

The use of antibiotics in food animals is of major concern as a purported cause of antimicrobial resistance (AMR) in human pathogens; as a result, alternatives to in-feed antibiotics such as heavy metals have been proposed. The effect of copper and CTC supplementation in weaned pigs on AMR in the gut microbiota was evaluated. Four treatment groups: control, copper, chlortetracycline (CTC), and copper plus CTC were randomly allocated to 32 pens with five pigs per pen. Fecal samples (n = 576) were collected weekly from three pigs per pen over six weeks and two *Escherichia coli* isolates per sample were tested phenotypically for antimicrobial and copper susceptibilities and genotypically for the presence of tetracycline (*tet*), copper (*pco*D) and ceftiofur (bla_{CMY-2}) resistance genes. CTC-supplementation significantly increased tetracycline resistance and susceptibility to copper when compared with the control group. Copper supplementation decreased resistance to most of the antibiotics, including cephalosporins, over all treatment periods. However, copper supplementation did not affect minimum inhibitory concentrations of copper or detection of *pco*D. While *tet*A and bla_{CMY-2} genes were associated with a higher multi-drug resistance (MDR), *tet*B and *pco*D were associated with lower MDR. Supplementations of CTC or copper alone were associated with increased *tet*B prevalence; however, their combination was paradoxically associated with reduced prevalence. These studies indicate that *E. coli* isolates from the weaned pigs studied exhibit high levels of antibiotic resistance with diverse multi-resistant phenotypic profiles. In a related study, total fecal community DNA (n = 569) was used to detect 14 *tet* genes and to quantify gene copies of *tet*A, *tet*B, *pco*D and bla_{CMY-2}. CTC and copper plus CTC supplementation increased both the prevalence and gene copies of *tet*A, while decreasing both the prevalence and gene copies of *tet*B, when compared with the control group. The diversity of *tet* genes were reduced over time in the gut bacterial community. The roles of copper supplementation in pig production and *pco*-mediated copper resistance in *E. coli* need to be further explored since a strong negative association of *pco*D, with both *tet*A and bla_{CMY-2}, suggests there exist opportunities to select for a more innocuous resistance profile.

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Acknowledgements

"Jesus Christ is the same yesterday, today and forever." Hebrew 13: 8

Amen!

Dedication

I dedicate this dissertation in memory of my father Ejeta Agga and older brother Tilahun E. Agga, whose words of wisdom sustained me through this long journey. Sadly, they did not live to see my academic success.

Chapter 1 - General introduction

Antimicrobial resistance (AMR) is a serious and growing global public health threat of the 21st century (1-3), making the treatment of bacterial diseases a challenging task. Antimicrobial resistance increases morbidity, mortality and costs of health care systems (4-6). In the United States alone, about 2 million people acquire serious infections with bacteria resistant to at least one antibiotic and at least 23,000 people die each year directly from antibiotic resistant infections (2). Studies have shown that the annual direct cost of antibiotic resistant infections to the United States health care system ranges from $21 billion to $34 billion per year with more than 8 million additional hospital days (7). The magnitude of the AMR problem is compounded by a rapid decline in the discovery of new antibiotics (5, 7). The widespread use and misuse of antibiotics in human medicine is generally accepted to be the principal cause of antibiotic resistance for humans (5, 6, 8). The use of antibiotics in food animals also poses an additional public health risk (8, 9).

Antibiotics form an integral part of animal production, especially in modern livestock and poultry production systems. A large proportion of the antibiotics used in the United States is administered to food animals (8, 10). Antibiotics are used for the treatment of diseases, to prevent or control the spread of diseases, and also to increase growth and improve feed efficiency. Of critical public health concern is the use of antibiotics for growth promotion in which antibiotics are administered at low doses and for long durations in a large group of animals (8). This practice is believed to cause prolonged selection pressures and thus animals can become important reservoirs of AMR. Antibiotic resistant bacteria can be transferred to humans through the food chain (11) or by direct contact with animals, which subsequently can become less responsive to treatment in humans over time (9). However, the actual contribution

of such agricultural uses of antibiotics to the antibiotic resistance problem in humans is not known (12); to some researchers, the actual contribution is believed to be small (13).

Tetracyclines are one of the oldest broad spectrum antibiotics with bacteriostatic activity, having been discovered in the late 1940s (14). Tetracyclines inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit at the tRNA acceptor site (A-site), thereby sterically blocking the binding of aminoacyl-tRNA (15). Chlortetracycline (CTC) and oxytetracycline are the most commonly used forms of tetracyclines in veterinary medicine (16-18). Tetracycline resistance is the most common form of antibiotic resistance observed in bacteria obtained from pigs (19-27) and in other animal species and man (28). Tetracycline resistance commonly occurs through efflux, ribosomal protection and enzymatic inactivation, with the first two being the two most clinically important resistance mechanisms (15, 28-31). Widespread tetracycline resistance is predominantly due to acquisition of new genes through horizontal gene transfer on mobile genetic elements such as plasmids, transposons, and conjugative transposons which may also carry resistance determinants to other antibiotics and heavy metals (30). To date, 43 tetracycline resistance determinants (*tet*) and 3 oxytetracycline resistance determinants (*otr*) have been characterized and published (31)[1].

Because of increased concern about the risk of AMR in humans (9), the use of antibiotics as antimicrobial growth promoters (AGPs) was banned in EU member countries in 2006 (9) and is currently being phased out in the United States (32) on the basis of the "precautionary principle" and the concept of "prudent use". From the Danish experience, the major initial consequences of banning AGP in swine include reduced productivity, poor growth rate and increased incidence of diarrheal diseases, particularly during the first two weeks of life (33).

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[1] http://faculty.washington.edu/marilynr/tetweb1.pdf

Later, these parameters were observed to return to and exceed baseline values (34). Limiting the use of antibiotics in animals for growth promotion purposes may reduce human health risks associated with antibiotic resistant bacteria (8). However, studies indicate that such restrictions increase the cost of production with decreased productivity and an expected increase in retail prices to consumers (8). A total ban of feed-grade antibiotics from swine production in the U.S. would increase cost of production by $2.8-$6.1 per animal produced which is associated with increased consumer costs for pork ranging from $180 million/year to over $700 million per year (8). Maintaining animal production in the absence of AGPs would require an increase in the total number of animals produced to get the same amount of meat. This would increase the need for more environmental resources, and potentially lead to further proliferation and dissemination of some diseases (9). Moreover, limiting or completely banning the use of antibiotics in animals can paradoxically lead to increased microbial load in the environment. To cope with these potential production losses, many alternatives to antibiotics are currently being evaluated (33, 35). The metals zinc and copper are commonly used for growth promotion purposes in pig production (33). Growth promotion effects of heavy metals are believed to be mediated through improved feed intake, improved growth rate and likely through their known antimicrobial activities (33, 36, 37).

In swine nutrition, copper (Cu), mainly in the form of copper sulfate ($CuSO_4$), is an essential trace mineral required for normal physiological activities. Copper is most commonly supplemented at 16 ppm (38), well over double the National Research Council (NRC) recommended required dose of 5-6 ppm (39). Much higher dietary levels, typically at 100-250 ppm of Cu (fed as $CuSO_4$), are also used to promote growth, increase feed intake and improve feed efficiency in weanling pigs in similar magnitude as that seen with antibiotics (40).

However, similar to antibiotics, plasmid-coded resistance to heavy metals is widespread (9, 41-44) and the use of copper has recently been shown to be associated with macrolide, tetracycline, and glycopeptide resistance in Gram positive enterococci (41-47). Copper salts have been suggested to select for antibiotic resistance in *E. coli* (48). Furthermore, heavy metal use is limited by its environmental toxicity of excreted metals as they are not further degraded (48). Unlike toxic organic compounds, inorganic forms of metals cannot be degraded (49, 50); subsequently, representing a long term selection pressure. Concerns surrounding the potential of metal contamination in maintaining a pool of AMR genes in the environmental and clinical settings are growing (50).

For bacteria, copper also is an essential trace element, though it is toxic at higher concentrations (51, 52). Thus, bacteria have developed various mechanisms for handling copper. Heavy metal homeostasis, versus resistance, is a delicate balance between maintaining cells with a supply of essential trace elements on one hand and protecting them from their toxic effects on the other (46). Copper resistance in *E. coli* can be selected as a result of agricultural use of copper (52). Wild type *E. coli* have developed several chromosomally encoded copper tolerance mechanisms and strains with plasmid borne copper resistance (*pco*) genes are able to survive under significantly higher levels of environmental copper and thus be termed as copper resistant (46, 53).

The *pco* determinant was first identified in 1983 by Tetaz and Richard (54) from *E. coli* strain isolated from the effluent of copper sulfate supplemented pigs in Australia. The *pco* gene is approximately 6 kilobase pairs and has been found on a conjugative plasmid designated as pRJ1004 (36, 54). The *pco* gene cluster consists of seven genes designated as *pco*ABCDRSE (46, 51, 55) which are arranged in two operons: *pco*ABCD and *pco*RS, along with a separate

*pco*E gene. The *pco*ABCD and *pco*E clusters are structural genes while the *pco*RS cluster

consists of regulatory genes. PcoA, PcoC and PcoE are periplasmic proteins; on the other hand,

PcoB and PcoD are, respectively, outer and inner membrane proteins involved in copper

transport. PcoRS is a two-component regulatory system that regulates the expression of

*pco*ABCD genes (55). The sensor protein is encoded by *pco*S and is phosphorylated by an

environmental signal (in this case, the presence of copper) which in turn phosphorylates and

activates the regulator protein encoded by *pco*R (51). The *pco*ABCD genes are expressed as a

polycistronic message from the same promoter under the *pco*RS regulatory system, with

stoichiometric production of the four gene products (56); it is believed that the expression of all

four genes is required for full resistance (55).

Ceftiofur is an extended-spectrum third-generation cephalosporin, first approved by the

U.S. Food and Drug Administration (FDA) in 1988 for the treatment of respiratory diseases in

cattle and pigs (57). However, its continued use in animals has been closely scrutinized due to

its cross-resistance with ceftriaxone, a closely related antibiotic that is used as a first-line

treatment for invasive life threatening diarrheal diseases in children such as can be caused by

Salmonella enterica (58). Resistance to ceftiofur is mediated primarily by a widely disseminated

plasmid-borne AmpC type β-lactamase, the bla_{CMY-2} cephamycinase gene (59, 60). *E. coli* can

act as an important reservoir of the AmpC bla_{CMY} genes, each of which can later be transferred

to other, more pathogenic bacteria such as *Salmonella enterica* (61).

A previous phenotypic study has shown that sub-therapeutic use of CTC in pigs results in

the co-selection of third generation cephalosporin resistance (ceftriaxone) among enteric bacteria

of pigs (23). It has been also reported that administration of ceftiofur in feedlot cattle is

associated with increased tetracycline resistance in *E. coli* (62) and, conversely, administration of

in-feed CTC in feedlot cattle is also associated with increased ceftiofur-resistant *E. coli* (63). Furthermore, a complete sequence of some IncA/C plasmids originating from cattle showed the presence of *bla*$_{CMY-2}$ gene along with multiple other antimicrobial resistance genes such as *tet*A (64); in addition, *bla*$_{CMY-2}$ was concomitantly reported with co-resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and other tetracycline resistance genes in *Salmonella* (65). Based on the above previously reported findings, it can be easily hypothesized that CTC supplementation in weaned pigs can select for tetracycline and ceftiofur resistances and also to other antibiotics. Secondly, copper supplementation favoring plasmid-borne *pco* genes also could select for antibiotic resistance such as against tetracycline and ceftiofur (50). Until recently, experimental studies evaluating the effects of feed-grade use of CTC along with elevated levels of copper supplementation in weaned pigs on AMR in their fecal flora have been lacking.

The major objective of this cluster-randomized experimental study was to investigate the impact of feed grade chlortetracycline and copper supplementation in weaned pigs on AMR in the gut bacteria, measured qualitatively both at phenotypic and genotypic levels in *E. coli*, and quantitatively at the microbial ecological level. Specific objectives were to investigate CTC and copper supplementation effects on:

1. the prevalence of phenotypic antimicrobial resistance of *E. coli* against copper and a panel of 15 antibiotics commonly used for the treatment of Gram negative infections,

2. the prevalence and distribution of tetracycline (*tet*), copper (*pco*D) and ceftiofur (*bla*$_{CMY-2}$) resistance genes in *E. coli*, and association of these genes with multidrug resistance and among themselves, and

3. the distribution and prevalence of 14 *tet* genes and the quantities of gene copies of *tet*A, *tet*B, *pco*D and *bla*$_{CMY-2}$ in the feces of weaned pigs supplemented with CTC, copper, both, or neither.

Chapter 2 - Antimicrobial resistance: review of epidemiology and challenges

Introduction

For over 70 years, the use of antimicrobial agents has reduced mortality and morbidity associated with bacterial infections in both humans and animals. As a consequence, many lives have been saved and food safety and security have been improved. However, due to their widespread use and misuse, the effectiveness of these 'wonder drugs' has declined due to the associated widespread occurrence of antimicrobial resistance (AMR). Antimicrobial resistance has been recognized as a major public health problem by international and national agencies (1, 66-68).

Enterococcus (*Enterococcus faecium*, *E. fecalis*) and *Escherichia coli* are the two major genera commonly used for AMR monitoring representing Gram positive and Gram negative bacteria respectively because: 1) they are ubiquitous, 2) they are common commensals in animals and humans, 3) they can easily acquire antimicrobial resistance determinants in response to selective pressures exerted by antimicrobials, and 4) they can assemble mobile genetic elements (plasmids, transposons, insertion sequences and gene cassettes) and transfer the resistance genes to other more pathogenic bacteria (19, 69). Thus, commensal bacteria are generally regarded to act as important reservoirs for AMR (70). Furthermore, they also can cause opportunistic or nosocomial infections on occasion, especially in hospital environments. Commensal bacteria were known to carry the same *tet* genes, plasmids, transposons, conjugative transposons as more pathogenic bacteria. Moreover, it has been observed that over time Gram

positive commensal bacteria carry multiple *tet* genes with the same mode of action (e.g., efflux or ribosomal protection) as those of more pathogenic bacteria (28).

Feed additives, with or without significant nutritional value, that are commonly added to swine diets include acidifiers, anthelmintics, antibiotics, carbohydrate degrading enzymes, carcass modifiers, flavors, high dietary levels of copper and zinc, fungicides, phytase, phytogenics, probiotics and prebiotics (71). Copper and antibiotics have been used as growth promoters in pig diets for more than 60 years (36). Early on, it was observed that pigs supplemented with antibiotics demonstrated improved average daily weight gain and feed conversion efficiency (36). The effect of antibiotics as growth promoters was serendipitously discovered in the late 1940s when the fermentation product of *Streptomyces aureofaciens* was added to the feeds of poultry and pigs, resulting in marked increases in their growth rate (9). This increased growth rate was hypothesized to be due to the presence of low levels of chlortetracycline in the fermentation products. This discovery coincided with the transitional replacement of pasture production systems with intensive animal production systems (9).

The use of in-feed antibiotics in animals, at both therapeutic and sub-therapeutic levels, to control infections caused by enteric organisms became widely practiced after the late 1940s (35, 71). Antibiotics are added to swine feed for therapeutic purposes at high doses, prophylactic purposes at intermediate doses, and for growth promotion purposes at sub-therapeutic doses (9, 40). Twelve antibiotics and five synthetic antimicrobial agents are approved by the Food and Drug Administration for use in swine feed (40) (Table 2.1). Chlortetracycline and oxytetracycline can also be used in combination with other drugs; for example, as chlortetracycline/penicillin/sulfamethazine (or sulfathiazole), neomycin/oxytetracycline) (40). With the exception of tetracyclines, sulfa drugs and carbadox which have broad spectra of

activity, most growth promoting antibiotics target Gram positive bacteria which predominate in the proximal part of the gut (72). A national swine survey providing data from 712 farms and collected by the National Animal Health Monitoring System (NAHMS) from 1989-1991 (18), indicted that 60% of the farms included antimicrobials in their swine diets. It was also reported that nursery pigs were more likely to be fed antimicrobial supplemented feeds than the later production phases, and for any purpose (9, 17, 18, 40). In the grower/finisher pigs antibiotics were usually fed only for disease prevention or therapy (17). An Animal Health Institute 2000 report indicated a decline in the sub-therapeutic use of antibiotics: it was estimated that 18% of all antibiotics used in animal production were for growth promotion purposes and 82% were for disease prevention and treatment (40). The proportion of antimicrobial agents used as AGPs fell from 17% in 2001 to 5% in 2004 (9).

Results of a recent study on the estimated use of in-feed antimicrobials in U.S. swine production similarly indicated that the proportion of antimicrobials used as AGPs in nursery pigs remains lower than that used for grower and finisher pigs (17). Chlortetracycline was found to be the most commonly used in-feed antibiotic in U.S. swine production system, followed by tilmicosin and oxytetracycline (17).

The mechanisms by which antibiotics improve growth have not been entirely elucidated. However, some mechanisms by which antibiotics improve growth could be associated with changes in intestinal flora including inhibition of subclinical infections caused by pathogenic bacteria, reduction of growth depressing microbial metabolites and microbial growth inhibition thereby increasing nutrient availability and uptake and utilization through the intestinal mucosa (9, 10, 40, 71, 72). Early studies had shown significant improvements in growth performance of pigs when antibiotics were added to swine feed (40, 71). However, in modern swine production

systems with improved production and herd health management practices, the magnitude of the growth promotional responses is usually lessened (73). This difference between early and current reports on the efficacy of AGPs is in part attributed to the hygienic status under which these sets of studies were conducted. More recent studies (73, 74), which were arguably conducted under better hygienic conditions when compared to studies conducted many decades ago, did not find any significant improvement in the growth rate of pigs supplied with tylosin or chlortetracycline. The growth promotion benefits of AGPs likely could be more pronounced under field (farm) conditions when compared to controlled research situations (9, 40, 73); perhaps as much as twice that obtained under research station conditions where bacterial load and environmental stress are lessened (40). At the farm level, antibiotics have been shown to improve growth rate by as much as 25-30% and feed efficiency by 12-15% in weaned pigs, and 8-10% and 4-5% in finisher pigs, respectively (40). Furthermore, the magnitude of growth promotion response varies with stage of pig growth. Different studies have shown that antibiotics are more effective in nursery pigs in promoting growth than in finishing pigs (9, 40, 71-73). The magnitude of response to AGPs varies with age (greater during weaning, breeding and farrowing), stage of production, and environmental conditions under which animals are kept (9). Therefore, limiting the use of AGPs only to the nursery phase and restricting to therapeutic use of antibiotics in the finishing phase with increased infection control measures should decrease the overall use of antimicrobials and thus reduce the risk of AMR under the current multisite swine production system paradigm (73).

Because of increased concern about the risk of AMR in humans (9), the use of AGPs has been banned in Europe since 2006 (9, 75) and is being phased out in the United States (32). From the Danish experience, banning AGPs was associated with initial increase in the use of

therapeutic antibiotics and mortality (33) however in the long term it did not negatively impact

swine production (34).   To cope with any potential production losses and to control disease,

many alternatives are being evaluated (35). Zinc and copper are commonly used for growth

promotion purposes in swine production (33). In the United States, the nonessential metal

arsenic is also used as a feed supplement for chickens, turkeys and pigs (46). The growth

promotion effects of heavy metals are thought to be provided through improved feed intake,

improved growth rate and antimicrobial activities (33, 36, 37) similar to the effects of antibiotics.

Zinc and copper are used for the treatment of post-weaning scouring in pigs (46). It has been

reported that copper supplementation in pigs, especially of $CuSO_4$, reduced the number of

streptococci and lactobacilli while coliforms either increased in numbers, or else remained

unaffected (33).

In swine nutrition, copper (Cu) is an essential trace mineral that is required for

hemoglobin synthesis, and for the synthesis and activation of oxidative enzymes that are

important for normal metabolism (39). A basal level of 5-6 ppm of copper is required in the

diets of neonatal pigs and at decreased levels in the later ages (39). However, the actual

nutritional requirements for copper and zinc are often inflated to account for diet related factors

that interfere with its absorption (46). The common forms of Cu salts with high bio-availability

include the sulfate ($CuSO_4 \cdot 5H_2O$), carbonate ($CuCO_3$) and chloride ($Cu_2[OH]_3Cl$); however, the

oxide and sulfide forms are poorly available to the pig (39, 76). The form of Cu that is most

commonly used as a feed additive for growth promotion in pigs is the sulfate salt (76).

Although copper is not generally classified as an antimicrobial, it has antibacterial

properties when fed at high levels in the diet (40). High dietary levels, typically at 100-250 ppm,

of Cu (fed as $CuSO_4$) promote growth, enhance feed intake and improve feed utilization

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efficiency in weanling pigs with a similar magnitude as that of antibiotics (40). These responses

to high Cu supplementation are either independent of, or else additive to, that obtained from in-

feed antibiotics; however, such increases are not seen when fed with zinc (37, 39, 76) and

growth promotion effects of copper decrease with the age of the pigs (37, 40). The maximum

tolerable level of Cu for pigs is 250 ppm of diet and excess dietary levels for prolonged periods

appear to be toxic (39, 46). Physiological mechanisms by which high dietary Cu stimulates

growth largely remain unknown; however, some of this has been attributed to its antibacterial

properties (39). The realization of the beneficial effects of copper (as $CuSO_4$) came mostly from

the observed association between elevated copper feeding (100-250 ppm) and increased growth

rates under experimental studies (77). In most European countries the maximum amount of

copper supplement allowed in swine feed is 170 to 175 mg/kg (170-175 ppm) for piglets (up to

12 weeks of age) and 25 to 35 ppm for growing and finishing pigs (46, 47). In the United States,

however, copper supplements are not limited by regulation and when supplemented at elevated

levels usually range from 100 to 250 ppm, independent of the age of pigs (46, 47).

Similar to the situation with antibiotics, heavy metal resistance encoded on plasmids is

widespread (9, 41-44). Copper salts have been purported to select for antibiotic resistance in *E.

coli* (48). Furthermore, heavy metal use is also limited by the environmental toxicity of excreted

metals since they are not degraded in the environment (48). In spite of control measures and

restricted use of antibiotics, the problem of antibiotic resistance still persists; this indicates the

need to better understand factors that govern the evolution, dissemination and perpetuation of

AMR beyond the use of antibiotics (50). Co-selection between metals and antibiotics occurs

either through co-resistance or cross-resistance (50). Co-resistance occurs as a result of physical

linkage of resistance genes co-located on the same genetic element such as a plasmid, transposon

or integron (50). It has been known for long time that metal and antibiotic resistance genes are linked, particularly on plasmids (50). Cross-resistance occurs when different antimicrobial agents attack the same target, have a common pathway or else share the same route of access to their target. Thus, it occurs in chemically related antimicrobial agents belonging to the same class, or between structurally dissimilar compounds through nonspecific efflux using the same mechanism (50). Furthermore, co-regulation is also another means for the maintenance and proliferation of antibiotic resistance determinants in which transcriptional and translational responses to metal or antibiotic exposure can be linked to form a coordinated response to either stressor (50).

Selective pressure associated with prophylactic or sub-therapeutic doses of antibiotics in pig feed has been shown to increase the rate of plasmid transfer during *in vivo* conjugation experiments. This practice increases selection of plasmid-containing bacteria in the intestinal flora since feeding with a single antibiotic agent selects for all genes associated with the plasmid specifying resistance to the agent (78). Such selection could indirectly also be exerted on any and all plasmid(s) carried by the same host cell. Thus, supplementation of $CuSO_4$ in pig feed as a growth promoter may lead to selection of copper resistant strains and indirectly to the proliferation of resistance or virulence plasmids (54, 79). When first identified, it was observed that a plasmid (pRJ1004) did not carry any other known antibiotic or heavy metal resistance genes (36). However, as has been since shown, feeding of copper to pigs may also serve to select *E. coli* resistant to antibiotics (50, 54, 80).

Associations between the transferable copper resistance (*tcr*B) gene in enterococci obtained from copper supplemented pigs and resistance to macrolide (erythromycin), glycopeptide (vancomycin) and tetracycline antibiotics have been reported (41, 43, 44, 81). This

suggests that *tcr*B is likely to be physically linked to *van*A, *erm*B or *tet*M genes on the same plasmid, depending on the jurisdiction. However, following the ban of the use of antibiotics as AGPs, erythromycin and vancomycin resistance declined suggesting that copper supplementation alone could not have maintained high levels of antibiotic resistance (45), or that the *tcr*B gene had not fully penetrated the enterococci population in swine and other food-producing animals. Holzel et al., 2012 (80) found a positive association between the level of detection of copper and elevated β-lactam resistance in *E. coli* isolated from liquid pig manure in Germany. However, metal resistance plasmids predated the use of antibiotics suggesting that the emergence of metal-antibiotic co-selection on genetic elements is a relatively recent phenomenon (50).

Antimicrobial classes, mode of action and mechanism of resistance

In veterinary medicine antimicrobials are generally used for three purposes: non-therapeutic uses such as for growth promotion, prophylactic uses to prevent and control infections and therapeutic uses for the treatment of sick animals (82, 83). The most commonly used antimicrobials in food animals belong to one of five major classes: beta-lactams, tetracyclines, macrolides, aminoglycosides, and sulphonamides, all of which are also used in humans (83).

Based on the importance of antimicrobials (defined as any antibacterial agent including both antibiotics and synthetic agents) used in medical practice, the World Health Organization (WHO) (84) categorizes antimicrobials into 3 categories based on two criteria: 1) when the antimicrobial agent is the sole therapy, or one of limited alternatives to treat serious human disease, and 2) when the antimicrobial agent is used to treat diseases caused by organisms that may be transmitted to humans from nonhuman sources or diseases caused by organisms that may

acquire resistance genes from nonhuman sources. Critically important antimicrobials are those that meet both criteria (Table 2.2). Further to this, the top 4 critically important antimicrobial classes prioritized by the WHO are 3rd and 4th generation cephalosporins, fluoroquinolones, glycopeptides and macrolides. Highly important antimicrobials are those antimicrobials that meet either of the two criteria (Table 2.3). Important antimicrobials are those antimicrobials which do not meet any of the criteria (Table 2.3). This classification is useful for risk analysis of bacterial resistance that can be attributed to the use antimicrobials in food animals (85, 86), to prioritize resources for mitigation strategies, to inform regulators and stakeholders for the types of antimicrobials to use and for better stewardship of currently available antimicrobials (86).

Similarly, the World Organization for Animal Health (OIE) has established list of important antimicrobials in veterinary medicine based on two criteria (87): 1) when the response rate indicating criticality from OIE member countries for antimicrobial agent exceeded 50%, and 2) when the antimicrobial agent is either the sole therapy for the treatment of serious animal disease or if it is one of the available alternative antimicrobials. Veterinary critically important antimicrobials are those that meet both criteria (Table 2.4); veterinary highly important antimicrobials are those that meet one of the criteria (Table 2.5); and veterinary important antimicrobials are those which meet neither of the criteria (Table 2.5).

Antimicrobials, especially the antibiotics, exert their antibacterial effect either through inhibition of bacterial cell wall synthesis, nucleic acid metabolism (folic acid metabolism, DNA gyrase, DNA transcription), or by inhibiting protein synthesis (82, 88). Antimicrobials that inhibit cell wall biosynthesis include the beta lactams (e.g., penicillins, cephalosporins, carbapenems and monobactams) and glycopeptides (e.g., vancomycin, avoparcin). Antimicrobial classes that inhibit protein synthesis include aminoglycosides, tetracyclines,

macrolides, chloramphenicol, clindamycin and oxazolidinones. Antimicrobial classes that affect

nucleic acid metabolism include quinolones, rifampicin, sulphonamides and trimethoprim (88).

With regards to AMR, bacteria display one of three phenotypes: susceptible, intrinsically

resistant or acquired resistance, with some organizations also utilizing an intermediate

classification (89). Intrinsic resistance is a natural phenomenon observed in all members of a

species whereas acquired resistance occurs only in certain isolates of a given species. For

example, Gram negative bacteria are intrinsically resistant to macrolides and glycopeptides since

due to their larger sizes these molecules cannot easily pass through the outer membrane (89).

Most clinically important AMR is acquired through genetic exchange mechanisms into a

completely susceptible population (89). Resistance to antibiotics can be conferred either on a

chromosome or else via mobile genetic elements (plasmids, transposons and integrons) (50).

Antimicrobial resistance can disseminate through clonal expansion of resistant bacteria in which

a resistant determinant is located on the chromosome and passed vertically into the offspring.

The most common means, however, is through horizontal transfer (through transformation,

transduction or conjugation) of the resistance determinants between bacteria. Bacteria generally

develop resistance through one or more of four mechanisms: 1) decreased antibiotic

accumulation as a result of decreased uptake or increased efflux; 2) target modification through

mutation; 3) enzymatic inactivation or modification; and 4) bypassing metabolic pathways (50,

82, 88, 89). Antibiotic sequestration has been also suggested as another potential resistance

strategy (50).

Aminoglycosides

Aminoglycosides are a major category of drugs used in human and veterinary medicine

and they were originally isolated from antibiotic producing bacteria: *Streptomyces* or

Micromonospora and thus named with the suffix -mycin or -micin respectively (16).

Aminoglycosides are bactericidal antibiotics mainly used to treat serious infections caused by aerobic Gram negative and Gram positive bacteria (90). This class includes streptomycin, dihydrostreptomycin, kanamycin, gentamicin, tobramycin, amikacin and neomycin, all of which are used both in human and veterinary medicine. Apramycin and fortimicin are only used in veterinary medicine (16). For example, apramycin has been used for the treatment of enteritis caused by Gram negative bacteria in farm animals.

Aminoglycosides are large molecules containing numerous amino acid groups which makes them basic poly-cations that are highly ionized at physiological pH (90). Chemically, they are composed of a hexose nucleus (6-carbon aminocyclitol ring) linked by glycosidic bonds to one or more amino sugars (16, 90). The action of aminoglycosides is bactericidal in a dose (concentration) dependent killing and their uptake by bacterial cells is possible only in the presence of oxygen. They inhibit bacterial protein synthesis by affecting the various steps in the translation process (initiation, elongation and termination) (16, 90). They bind to the 16S rRNA within the 30S ribosomal subunits; specifically, to the aminoacyl site (A-site) for the 4, 6-substituted 2-deoxystreptamine (2-DOS) (16).

The major resistance mechanism to aminoglycosides is enzymatic inactivation by plasmid specified enzymes broadly classified as phosphotransferases, acetyltransferases and adenyltransferases (16, 90, 91). The aminoglycoside acetyltransferases are usually named as *aac* followed by a numeral in the parenthesis to indicate their enzymatic target on the aminoglycoside molecule (eg *aac*C(3')). The *aac* genes confer resistance to gentamicin, tobramycin, and kanamycin. Aminoglycoside phosphotransferases (named as *aph*) confer resistance to kanamycin and neomycin. Some *aph* genes are also designated as *str*A and *str*B which encode

resistance to streptomycin. Aminoglycoside nucleotidyltransferases include *aad* and *ant* groups

of genes and confer resistance to gentamicin, tobramycin or streptomycin (92). Plasmid

mediated resistance to aminoglycosides is transferable and a single plasmid can facilitate cross-

resistance to multiple aminoglycosides and co-resistance to other structurally unrelated

antimicrobials, thus easily contributing to the problem of multidrug resistance (90).

Because of its unique structure, apramycin overcomes plasmid mediated degrading

enzymes. Resistance to apramycin is thus far encoded through a single enzyme: aminoglycoside

3-N-acetyltransferase IV. This unique feature has been used as a marker to investigate the rate

of transfer of resistance genes from the use of apramycin in animals to human bacterial isolates

(90). Other resistance mechanisms can also occur through chromosomally mediated target

alteration due to mutations in the 16S RNA. This is mainly important for streptomycin and

dihydrostreptomycin resistance, which occurs as a result of a single step mutation conferring

high level resistance (90). The third mechanism of resistance to aminoglycosides is reduced up

take or export (16, 90). The aminoglycoside resistance genes that have thus far been reported

among *E. coli* isolated from food animals in the United States are *aac*(3'), *aac*(6'), *aad*A, *aad*E,

*str*A/B, and *aph* (92).

Beta-lactams

This class of antimicrobials includes narrow spectrum penicillins and cephalosporins,

expanded spectrum cephalosporins, and potent broad spectrum carbapenems, monobactams and

other penams (93). The first drug in this class to be discovered in 1929 was penicillin G, which

is the natural product of *Penicillium notatum*. This class consists of both narrow spectrum

antibiotics acting only against Gram positive bacteria and broad spectrum drugs that also act

against aerobic and anaerobic Gram negative bacteria (93, 94). Although the originally

discovered beta-lactams were natural products, most other beta-lactams are semisynthetic products that originate either from 6-aminopenicillanic acid or 7-aminocephalosporanic acid, which represent the active moiety essential for their antibacterial activity (93, 94). Structurally, all beta-lactams share a unique four membered beta-lactam ring and rupture of this ring, as a result of enzymatic action, results in loss of antibacterial activity of this class (93). New beta-lactams have been progressively synthesized through chemical modification of the core beta-lactam ring. These drugs include modified penicillins (methicillin and oxacillin), cephalosporins (cephalothin, cefoxitin, ceftriaxone and cefipime), and carbapenems (imipenem and meropenem) (93, 95, 96). Since their introduction into clinical practice in 1975, 4 generations (1st, 2nd, 3rd and 4th) of cephalosporins have been synthesized through chemical modification of the parent drugs (95).

Beta-lactam antibiotics block cell wall synthesis by interfering with the final stage of peptidoglycan synthesis. This occurs through inhibition of the activity of enzymes collectively known as penicillin binding proteins (PBPs), including transpeptidases and carboxypeptidases, by specifically binding to terminal D-Ala-D-Ala in the elongating peptidoglycan thereby preventing the cross linking of the cell wall (93, 94). The PBPs catalyze cross linkage of the glycopeptide polymers that form the integral part of the bacterial cell wall (93). Bacterial cell wall synthesis is a complex pathway that involves enzymatic linkage of precursors in the cytoplasm and transportation onto the outer surface of the cytoplasmic membrane. The resulting N-acetylmuramyl-pentapeptide is incorporated into the growing peptidoglycan structure to form a cell wall by specific transglycosylases and transpeptidases for cross linking of the cell wall components (94). Beta lactam antibiotics are bactericidal drugs and their optimal bacterial efficacy is time dependent (93). The difference in susceptibility between Gram positive and

Gram negative bacteria to the beta lactams could be attributed to differences in PBP receptor sites, amount of peptidoglycan, ability of the drugs to penetrate the outer cell membrane of Gram negative bacteria, and molecule resistance to different types of beta-lactamase enzymes produced by bacteria (93).

Three resistance mechanisms have been recognized for beta-lactams: target modification (acquisition of new PBPs), reduced permeability and increased efflux, and enzymatic inactivation through beta-lactamases (95). In Gram positive bacteria, the most common mechanism of resistance involves the acquisition of new PBPs that have low affinity for common beta-lactams. One notable example is methicillin resistant *Staphylococcus aureus* that possess the PBP2a encoding *mec*A gene used to produce a low affinity PBP. Another mechanism of resistance in Gram positive bacteria is the production of penicillinase, a specific beta-lactamase enzyme against penicillins that degrades the beta-lactam ring.

Gram negative bacteria are inherently resistant to penicillins due to low permeability of their cell wall, lack of PBPs, and production of wide spectrum of beta-lactamase enzymes (93). In Gram negative bacteria the major resistance mechanism is the production of β-lactamases. Gram negative bacteria inherently express low levels of chromosomally mediated β-lactamases that are exported to the periplasmic spaces. However, the most common β-lactamases that confer high level resistance are those borne on plasmids. More than 1,000 unique β-lactamases have been described with varying molecular and functional properties. At the molecular level, β-lactamases that utilize serine at their active site are classified as classes A, C and D while those that require one or two zinc ions to facilitate hydrolysis of the β-lactam ring are classified as class B β-lactamases (i.e., metallo β-lactamases) (94). The β-lactamases that are of current concern are the extended spectrum β-lactamases (ESBLs) which hydrolyze penicillins, early

cephalosporins, and expanded spectrum cephalosporins and monobactams (such as aztreonam),

serine carbapenemases which hydrolyze all β-lactams and the metallo β-lactamases (MBLs)

which can hydrolyze all β-lactams except monobactams. These β-lactamases are widespread

among Gram negative bacteria on mobile genetic elements such as integrons and transposons;

these, in turn, are commonly carried on transferable plasmids that also convey resistance genes to

multiple antibiotics (93).

Folic acid biosynthesis inhibitors

In 1932, sulfonamides were the first antimicrobials to be approved for clinical use (14).

Sulfonamides are broad spectrum bacteriostatic antimicrobials acting against both Gram positive

and Gram negative bacteria (97, 98). Sulfonamides interfere with bacterial folic acid

biosynthetic pathways by competitively blocking the incorporation of para-aminobenzoic acid

(PABA) into the folic acid molecule. Sulfonamides specifically compete with PABA for the

dihydropterate synthetase (DHPS) enzyme (97, 98). Trimethoprims, however, inhibit

dihydrofolate reductase (DHFR) enzymes thereby inhibiting the reduction of dihydrofolic acid

(DHF) to tetrahydrofolic acid (THF); this latter compound is an essential precursor in the

thymidine pathway leading to inhibition of bacterial DNA synthesis (92). Resistance to

sulfonamides can occur as a result of chromosomal mutation which can result in reduced drug

penetration, production of insensitive DHPS or hyper production of PABA (97). However, the

most common resistance is due to the acquisition of plasmid or integron borne resistance genes:

*sul*1, *sul*2, *sul*3, each of which encodes an insensitive DHPS enzyme (92, 97). In the U.S., most

of the reported bacterial resistance to sulfonamides is as a result of *sul*1 and *sul*2 (92).

Resistance to trimethoprim is through the acquisition of the dihydrofolate reductase (DHFR)

encoding genes, *dhf*r or *dfr* (92). Sulfonamide resistance is widespread among bacteria isolated

from animals, which could easily be related to their more than 80 years of use, and also as a result of the linkage of the *sul* genes to other resistance genes such as trimethoprim and streptomycin (97). There is also complete cross-resistance among the different members of the sulfonamide class (97). Sulfonamides are also commonly used as in-feed antimicrobials in pigs combined with other antimicrobials such as with chlortetracycline, penicillin G and tylosin (17). To potentiate their efficacy, sulfonamides are usually used in combination with trimethoprim, and resistance to this combined formulation (trimethoprim/sulfamethoxazole) is relatively rarely reported in the U.S. (92).

Macrolides

Macrolides are large molecules characterized by a 12-17 member central lactone ring attached to two or more sugar moieties (16, 99). Natural macrolides are produced by *Streptomycetes* by the action of various polyketide synthase enzymes (16). Erythromycin was the first macrolide to be discovered in 1952 (31). The 14-membered lactone ring family includes clarithromycin, erythromycin and telithromycin, the 15-membered ring family includes azithromycin, while the 16-membered ring compounds include spiramycin, tylosin and tilmicosin (16, 99). Macrolides are generally bacteriostatic and their mode of action is through inhibition of protein synthesis by reversibly binding to 23S rRNA, adjacent to the peptidyl transferase center of the 50S subunit, thus blocking elongation of the peptide chain leading to premature dissociation of peptidyl-tRNA (16, 31, 99).

Three acquired resistance mechanisms to macrolides (and to lincosamides and streptogramins-MLS) have been characterized. The first mechanism is target site modification, either through 23S rRNA methylation or mutation in the 23S rRNA or 50S ribosomal subunit mediated by rRNA methylases, each of which is encoded by an erythromycin resistant methylase

(*erm*) gene. Currently, 33 *erm* genes have been reported, each of which methylates RNA by adding one or two methyl groups to a single adenine in 23S rRNA. The modification of these prevents the binding of macrolides, lincosamides and streptogramins (MLS) to the ribosome to prevent protein synthesis (31). The *erm* gene confers resistance to MLS. The second mechanism of resistance involves two types of efflux pumps (ATP binding (ABC) transporters, and major facilitator superfamily (MFS)) that pump the drugs out of the cell. The most commonly found efflux protein coding genes are the *mef* and *msr* genes (16, 31, 99). The third resistance mechanism against erythromycin occurs via enzymatic degradation of the lactone ring as mediated through esterases and phosphotransferases encoded by *ere* and *mph* genes respectively (16, 31).

Phenicols

Chloramphenicol, and the related drugs florfenicol and thiamphenicol, inhibit microbial protein synthesis by irreversibly binding to the 50S ribosomal subunit (100). Chloramphenicol was the first broad spectrum antibiotic to be discovered (14). It is a bacteriostatic antibiotic active against a wide range of Gram negative and Gram positive bacteria (100). Though chloramphenicol use in humans and animals is nearly completely banned in the developed world because of associated rare toxicity (aplastic anemia), it is still used in developing countries (100). The most frequently encountered chloramphenicol resistance is enzymatic inactivation through acetylation by chloramphenicol acetyltransferases (CATs) which inhibit binding of the drug to the 50S bacterial ribosomal subunit (100). The *cat* genes (*cat*1) are commonly carried on plasmids in Enterobacteriaceae which also usually carry ≥1 other resistance genes (100). Resistance to chloramphenicol also occurs through efflux pumps mediated by *flo*R (conferring resistance to florfenicol and chloramphenicol) and *cml*A which have been detected from the U.S.

National Antimicrobial Resistance Monitoring System (NARMS) animal isolates (92). It has been reported that a single dose of florfenicol treatment in feedlot steers can result in a dramatic increase in multidrug resistant *E. coli* (101), likely as a result of selection for plasmids carrying the *flo* gene which is linked with other resistance genes (100).

Quinolones

The fluoroquinolones, also commonly known as quinolones, are synthetic antimicrobial agents in which the quinolone nucleus possesses fluorine (102). Nalidixic acid (a quinolone) was the first member of the class to be discovered and approved for clinical use in 1965 (102). Norfloxacin was the first fluoroquinolone approved for clinical use, followed by ciprofloxacin. The first fluoroquinolone approved for use in animals was enrofloxacin (in 1988) in companion animals (102). The first use of fluoroquinolones in food animals was approved by the FDA in 1998 (83) in cattle. Because of the shared mechanism of action, bacteria resistant to fluoroquinolones that are used in animals are also resistant to those used in humans through cross resistance (92, 102). Because of their usefulness in humans, these classes require a stringent approval procedure with post approval monitoring for the development of resistance (83). The use of enrofloxacin in poultry was banned in the U.S. in 2000 due to increased fluoroquinolone resistant *Campylobacter* species associated with the use of this drug in poultry (103).

The quinolones and fluoroquinolones inhibit DNA replication and transcription by inhibiting the functions of bacterial type II topoisomerases: DNA gyrase and topoisomerase IV (92, 98, 102). Topoisomerase II (also known as DNA gyrase) catalyzes the supercoiling of double stranded DNA and consists of two subunits, GyrA and GyrB (102). The 4-quinolone molecule interrupts the DNA breakage-reunion of the DNA supercoiling stage by binding to the DNA gyrase-DNA complex (102). DNA topoisomerase IV (Topo IV), a bacterial type II DNA

topoisomerase, is the second target for fluoroquinolones and is a multimeric protein consisting of two ParC and two ParE subunits; these, in turn, also exhibit sequence homology to GyrA and GyrB respectively (102). Topo IV mediates the relaxation of duplex DNA and decantation of daughter chromosomes after replication (102). Fluoroquinolone resistance occurs through target modification, decreased permeability, efflux pumps and target modification (98, 102).

The main mechanism of quinolone resistance is associated with chromosomal mutations in type II topoisomerase structural genes commonly involving *gyr*A, *par*C, or rarely *gyr*B and *par*E (92, 98, 102). These mutations usually occur on a chromosomal region called the quinolone resistance determining region (QRDR). In most Gram negative bacteria, quinolone resistance develops in a stepwise manner as a result of successive mutations occurring in the QRDR (102). However, in *Campylobacter* species that lack topoisomerase IV, a single mutation in *gyr*A is often sufficient to confer high levels of resistance (102). This explains the higher prevalence of quinolone resistance in *Campylobacter* compared to *E. coli* originating from food animals (104).

Tetracyclines

Tetracyclines represent a structurally related class of antibiotics with a broad spectrum of activity. Tetracyclines are one of the oldest antibiotics and the second broad spectrum antibiotic class (next to chloramphenicol) to be discovered (14). They are active against both Gram positive and Gram negative, aerobic and anaerobic, bacteria both in humans and animals. They are also active against intracellular organisms such as *Chlamydia*, *Mycoplasma*, *Brucella* and *Rickettsia* and several protozoan parasites (28, 105). Apart from their therapeutic uses in animals, tetracyclines are also widely used as feed additives in livestock production as growth promoters, and also in plant agriculture and aquaculture (28). The most commonly used

tetracyclines in the veterinary medicine are chlortetracycline and oxytetracycline (16). These natural tetracyclines were isolated from *Streptomyces* spp. by bacterial type II polyketide synthases (16). Based on the timeline of their discovery, tetracyclines can be grouped into 3 generations. The first generation tetracyclines (1948-1963) include chlortetracycline, oxytetracycline and tetracycline; the 2nd generation (1965-1972) includes minocycline and doxycycline; and the 3rd generation (after 1972) is represented only by tigecycline, a glycylcycline antibiotic (15, 28).

Structurally, tetracyclines are composed of four linearly fused tetracyclic nucleus rings, designated as A, B, C and D, to which functional groups are attached (15, 28). Tetracyclines are strong metal chelating agents and contain divalent metal chelation site characteristics of the class; these are essential both for their antimicrobial activities and pharmacokinetic properties (15, 28, 105). There is a structure and activity relationship for tetracyclines. Each of the four rings in the linearly fused tetracyclic nucleus must be 6-membered and purely carbocyclic to retain full antibacterial activity, with the exception of 6-thiatetracycline, which possesses sulfur at position 6 of the C ring (28). Many drugs have been synthesized through structural modification of the parent molecules (28). The 3rd generation tigecycline, for example, is a result of meta-substitution of ring D (15).

Tetracyclines are bacteriostatic antibiotics that inhibit protein synthesis (15, 16, 28, 105). In Gram negative bacteria, tetracyclines traverse the outer membrane through OmpF and OmpC porin channels as a positively charged cation (mainly with magnesium) tetracycline complex. This is followed by diffusion of uncharged tetracyclines through the inner membrane after being liberated in the cytoplasm. In Gram positive bacteria, tetracyclines diffuse freely as an electroneutral molecule through the cytoplasmic membrane (28). Uptake of tetracyclines across

the cytoplasmic membrane is an active process in which a molecule of ATP is spent for every proton molecule that is pumped in (28). In the cell, tetracyclines bind to the 30S ribosomal subunit at the tRNA acceptor site (A-site). This sterically blocks aminoacyl-tRNA binding, leading to inhibition of protein synthesis (15).

Mechanisms of tetracycline resistance

Tetracycline resistance is the most common form of antibiotic resistance among bacteria (20). The "resistome" concept refers to the aggregate of all antibiotic resistance mechanisms (15). Resistance to tetracycline can occur through various methods, such as active efflux of tetracycline out of the cell, production of ribosomal protection proteins, decreased drug permeability, target mutation and enzymatic degradation. However, the most common mechanisms of resistance are efflux, ribosomal protection and enzymatic inactivation, with the first two being the two most clinically important resistance mechanisms against tetracyclines (15, 28-31). Tetracycline resistance is predominantly due to acquisition of new genes through horizontal gene transfer on mobile genetic elements such as plasmids, transposons, and conjugative transposons; these, in turn, may also carry resistance determinants to other antibiotics and heavy metals (30). The differences in the host range of the different tetracycline resistance determinants can partly be due to the type of element each *tet* gene is associated with. Generally, conjugative transposons have less host specificity than plasmids and thus can be transferred to unrelated species and genera. The *tet* genes have not been found within integrons and gene cassettes ─the mobile elements frequently found in Gram negative bacteria (30, 106, 107). Generally, integrons and gene cassettes serve as a gene capture mechanism for multiple resistance genes to be linked. Currently, 43 tetracycline resistance (*tet*) determinants and 3

28

oxytetracycline resistance (*otr*) determinants have been characterized[2] (Table 2.6) that are known

to confer tetracycline resistance to bacteria. The protein products of these genes were first

detected and subsequently given the name *tet* by Levy and McMurry (108).

A tetracycline resistance determinant is defined as "a naturally occurring, generally

contiguous genetic unit which includes all structural and regulatory genes involved in resistance"

(109). DNA-DNA hybridization is currently considered a standard method to differentiate

different *tet* genes. Two genes are considered related and thus belong to the same class if they

show ≥80% amino acid sequence similarity under stringent DNA hybridization conditions.

Thus, two genes with ≤79% amino acid sequence similarity are considered two different genes

(28). The nomenclature for tetracycline resistance determinants was standardized by Levy et al.,

1989 (109); 1999 (110). Accordingly, each tetracycline resistance class is designated by a

capital letter or number and placed into the parenthesis with space between Tet and the class

(example Tet (A). The first structural gene of a given class is designated as *tet*A and the

subsequent structural genes would be designated as *tet*B as for class P determinant which has

two structural genes that are designated as *tet*A(P) and *tet*B(P). However, all other classes of

tetracycline resistance determinants characterized to date have only a single structural gene.

Accordingly, the structural gene from the Tet (A) determinant can be written as *tet*A(A), *tet*(A)

or *tet*A (29). The latter designation was followed throughout the thesis. Tetracycline resistance

is inducible with a sub-inhibitory concentration of tetracycline (111). Furthermore, the presence

of tetracycline increases horizontal gene transfer of tetracycline resistance genes (16).

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[2] http://faculty.washington.edu/marilynr/tetweb1.pdf

Efflux pumps

Currently 30 efflux genes have been characterized and comprise 65% of all *tet/otr* genes. They encode membrane associated, energy dependent proteins (approximately 46 kDa) which export tetracycline out of the cell; thus, reducing the intracellular concentration of tetracycline. A proton is exchanged for a tetracycline magnesium ion that is exported out against a concentration gradient. Though efflux genes are found in both Gram negative and Gram positive bacteria, they are the most commonly found *tet* genes among Gram negative bacteria. The *tet*B gene is the most common of all efflux genes that has been identified among Gram negative bacteria (31). Most of the efflux proteins confer resistance to naturally occurring tetracyclines (tetracycline) but not to the semisynthetic second (minocycline) and third (glycylcyclines) generation tetracyclines; that is, except *tet*B which confers resistance to both tetracycline and minocycline (28).

Although tetracycline efflux proteins share amino acid and protein structure similarity with other efflux proteins involved in MDR, quaternary ammonium resistance, chloramphenicol and quinolone resistance, they are specific for the export of tetracycline (29, 112). Nonspecific efflux systems, such as those belonging to the resistance nodulation cell division (RND) family, also confer tetracycline resistance as a result of overexpression due to mutation in their regulatory sequences (16). Bacteria have nonspecific innate chromosomally encoded efflux proteins which transport molecules in and out of the cell. These are broadly divided into the major facilitator super family (MFS), the resistance nodulation cell division (RND) family, the small multidrug resistance (SMR) family and the ATP binding cassette transport (ABC) family. Some of these efflux pumps confer lower-level tetracycline resistance. The RND efflux pumps have 12 predicted transmembrane sequences (TMS). They are mainly found in Gram negative

bacteria and are involved in the export of multiple molecules such as antibiotics and toxic metals. A chromosomally mediated tetracycline efflux pump has been described in *E. coli* which is encoded by the multiple antibiotic resistance (*mar*) locus (113).

Most of the tetracycline efflux pumps are integral membrane proteins and belong to the major facilitator super family (MFS) with 12-14 TMS; that is, based on the number of times they span the lipid bilayer of the inner cell membrane. Few efflux genes which code for a predicted ABC transporter family have been characterized. These include, for example, *otr*C and the newly identified *tet*AB(46) efflux genes (15, 112). Efflux genes that are exclusively found in Gram negative bacteria are *tet*(A, B, C, D, E, G, H, 30). In addition to mobile genetic elements, the *tet*B gene can also be found on chromosomes (114, 115). The *tet*E gene differs from *tet*A-D genes in that it is associated with large non-conjugative and non-mobile plasmids or else is found on the chromosome (28, 114). Efflux genes that are exclusively found in Gram positive bacteria include *tet*Z, *otr*B and *otr*C. The *tet*K and *tet*L genes are predominantly Gram positive efflux genes but they are also reported from some Gram negative bacteria (28). The Gram negative efflux genes are widely distributed and mostly associated with large conjugative plasmids (28) of various incompatibility groups (116). The Gram negative *tet* genes have higher G+C contents (>40%) than those of Gram positive origin, the latter of which have <35% G+C content (28). The *tet*K and *tet*L genes are generally associated with small transmissible plasmids; however, on occasion they can also be found integrated into the chromosomes (28). In addition to tetracycline resistance determinants, most tetracycline resistance gene carrying plasmids also carry genes encoding resistance to other antimicrobials, heavy metals and virulence factors. Thus, co-selection as a result of selection pressure by any of these factors can have tremendously

contributed to the widespread occurrence of tetracycline resistance in association with MDR, likely since soon after the beginning of the antibiotic era (28).

Gram negative efflux determinants consist of two divergently oriented genes: one coding for an efflux protein and the other for a repressor protein (15, 20, 28). It has been shown that in the absence of tetracycline constitutive expression of Tn10 (encoding *tet*B) in *E. coli* is disadvantageous in growth competition with non-expressing cells (20). Thus, efflux encoding determinants are tightly regulated and are inducible. Both the *tet* efflux and the *tet*R genes are regulated by tetracycline. TetR is a tetracycline inducible repressor protein that shuts down the transcription of the efflux genes and its own expression in the absence of tetracycline and allows the expression of both genes only in the presence of tetracycline (20). Tetracyclines inhibit translation but paradoxically they also induce expression of the efflux proteins. This is balanced by a highly efficient transcriptional regulation system which is only induced in the presence of sub-inhibitory concentrations of tetracycline (20). The two genes share a central regulatory region with overlapping promoters and operators. TetR is a dimeric DNA binding protein with high sequence specificity for the *tet* operator in the absence of the inducer tetracycline (20). It binds to the upstream operator region of the efflux genes and negatively regulates the expression of efflux proteins (15, 111). In the absence of tetracycline, TetR occurs as a homodimer binding to two tandemly oriented *tet* operators (28). This blocks the transcription of the structural genes, both for the repressor and the efflux protein (28). The system is induced when a tetracycline-Mg^{2+}complex binds to the repressor protein and this only requires nanomolar concentrations of tetracycline (20, 28).

The regulation of efflux *tet* genes is one of the most sensitive and effective effector-inducible transcriptional regulation systems ever described (20, 28). The binding of TetR with

tetracycline-Mg^{2+}complex causes a conformational change weakening its interaction with DNA; this then leads to its dissociation from the operator region allowing the expression of the efflux proteins (15, 28). The *tet* genes are differentially regulated in such a way that TetR expression precedes that of the efflux proteins (20). The repressor protein binds to the DNA only when the intracellular tetracycline concentration (< 1 nM concentration) is insufficient (28).

Repressor proteins have not been found in the Gram positive efflux genes *tet*K and *tet*L and therefore they are believed to be regulated by translational attenuation (28, 111). This translational attenuation is due to the presence of a leader peptide with stem loop mRNA structures with two ribosomal binding sites (RBS: RBS1 and RBS2). The RBS1 overlaps the leader peptide and the RBS2 is downstream of the leader peptide and hides the RBS for the structural gene. In the absence of tetracycline, the ribosome binds to RBS1 and a short leader peptide is translated which ends before it reaches RBS2. However, in the presence of tetracycline, a second stem loop structure in the mRNA forms which uncovers the RBS2 site allowing translation of the efflux protein (28).

Ribosomal protection

Twelve ribosomal protection genes have been characterized and they code for ribosomal protection proteins (RPPs) (Table 2.6). These proteins are cytoplasmic proteins that protect the ribosome from the action of tetracycline and confer resistance to 1st and 2nd generation tetracyclines (doxycycline and minocycline) but not to the 3rd generation tetracycline (tigecycline). Generally, RPPs confer a wider spectrum of tetracycline resistance than efflux genes, with the exception of *tet*B. RPPs are approximately 72.5 kDa in size and share high homology with the translation elongation factors EF-Tu and EF-G GTPases (15, 28). By binding to the ribosomes, the RPPs weaken the interaction between tetracycline and the ribosome,

resulting in the release of tetracycline so that aa-tRNA can bind to the A-site and protein synthesis continues normally (15). The *tet*M gene has the widest bacterial host range of all the *tet* genes, presumably because of its association with conjugative transposons (such as Tn916 and Tn1545) with a very wide host range (30). Ribosomal protection genes are associated with plasmids, transposons and conjugative transposons. However, they have not been identified in integrons which capture and allows multiple resistance genes to be linked (107).

Generally, the ribosomal protection genes are rarely found in enteric Gram negative bacteria (117, 118). This could be because of the lower level of resistance conferred by ribosomal protection versus that is conferred by the efflux pumps (28, 114) which does not offer a survival advantage in the presence of tetracycline for the enteric bacteria. The *tet*M gene has been documented in *E. coli* obtained from pigs and chicken (117). However, the ribosomal protection genes have been more often detected in non-enteric Gram negative bacteria (28, 30). Ribosomal protection is regulated by transcriptional attenuation (16).

Enzymatic inactivation

Only three genes have been characterized that are capable of inactivating tetracyclines (Table 2.6). In 1989, the *tet*X gene was the first inactivating gene to be identified from Tn4351 and Tn4400 transposons harbored by the obligate anaerobe *Bacteroides fragilis* (119). Interestingly, its tetracycline resistance activity was discovered when the gene was transferred to aerobically growing *E. coli* that resulted in the destruction of the antibiotic and darkening of the growth medium (120, 121). Tet(X) is a flavin dependent monooxygenase enzyme that regioselectively hydroxylates tetracyclines to C-11a hydroxyl-tetracyclines (15, 119-121). This hydroxylation causes an unstable compound that undergoes non-enzymatic degradation (119). It acts on the first and second generation tetracyclines. Tet(X) requires flavin in the form of flavin

adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH), Mg^{2+} and

oxygen for its enzymatic activity (119, 120). Paradoxically, because of its oxygen requirement

for its enzymatic activity, TetX expression in the source organism *B. fragilis* does not confer

resistance to tetracycline (121). Though *tet*(X) has not been isolated from clinically resistant

bacterial strains, it has been reported to inactivate tigecycline, a 3rd generation tetracycline (15,

120). Semisynthetic glycylcyclines that were derived from the modification of tetracyclines at

position 9 were produced in the 1990s (120). They are active against tetracycline resistant

bacteria carrying the *tet* genes for ribosomal protection and efflux pumps (120). To date, the

minocycline derivative tigecycline is the only glycylcycline class of tetracycline that has been

approved by FDA (in 2005).

Copper handling mechanisms in *E. coli*

Copper (Cu) is a divalent heavy metal cation that provides important physiological

functions for living cells, including bacteria (49, 122). The most important function of Cu is in

the oxygen-dependent terminal oxidases, such as cytochrome C oxidase, and related enzymes

which are involved in the respiratory chain of many organisms (49, 122). At higher

concentrations, Cu becomes toxic to the cell by forming reactive oxygen species such as

hydrogen peroxide radicals and through its interaction with cell membranes (49) leading to lipid

peroxidation, disruption of the cell membrane and protein oxidation and inactivation (79). Cells

utilize two different types of uptake systems for heavy metals. The nonspecific uptake system is

a constitutively expressed rapid system in which various substrates diffuse across cytoplasmic

membrane of bacteria across a chemiosmotic gradient. The second type is a highly substrate

specific, slower process, using ATP hydrolysis as an energy source and induced only during

periods of need (49). Because of the constitutive expression of the nonspecific system, the gate

is always open- the first reason that makes heavy metals toxic to cells (49). As adaptation to high concentration of heavy metals, bacteria with heavy metal ion homeostasis and metal resistance determinants evolved. Copper uptake in *E. coli* is cation specific to ensure adequate supply of Cu in the presence of related cations such as zinc or nickel (122).

High concentrations of Cu in the gastrointestinal tract (GIT), particularly in the stomach and duodenum of mammals, coupled with an acidic condition that prevails in this part of the gut makes Cu even more toxic to enteric bacteria (46, 52, 122). Consequently, intestinal bacteria have developed elaborate mechanisms for copper homeostasis in adaptation to their specific ecological niche of the animal gut (46, 122). *E. coli* is a facultative aerobic bacterium that resides in the GIT of animals and humans (122). Bacteria utilize a combination of three possible mechanisms for heavy metal resistance (49): 1) active efflux of the heavy metal ion out of the cell, 2) segregation of metal ions as complexes, often with sulfur, and, 3) reduction of toxic metals to a less toxic oxidation state. For detoxification by reduction, the redox potential of a heavy metal should be within a physiological redox range (-421 mV to +808 mV) of most aerobic cells. The electrochemical potential of Cu (2+)/Cu (+) is -268 mV which renders it to be reduced to a less toxic state (Cu (I)). Since a reduced metal compound should also be diffusible out of the cell to prevent re-oxidation, an efflux system should be present if reduction is also used as a detoxifying mechanism (49).

Unlike toxic organic compounds, heavy metals cannot be degraded (49, 50) subsequently representing a long term selection pressure. Concerns surrounding the potential of metal contamination in maintaining a pool of AMR genes in the environmental and clinical settings (50) are growing. For bacteria copper is an essential trace element that is required for growth; but it also becomes toxic at high concentrations (51, 52). Thus bacteria with various

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mechanisms have evolved to for copper handling within bacterial cells. Copper resistance in *E. coli* can be selected as a result of agricultural use of copper (52).

Copper homeostasis systems in E. coli

Generally, since Cu is toxic at higher concentrations than dosages provided to meet normal physiological needs, the intracellular concentration of heavy-metal ions needs to be tightly controlled. In fact, copper resistance is likely just one of many cases reflecting the general need of every living cell for heavy metal homeostasis (49). Heavy metal homeostasis versus resistance is a delicate balance between maintaining cells with a supply of essential trace elements on one hand, and protection from an excess on the other hand (46). Since most of the early studies were carried out with plasmid encoded systems, there has been a misperception that metal resistance and intrinsic metal homeostasis are different; in fact, it is likely that they are two sides of the same coin (46).

Four chromosomally mediated copper homeostasis systems have been described in *E. coli*. The copper uptake and transport (*cut*) system is an early attempt to elucidate Cu handling in *E. coli*. It consists of six structural genes (*cut*ABCDEF) and a regulatory gene *(cut*R) which are involved in the uptake, storage and transport, export and regulation of normal copper homeostasis and resistance to excess amounts (49, 122). Copper uptake involves two copper uptake genes: *cut*A, which also uptakes zinc, and *cut*B which is specific for copper. Copper storage/transport is achieved through *cut*E and *cut*F genes. Excess Cu is exported by *cut*C and *cut*D genes. The expression of the *cut* genes is regulated through *cut*R to maintain intracellular Cu homeostasis. In addition to their direct involvement in Cu transport and metabolism, these genes also interact with genes involved in copper resistance (79). Therefore, it has been hypothesized that the *cut* genes are only indirectly involved in Cu homeostasis (122).

The second homeostasis system is the *cop* operon which centers on CopA, a Cu translocating P-type ATPase that forms the central component of copper homeostasis in *E. coli*. CopA is required for intrinsic Cu resistance under both aerobic and anaerobic conditions (55, 122). The expression of *cop*A and *cue*O genes is regulated by a copper responsive *cue*R, which is activated by intracellular Cu (I) levels. The superfamily of P-type ATPases consists of various proteins involved in the transport of charged substrates across biological membranes (122). Well studied prokaryotic ATPases include the CopA and CopB of *Enterococcus hirae* and CopA of *Helicobacter pylori* and *E. coli*. In *E. coli*, CopA is responsible for cytoplasmic copper homeostasis that transports excess Cu (I) from the cytoplasm to the periplasm where it is oxidized by a multicopper oxidase enzyme, CueO (55). In Gram positive bacteria (*E. hirae*), copper metabolism is much clearer and involves a *cop* operon with *cop*A and *cop*B structural genes both encoding for P-type ATPase. CopA is responsible for copper uptake and nutrition while CopB is responsible for copper efflux and detoxification (49).

The *cus* (copper sensing) system is a proton driven independent copper efflux system on the *E. coli* chromosome that confers resistance to Cu and silver (122, 123). The *cus* determinant consists of two operons, *cus*RS and *cus*CFBA that are transcribed in opposite directions (122, 123). The *cus*RS operon is a two-component signal transduction regulatory system that codes for a histidine kinase, CusS sensor protein and a response regulator CusR (122-124). CusRS activates the expression of the *cus*CFBA operon in response to elevated concentrations of copper (123, 124). The Cus system transports Cu (I) directly from the periplasm (55, 122, 123, 125). The *cus*CFBA operon consists of a tripartite cell wall associated proteins as proton-substrate antiporter, CusCBA and a copper (Cu (I)) binding protein, CusF (55). The Cus system belongs to CBA-transport systems, a family of related transport systems, exclusively found in Gram

negative bacteria (122). The CBA transport systems usually transport metals and drugs, and consist of three structural proteins encoded by a single operon. The central pump protein belongs to the resistance nodulation division family (RND) (122). The RND is a protein family of membrane bound proton driven transporters (122, 123). The second component of the CBA transporters are the membrane fusion proteins (MFP) which consist of a family of periplasmic efflux proteins and periplasmic adaptor proteins (122). CusA belongs to RND protein superfamily (122, 124). CusC is an outer membrane protein with similar homology to TolC stress-responsive protein (124). CusB belongs to the MFP family which is anchored in the inner membrane with a long periplasm spanning domain (124). The CusCBA transport complex is proposed to transport Cu from the cytoplasm to outside of the cell by using proton motive force as energy source (124). The *cus* system is particularly important under anaerobic conditions (46).

The 4th system is the *cue* (Cu efflux) system, consisting of a copper responsive metalloregulatory protein CueR that upregulates the expression of *cop*A and *cue*O genes. CueO is a periplasmic multicopper oxidase (124, 126) enzyme which is primarily involved in aerobic system for Cu tolerance in *E. coli*. The *cue* confers Cu tolerance under moderate to high copper concentrations while the *cus* augments Cu tolerance under extreme Cu stress conditions (124). CueO is not active in the absence of oxygen (46).

To summarize, there are clearly a number of different systems available to bacteria to deal with homeostasis and resistance needs. While this is evolutionarily and ecologically desirable, it can make epidemiological studies difficult to interpret.

Plasmid-borne copper resistance in E. coli

Though wild type *E. coli* have several chromosomally encoded copper tolerance loci, as mentioned above, strains with plasmid based resistance genes should theoretically be able to survive under significantly higher levels of environmental copper and so have been termed as copper resistant (46, 53). These plasmid-borne genes are involved in elaborate periplasmic Cu handling and provide additional Cu resistance capacity to those chromosomal systems outlined above (46). On the other hand, plasmid-borne systems remain dependent on the presence of Cu homeostasis mechanisms (46).

Copper resistance in *E. coli* is believed to be mediated mainly through a plasmid borne copper resistance (*pco*) gene determinant. The *pco* gene was first identified in 1983 by Tetaz and Richard (54) from *E. coli* strain isolated from the effluent of copper sulfate supplemented pigs in Australia. The *pco* gene is approximately 6 kilobase pairs and has been found on a conjugative plasmid designated as pRJ1004 (36, 54). The *pco* determinant of *E. coli* plasmid pRJ1004 has been cloned and sequenced (52). The pRJ1004 was found to be incompatible with plasmids belonging to two incompatibility groups: IncI1 and IncK groups. Thus it has been hypothesized that it could be a hybrid plasmid formed as a result of illegitimate recombination between those two, or that pRJ1004 represents an ancestral type of those two that evolutionarily diverged to become compatible to each other (54). Williams et al., 1993 (36) concluded that copper resistance among enteric bacteria (*E. coli*, *Salmonella* and *Citrobacter freundii* isolates) isolated from different geographic locations was caused by closely related resistance determinants present in non-identical plasmids.

The *pco* gene cluster has seven genes designated as *pco*ABCDRSE (46, 51, 55) which are arranged in two operons: *pco*ABCD and *pco*RS, along with a separate *pco*E gene. The

*pco*ABCD and *pco*E clusters are structural genes, while the *pco*RS cluster consists of regulatory genes. PcoA, PcoC and PcoE are soluble proteins that are expressed into the periplasm; PcoB and PcoD are the outer and inner membrane proteins, respectively, that pump copper out of the periplasm of the cell. PcoRS is a two-component system that regulates the expression of *pco*ABCD genes (55). The *pco* resistance system is inducible by copper (36, 51). The sensor protein is coded by *pco*S that is phosphorylated by an environmental signal (in this case presence of copper); this, in turn, phosphorylates and activates the regulator protein encoded by *pco*R (51). Since the expression of *pco* gene has also been found to be regulated in *pco*R mutants, the presence of regulatory interaction between the plasmid borne *pco* genes and the presence of other chromosomal, or other plasmid, regulatory factors in *E. coli* has been speculated (49, 51, 79). Furthermore, based on the homology observed between *cop* plasmid genes and chromosomal DNA of sensitive strains of pseudomonas species, it has been suggested that the copper resistance operon could have evolved from chromosomal genes (36).

The mechanism of copper resistance in *E. coli* bearing pRJ1004 is through energy dependent active efflux of copper in a modified form which renders copper unavailable to the copper uptake system in a bacterial cell wall (36, 51, 79). This is in contrast to sequestration of copper and high accumulation by *cop* encoded resistance observed in *Pseudomonas syringae* pv. tomato (36). It was found that expression of all four *pco* proteins PcoABCD is required for full copper resistance and disruption of any of these genes could result in loss of copper resistance, or even increased copper susceptibility (55). On the account of accumulating information, Zimmermann et al., 2012 (55) have proposed a model for *pco*-based copper resistance in *E. coli*. PcoC is a periplasmic copper chaperone with two separate binding sites specific for Cu (I) and Cu (II). PcoA is a multicopper oxidase that oxidizes PcoC-bound Cu (I) to a less toxic form Cu

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(II). The *pco*E gene is not strictly required for full copper resistance but it reduces the time required to recover from copper induced stress. Like the other genes, it is induced by copper salt but unlike *pco*ABCD structural genes, it is controlled by a separate chromosomal two-component copper tolerance system *cus*RS. It acts as a sponge that sequesters toxic copper ions in the periplasmic space (122, 126). This gives time for the expression of other genes which are under the control of the *pco*RS regulatory system. PcoE relays the sequestered Cu (I) to PcoC to be imported into the cytosol through the inner membrane (IM) pump PcoD to provide nutrient copper for the activation of newly synthesized PcoA, or for the oxidation of Cu (I) to Cu (II) by cuprous oxidase PcoA; subsequently, this is then exported out of the cell by the outer membrane (OM) PcoB protein. While Pco proteins are mainly involved in the periplasmic copper efflux in *E. coli* (126), it has also been shown that PcoA can substitute for the chromosomally encoded CueO protein (126).

The *pco* determinant in *E. coli* is regulated by a plasmid and a chromosomally encoded component regulatory system: *pco*RS and *cus*RS, respectively (126). On one hand, the copper resistance genes must be carefully regulated to ensure their expression is sufficient to protect against the toxic effects of increased ambient copper concentrations in the environment, and on the other hand must not be so high that the bacterial cell is depleted of copper (79). Therefore, it has been predicted that the induction profile for copper is a linear, or slightly sigmoidal, curve against the copper concentration as opposed to that of mercury which is sigmoidal curve (79). The *pco* dependent copper resistance acts in the presence of high external copper (79). Therefore, it has been suggested that copper metabolism and resistance genes are coordinately regulated (79). A chromosomal gene which can replace *pco*R function encodes a two component system and the corresponding sensor component is encoded on the chromosome (79). Thus, it

has been concluded that plasmid borne copper resistance in *E. coli* is closely linked with chromosomal systems for copper management (52). It has been proposed that copper resistance in *E. coli* requires both plasmid and chromosomal genes to contribute to resistance in an integrated fashion so that resistance to high copper concentrations is expressed, while homeostasis of intracellular copper concentration is maintained at physiological levels (36). The *pco* determinant likely can also function in a limited range of bacterial genera which are closely related to *E. coli*, such as *Citrobacter*, *Salmonella* and *Shigella* (36).

It was reported that many other bacteria are more sensitive to copper than *E. coli* (127). The copper resistance determinants have been found to be homologous in *E. coli* and *Pseudomonas* species, but they are phenotypically distinct; that is, while resistant *E. coli* are colorless, *Pseudomonas* appear blue, suggesting that *E. coli* export excess copper out of the cell while *Pseudomonas* accumulates or sequesters excess copper (49, 51).

Ecology of antimicrobial resistance: mechanism of dissemination and persistence

Antimicrobial resistance is largely an ecological problem, as opposed to being traditionally viewed as an individual human/animal patient problem (128, 129). The antibiotic resistance phenomenon is a function of the selective pressure imposed by the antibiotics, and the presence of resistance genes (130, 131). Agricultural and clinical settings are two environments that are important for significant proliferation, maintenance and source of antibiotic resistance (50, 128). Antimicrobial treatment often kills the targeted pathogens, along with most of the susceptible commensals in the ecosystem, while paradoxically expanding resistant commensals that could harbor resistance genes (128). The majority of scientific studies of co-selection at the population and community level are, however, observational and have relied heavily on culture

dependent phenotypic analysis of isolates, making the quantification of co-selection pressures in microbial communities difficult (50). Antimicrobial resistance determinants can be maintained in larger elements, such as integrons within transposons and within plasmids; thus, the transfer of the larger element also leads to the transfer of the smaller integral elements (50).

Antibiotic resistance in targeted pathogens was observed very soon after the introduction of most of the antibiotics into clinical use (132). Antibiotic resistance can be as a result of spontaneous mutation, which can be transferred vertically, or else be acquired from other bacteria and transmitted horizontally (12, 128, 132). Mutation can simply be an initial step in the development of high levels of resistance observed; however, it is also an important mechanism for some antibiotics such as streptomycin resistant *Mycobacterium tuberculosis*, and for the widespread development of fluoroquinolone resistance in both Gram positive and Gram negative bacteria (132). But the majority of antibiotic resistance is through acquisition of resistance genes from exogenous sources (132). Thus, horizontal gene transfer plays a primary role in the dissemination of antibiotic resistance genes (132). Multidrug resistance, in particular, results from acquisition of tandem arrays of genetically linked resistance genes which often are borne by integrons or other transposons that can reside in the chromosome, or else on conjugative or mobilizable plasmids (128).

The use of a specific antibiotic not only affects the targeted organism but also all other members of the resident microflora, both cultivable and non-cultivable, as a collateral effect (128, 132). Evidence from the analysis of pre-antibiotic era bacterial populations, in which antibiotic resistance strains were largely absent, and analysis of the development of antibiotic resistance to antibiotics (such as tetracycline) following their introduction, have suggested a direct association between antibiotic use and an increase in the proportion of antibiotic resistant

bacteria (132). Conversely, prevalence of AMR is often reduced, depending on the duration of

the antibiotic has been marketed, when the use of antibiotics is restricted; however, it never

disappears (132). Besides selecting for resistant bacteria, antimicrobial therapy can affect a

given microbial ecology by changing the types of organisms that comprise it, leading to the

occurrence of new opportunistic bacteria intrinsically resistant to the antibiotic in use. For

instance the use of 2nd and 3rd generation cephalosporins has selected for enterococci which are

intrinsically resistant to these agents, perhaps with the concomitant emergence of vancomycin

resistant enterococci (VRE) (129).

The most significant aspect in the development of antibiotic resistance is tandem

assembly of resistance genes within a single, mobile genetic element to generate multidrug

resistance (MDR) clusters (12, 128, 130, 132). Multidrug resistance can develop as a result of

one of the following mechanisms (128, 132): 1) a single plasmid or transposon encodes

resistance to different antibiotics in a cluster, 2) a single gene encodes for similar mechanisms

that renders resistance to a class of related antibiotics (cross resistance); for example, *erm* for

macrolides, *bla* for β-lactams, 3) a single resistance gene encodes for a group of structurally

unrelated antibiotics; for example, *aad* confers resistance to streptomycin (aminoglycoside) and

spectinomycin (aminocyclitol antibiotic), 4) the use of non-antibiotics such as heavy metals

(mercury, copper and zinc) selects for the establishment of genetically linked antibiotic

resistance, and 5) mutations occur stepwise in multiple independent target genes. The most

common mechanism by which Enterobacteriaceae assembles tandem arrays of antimicrobial

resistance genes is through gene capture by integrons (12, 132).

Horizontal gene transfer (HGT) in bacteria occurs through one of three mechanisms (12,

130, 133). Transformation involves the uptake of naked bacterial DNA from the environment,

followed by homologous recombination and incorporation into the chromosome of the recipient bacteria if the donor and the recipient strains are related (12). However, unlike transformation, transduction and conjugation involve specialized entities, bacteriophages and plasmids, respectively, for their transfer (12). Transformation and bacteriophage mediated transduction usually transfer alleles of homologous genes among closely related bacteria (12). However, DNA transferred by conjugation can recombine with the recipient chromosome if it is homologous or remains associated with self-replicating plasmid (12). In conjugative transposons, the transfer of a resistant element can be induced by antimicrobial exposure (128). An experimental study conducted in pigs has shown that the use of sub-inhibitory or sub-therapeutic concentrations of antimicrobials increases *in vivo* transfer of AMR or virulence genes from commensal *E. coli* to pathogenic bacteria such as *Salmonella*, *Yersinia*, *Proteus* and *Shigella* (134). It was observed that sub-inhibitory concentrations of β-lactams enhanced the transfer of tetracycline resistance plasmids in *S. aureus* by up to 1000 fold (133). Stress, particularly the SOS response (a global response to DNA damage), which is induced by DNA damaging agents such as mitomycin C and antibiotics (fluoroquinolones and dihydrofolate reductase inhibitors), also has been shown to increase horizontal transfer of antibiotic resistance genes by more than 300 fold by inducing the mobility of transposons and insertion sequences (133). Thus, the use of SOS response inducing antibiotics such as fluoroquinolones may co-select for other antibiotic resistance genes that are physically linked on a mobile genetic element (MGE) (133).

Plasmids can carry antimicrobial resistance determinants; most notably, in the form of transposons and integrons (12). Transposons and integrons are segments of DNA that encode genes that promote two different types of specialized recombination (12). Unlike phages and

plasmids, they are not self-replicating and do not carry genes encoding for intercellular transfer (12). They move from one cell to another through phages, or more commonly through plasmids (12). Unlike transposons which can move from one place to another in the same cell, integrons cannot move themselves; however, they can move relatively small pieces of DNA called gene cassettes into their insertion site (*attl*). Integrons provide a genetic platform for capturing and allowing the expression of random segments of DNA (gene cassette) which often carry antimicrobial resistance genes (12). Each gene cassette can provide resistance to a chemically distinct class of antimicrobial agent (128). Multiple resistance transfer elements (plasmids, transposons and integrons) can collect and recombine resistance gene cassettes in any order (128). Thus, treatment with any antimicrobial agent can select for bacteria resistant not only to the specific agent but also to other unrelated antimicrobial agents through genetic linkage (128). Selection by treatment with one antimicrobial agent will enrich a population of strains resistant to all antimicrobial agents whose resistance genes are genetically linked to that of the antimicrobial agent used (128). In an experimental study conducted in chickens in the mid-1970s, 70% of *E. coli* isolates from a tetracycline supplemented group eventually became resistant to more than two antibiotics, including ampicillin, sulphonamides and streptomycin (135). Non antimicrobial environmental toxicants such as heavy metals can also select for multidrug resistant plasmids (128). Thus, any use of antimicrobial or other selective agents potentially could select for all resistance genes in the genetic arrays as well as for the plasmids residing in the bacteria (128).

Independent of the HGT of the resistance genes, antibiotic resistant bacteria are also widely distributed in different macro ecological niches such as human and veterinary health care institutions, animal production and global transfer through trade and movement (128, 132).

Interactions between human, animal, plant and the environmental bacterial populations lead to increase in the incidence of antibiotic resistant pathogens in various populations and reach humans through the food chain (132). One of the first consequences of the use of antibiotics in animals was the transfer of resistance genes from bacteria of animal origin to human pathogens such as *Salmonella* and *E. coli* 0157:H7 (132).

Any potential for the reversibility of AMR, once developed, is determined by two factors: dilution and the fitness cost depending on whether the system is closed or open (6). In open systems, such as hospital settings and farm environments, with a continuous influx and efflux of humans and animals, the incoming bacterial population has lower frequency of AMR than the resident population (i.e. more selective pressure from antibiotic use). If antibiotic use is reduced at hospital/farm environment, the driving force for reversibility is the dilution effect of resistant population by incoming susceptible population. However, in a closed community, where movement in and out is minimal, the driving force for reversibility is the biological fitness cost associated with resistance that is any effect the resistant mechanism has on reducing the ability of the pathogen to reproduce and spread in the population (6). Antimicrobial resistance can persist through 1) direct selection for resistance marker in the presence of antibiotic selection pressure (both at antibiotic concentrations above the MIC and sub-MIC), 2) co-selection between resistance markers (within genetic elements and within clones), 3) cost free resistance, 4) when fitness increases resistance, 5) compensatory evolution that reduces costs and allows maintenance of resistant strains even in the absence of selective pressure and plasmid persistence (6, 133). Under antibiotic selective pressure, the rate of appearance of antibiotic resistant bacteria in the susceptible population is a function of the combined rates of *de novo* mutation and HGT of the resistance determinants (6). The most important measure is the rate at which the

existing resistant strains increase in frequency as a function of the level of drug exposure in bacterial population (6).

The release and distribution of large volumes of antibiotics into a larger physical environment such as the gut selects for resistance and resistance transfer by HGT (6, 130, 133). Large reservoirs of resistance genes in the human and animal microbial community can serve as donors for transfer of resistance to other resident bacteria, including pathogens (128, 136). Commensal bacteria serve as both the reservoirs of antibiotic resistance genes (128, 133) and as a dynamic breeding ground for evolutionary selection. This occurs subsequent to the generation of novel and diverse resistance genes, such as the mosaic resistance determinant *tet*O/32/M (133), which is believed to have arisen as a result of recombination of two different *tet* genes.

Co-selection is a very common feature of resistance that is acquired by HGT through mobile genetic elements (MGEs) such as plasmids, transposons and integrons; also through multidrug resistant clones (136). Because of genetic linkages, resistance to one antibiotic can remain stable, or even increase, in the absence of that specific antibiotic use (6, 128, 133). This is because many antibiotic resistance genes reside on large self-transmissible genetic elements such as conjugative plasmids and transposons, each of which can carry multiple genes encoding resistance to antibiotics, heavy metals and biocides (133). Acquisition of antibiotic resistance genotype can actually increase bacterial fitness, even in the absence of antibiotic selective pressure, allowing for the rapid emergence and dissemination (6, 133) of resistant strains in a given bacterial population. Thus, amelioration of the fitness cost of antibiotic resistance carriage is one reason for the persistence of antibiotic resistance genes in the apparent absence of selection pressure from antibiotic use (133).

Although a decline in the prevalence of bacteria resistant to specific antimicrobial agents can sometimes be seen following restricted or discontinued use of an antimicrobial agent, a return to pre-introduction levels does not usually occur (128), or else the decline occurs at a slower rate (compared to rate of increase), if at all (129). The observation that there may appear to be little or no correlation between changes in the levels of consumption of any particular antibiotic, and the changes detected in the frequency of resistance, has mainly been attributed to co-selection (6) or persistence of resistant strains in the commensal microbiota (128). In one two-year prospective intervention study in Sweden, effected through voluntary withdrawal of trimethoprim use in a community setting, it was observed that despite an 85% reduction in the use of trimethoprim, resistance rates in *E. coli* and other bacteria were not meaningfully affected (137). Similarly, in Great Britain a 97% reduction in sulphonamide use in humans from 1991-1999 did not result in a significant reduction in sulfamethoxazole resistance (138).

Microbiological approaches for antimicrobial and copper susceptibility testing

Antibiotic susceptibility testing

Culture-based antimicrobial susceptibility testing (AST) methods involve the isolation and then characterization of bacteria that are tested for phenotypic sensitivity or else the presence of resistance genes by molecular methods. In vitro AST results are reported either qualitatively or quantitatively (139). Qualitative results are reported as susceptible, intermediate, or resistant based on either clinical or epidemiological breakpoints. Quantitative results are reported as minimal inhibitory concentrations (MIC), expressed as μg/ml or mg/L (139), for broth dilution methods, or zones of inhibition (mm) for disk diffusion assays. For accuracy and comparison of

results, ASTs are usually performed in adherence to one of several possible standardized

methods (4, 139). The Clinical and Laboratory Standards Institute (CLSI) and the European

Union Committee on Antimicrobial Susceptibility Testing (EUCAST) are the two major

international standard setting organizations for AST (140, 141). Most ASTs are performed on

aerobic and facultative anaerobic bacteria with inhibition of bacterial growth, rather than

bacterial killing, as a measured end point (139). Phenotypic susceptibility testing methods are

performed by two methods: diffusion (disk diffusion or concentration gradient) or dilution (agar

or broth: microdilution or macrodilution) methods.

The disk diffusion and the broth microdilution methods are the most commonly used

AST systems in veterinary medicine (139, 140, 142). The agar disk diffusion method (143) is

the most widely used method in veterinary medicine because of its flexibility in the type and

number of antimicrobial agents that can be tested, ease of use, and low cost (139, 142, 144). The

disk diffusion test is based on the diffusion of the antimicrobial agent, by form of concentration

gradient, from an antimicrobial impregnated disk placed on the surface of agar media (CLSI

recommended medium is Mueller-Hinton agar) that has previously been inoculated with an

approximate inoculum size of 1 to 2 x 10^8 colony forming units (CFU)/ml of a pure culture of the

test bacterium (139, 144, 145). When the concentration of the antimicrobial agent becomes too

diluted to inhibit bacterial growth, a zone of inhibition (usually expressed in millimeters from the

disk) is formed. This zone of inhibition is inversely proportional to the MIC of the test

organism: the larger the zone of inhibition, the smaller the concentration of the drug required to

inhibit the organism (139, 144). The major drawback of the disk diffusion test is that it gives

only qualitative results: susceptible, intermediate or resistant (SIR) (139, 142, 144, 145) and it

can be difficult to make accurate inter-laboratory comparisons. However, besides the qualitative

determination of AST, periodic examination of the distribution of the zones of inhibition for any given bacterial population, or the MICs in the case of broth dilution, can suggest early trends in emergence of resistance, or reduced susceptibility (4).

The Epsilometer test (E-test®, AB BIODISK, Solna, Sweden) with a concentration gradient strip, is a modification of the disk diffusion test that generates quantitative end points (139). It depends on the diffusion of a continuous concentration gradient of antimicrobial agent laterally from a plastic strip into an agar medium inoculated with a pure culture of the test bacterium (139). The plastic strip consists of a defined concentration of the drug on one side, in a continuous gradient from top to bottom, and a continuous MIC interpretative scale on the other side (139, 145). The MIC is determined by reading the concentration on the strip where the zone of inhibition intersects the strip (139, 145). Since the E-test uses a concentration gradient, MIC values between the standard two-fold dilutions can be obtained (139). Unlike the disk diffusion test this test is relatively expensive (139). It is best suited for the determination of MIC of only one or two drugs, or when a fastidious organism such as *Campylobacter* is tested (145). Neither of these agar diffusion methods is suitable for high thorough put such as for large-scale surveillance programs (139).

Quantitative susceptibility testing with the dilution methods (agar, broth macrodilution, or broth microdilution) are based on serial two-fold dilutions indexed to the log base 2 (139). The agar dilution method is considered as a "gold standard"; however, both agar dilution and broth macrodilution are cumbersome, especially when large numbers of samples have to be processed (139). The broth macrodilution (tube dilution) method involves the preparation of two-fold serial dilutions of each antimicrobial agent in a liquid growth medium, which is then dispensed into test tubes (145). These tubes are then inoculated with a standardized

concentration of bacterial suspension (often, of 1 to 5 x 10^5 CFU/mL), incubated at 35°C overnight; thereafter, the tubes are examined for visible bacterial growth as measured by turbidity. The MIC is determined as the lowest concentration of the antimicrobial that inhibits visible bacterial growth (145).

Broth microdilution is increasingly used in diagnostic laboratories and surveillance programs (139, 145). Unlike the diffusion methods, it allows for high sample throughput and quantitative inference; however, it generally is more expensive (144). Broth microdilution can be performed with commercially automated susceptibility testing systems such as the Sensititre® system (Trek Diagnostic Systems, Westlake, OH) (142). It is performed in microtiter plates with round or truncated V-bottom wells using antimicrobial agents of known potency following progressive two-fold dilutions (139). Commercially available plates can be purchased in a dehydrated form, providing a longer shelf life at room temperature (one to two years) or else as frozen trays with a shelf life span of approximately six months when stored at -10°C (139). Generally, microdilution tests are more expensive than the disk diffusion test and clearly lack flexibility in terms of the type and number of antimicrobial agents to be tested (139); that is, they are restricted to the number of available wells (e.g., 96) and thus the number of antimicrobials and dilutions tested must be carefully considered. The automated processes for incubation and reading using such devices as the Sensititre ARIS® system (Trek Diagnostic Systems, Westlake, OH) mean it can be used for high throughput testing (144, 145), and inter-laboratory comparisons are facilitated with surveillance systems through use of common control strains and common templates for the microtiter plates.

In the Sensititre® system, the broth microdilution test is performed by making a bacterial suspension from an overnight culture, diluted in demineralized water and turbidity adjusted to a

0.5 McFarland turbidity standards which gives approximately 1 to 2 x 10^8 CFU/ml of inoculum size. This suspension is further diluted in Mueller-Hinton broth to give a final bacterial concentration of 5 x 10^4 CFU per well. The suspension is then used to inoculate a Sensititre® plate containing serial two-fold dilutions of the antimicrobial agents to be tested. The trays are then incubated in a stack of two-four trays at 35°C for 16-20 h. The Sensititre® plates can be read manually, using SensiTouch® Vizion® and SWIN® (Sensititre for Windows) for computer assisted reading or automatically using the Sensititre® Automated Reading and Incubation System (ARIS®, Trek diagnostic system) (145, 146). The Sensititre ARIS is an automated system that detects fluorescence liberated by bacterial enzymes that cleave the fluorogenic substrate in the broth, which then is converted to an MIC estimation via a computer algorithm (145, 146). The MIC is recorded as the lowest concentration of antimicrobial agent that completely inhibits visible bacterial growth (139, 147). However, the true MIC of the drug lies somewhere between the last concentration with visible growth inhibition and the first concentration with no visible growth (i.e. between two consecutive $\log_2$ dilutions; that means the reported MIC merely represents a range of MIC values (139, 147).

Breakpoints (interpretative criteria) are usually used to define susceptibility or resistance of bacteria to antimicrobials, either expressed as a concentration (mg/L or µg/mL) or a zone of inhibition (diameter in mm) (141). In the United States, the interpretative criteria are based on clinical breakpoints according to CLSI efficacy expectations for human pathogens or else based on the CLSI's subcommittee on Veterinary Antimicrobial Susceptibility Testing (CLSI-VAST) which is specific for intended drug/bacterial pathogen combinations in the targeted animal species (139, 147). Three different terminologies are commonly used in the literature, which often creates confusion in the interpretation of AST results. The first is wild-type breakpoint

(also called microbiological breakpoints or epidemiological breakpoints), commonly used by

The European Committee on Antimicrobial Susceptibility Testing (EUCAST). This refers to the

MIC of an antimicrobial that distinguishes wild type populations of bacteria from those with

acquired or selected resistance mechanisms. A wild type strain is defined as a bacterial strain

that does not harbor any acquired or selected resistance to the particular antimicrobial being

tested (141). It is established based on data generated from moderate to large numbers of *in vitro*

MIC tests, deemed sufficient to describe the wild type population (141). The population that

departs from the often normally distributed population (wild type) is categorized as non-wild

type (140). The second term used is clinical breakpoint which refers to MIC concentrations that

separate strains with a high likelihood of treatment success from those bacterial strains for which

treatment is more likely to fail (141). These breakpoints are derived from prospective clinical

trials comparing outcomes with the MICs of the infecting pathogen (141), such data are

combined with expected concentrations at the target site. The third system is based on PK/PD

breakpoints, referring to antibacterial concentrations calculated from pharmacokinetic (PK) and

pharmacodynamics (PD) parameters that predict the efficacy of the antimicrobial *in vivo*. PK/PD

breakpoints are derived from data that are generated in animal models and then extrapolated to

humans (or animals) using mathematical or statistical methods (141).

According to CLSI clinical guidelines, either an MIC or zone diameter is used to indicate

susceptible, intermediate and resistant (SIR) categories (140, 147). A susceptible categorization

suggests the infection can be treated with the recommended dosing regimen of the antimicrobial

agent. An intermediate category implies that infection can be appropriately treated in body sites

where the drug is physiologically concentrated, or at a higher dosage; however, it also suggests a

buffer be employed to prevent small, uncontrolled technical factors that may cause major

variations in the interpretation and expected results. Resistant isolates are not inhibited by the usually achievable concentrations of the agent with the normal dosage schedule, or else fall in the range where specific microbial resistance mechanisms are likely to impede successful outcomes (147).

Of particular note, when the MIC of a WT population is plotted on a histogram, it typically follows a log-normal distribution (i.e., WT MICs are normally distributed on a logarithmic scale such as logarithm to base 2). Strains with the same type of acquired resistance also often have a log-normal distribution of MICs. Therefore, in species where a single resistance mechanism to a specific antibacterial predominates, it is common to see a bimodal distribution (147). In many cases the breakpoints established by use of epidemiological cut-off values and those from clinical cut-offs will be similar; however, in some cases they differ, leading to disagreement as to the appropriate values to be interpreted.

Genotypic approaches using molecular techniques to detect previously known and new resistance genes have been used to supplement traditional AST phenotypic methods (139). Determination of genotypic resistance allows for rapid and accurate detection of single and multiple resistance genes (and variants) in a single test, which can help reduce the emergence of antimicrobial resistance by enabling the most appropriate therapy (139). Large numbers of antimicrobial resistance genes can be studied by probing bacterial species, which yields a great advantage for surveillance and monitoring purposes (139). PCR methods have been used for the detection of various resistance genes from bacterial isolates (92, 139). One major disadvantage of the genotypic methods includes they can only detect resistance capacity, not determine susceptibility; other disadvantages include that these tests can be expensive, labor intensive, and are often non-standardized.

On the other hand, culture based methods of antimicrobial susceptibility testing have several limitations: 1) they are time and labor intensive, 2) the use of selective media for different bacterial species causes *a priori* selection bias on the type of bacteria, and 3) at this time, most bacteria in the mammalian gut cannot be cultivated in the laboratory (72). Only 20 - 40% of bacterial species from the mammalian gut can be cultured and identified with the use of the currently available cultivation techniques; thus, overlooking the greater proportion (60-80%) of intestinal bacterial species (72). It seems clear that these non-cultivable bacteria could act as important reservoirs of antimicrobial resistance genes (136). Most often, only infection related strains of bacteria, or sometimes commensal indicator bacteria, are identified and studied for susceptibility to antibiotics in epidemiological studies (132). Thus, the overall extent and change of antibiotic susceptibility in microbial populations cannot fully be analyzed using culture-based methods (132). Therefore, molecular techniques targeting overall microbial ecology allow for the identification of difficult to culture pathogens and their resistance genes, perhaps facilitating targeted and narrow spectrum use of antibiotics (4). Metagenomics represents a culture-independent genomic analytic approach to a population of microorganisms under their natural environment. A typical metagenomic approach involves isolating DNA from an ecological sample, cloning the DNA into a suitable vector, transforming the clones into a host bacterium, and screening the resulting transformants for phenotypic features (148). At this time, this approach remains time consuming and expensive for the surveillance and monitoring of antibiotic resistance. In contrast, targeted analysis of total community DNA is a rapid and relatively cheaper method for the examination of specific resistance genes extracted from complex samples such as feces (149). This method has been used for the detection of resistance genes (150-152) and quantification of antibiotic resistance genes (153-155). However, with this

method it is impossible to ascribe the resistance genes to specific bacteria in the bacterial community sample (149) as this would clearly present the phenomenon of ecological fallacy. Ecological fallacy occurs when inferences are made at individual level from data collected at a group level (such as the total community DNA).

Determination of copper resistance

Bacterial metal susceptibility measurements in the laboratory are expressed in terms of inhibition of bacteria growth as a function of mg of copper per L of medium, or else in mM units (mM per L); for example, 1 mM of copper equals 63.5 mg of copper per L (46). Determination of bacterial copper susceptibility depends on the species or strain of microorganism, the type of growth medium, and pH of the medium (46). Complex media can readily bind copper and make it unavailable to bacteria; on the other hand, minimal media can increase bacterial sensitivity to copper (46). Mueller-Hinton medium (broth, or agar), which is commonly used to determine resistance to antibiotics, is reported to be the optimal growth medium in which to determine copper resistance (46). Since cupric oxide (Cu (II)) is a weak acid, it causes a drop in the pH of the medium when added. Therefore, the pH of the medium has to be adjusted to 7.0 for optimal growth of bacteria (46, 54). Low pH (~6.0) increases the fraction of unbound Cu (II) availability to bacteria, while pH values above 7 favor the formation of insoluble copper hydroxides, which also reduce the amount of copper available to bacteria (46).

Generally speaking, three different assay methods have been used for copper susceptibility testing: 1) gradient agar plates (36), 2) an agar dilution assay based on CLSI procedures (156, 157), and 3) a broth dilution assay (158). Disc diffusion methods with impregnated discs are rarely used for copper susceptibility testing (46). In gradient agar diffusion two different solid agar layers, in which the first contains pH-adjusted elevated

concentrations of Cu, and the second plate contains no Cu, are used. Bacterial suspensions at 0.5

McFarland Standard are then applied across the metal gradient by using a inoculating wire after

the second layer solidifies (46). The latter two methods are based on serial copper dilutions

dissolved in agar or broth media, in which linear serial dilutions of copper are made; thereafter,

the MIC is determined in a method similar to that which is used for antibiotic susceptibility

testing. Unlike for antibiotics, there are no universally acceptable resistance breakpoints for

metal ion concentrations that can be used to designate microbial resistance (tolerance) versus

susceptibility. Therefore, the few studies conducted to determine the MIC of copper (and other

metals) have been based on the MIC distribution of the tested organisms (and thereafter, the

epidemiological cutoffs) (156).

Statistical analysis of antimicrobial susceptibility data

Minimum inhibitory concentration data from antimicrobial sensitivity test results are

most often categorized, using established breakpoints, into binary outcomes such as resistant

versus susceptible (including the intermediate category) for ease of interpretation and to guide

clinical therapy. This binary outcome is then analyzed by simple cross tabulation or through the

use of regression models. Logistic regression, without accounting for clustering, generalized

estimating equations (24, 25) for two-level data, or multilevel logistic regression, are used to

account for multivariable effects and clustering (23). Multi-level models are often used to

account for an existing hierarchical data structure (clustering of observations) and also to

simultaneously model covariates measured at multiple hierarchical levels which decomposes

variability across the hierarchical levels (159-161). Multivariate analysis of multidrug resistance

is used to account for pharmacological, biological or genetic dependence among multiple binary

outcomes, though it has rarely been used (25). For example, a multivariate probit regression

model can be used to assess the impact of a set of risk factors simultaneously on multiple binary resistance outcome variables. To model only two binary outcomes together, a simpler bivariate probit regression can be used (162).

The similarity of different antimicrobial phenotypes among bacterial isolates has been examined using cluster analysis (163, 164). Cluster analysis is a classification method that can be used to describe multidimensional antimicrobial resistance in bacteria and to classify the bacterial isolates according to their similarities, or dissimilarities, to each other depending on the binary resistance profiles (164). Cluster analysis can later be used for risk factor analysis that can then ideally be employed for the prevention and reduction of AMR (164, 165). The occurrence of multiple resistance patterns among bacteria of the same species tested against a panel of antimicrobial agents is a multivariate problem (165) thus requiring a multivariate analysis. Understanding this complex multivariate analysis is a challenging task. A univariate analysis, considering just one antimicrobial agent at a time (multiple binary outcomes) risks underestimating the true situation by ignoring multiple resistances. On the other hand, analyzing unique resistance profiles separately or as multivariate data with a multinomial distribution, often suffers from a lack of power as the number of isolates with each unique profile rapidly becomes sparse, even with thousands of isolates under examination (165). As a result, it is often not possible to fully assess the benefit of any particular control effort on AMR, especially considering the multiple resistances.

Cluster analysis to deal with the multivariate nature of AMR is made feasible by classifying the resistance profiles into clusters (groups) and modeling them either by univariate or multivariate regression with multinomial logistic regression (which accounts for the co-dependence of the resistance profiles in order to identify risk factors that are responsible for their

occurrence) (165). However, the multinomial logistic regression often suffers from a lack of

power and model instability due to sparse cells. The classical multivariate analysis approaches

such as factor analysis suffer from dimensional reduction which makes the interpretation very

challenging (165). Bayesian network analysis has been recently applied for the analysis of AMR

in veterinary medicine (165). Bayesian networks are graphical statistical frameworks used to

infer a probabilistic model that best describes the joint probability structure (co-dependence)

within observed data (165). Additive Bayesian network (ABN) modeling is comprised of a

collection of conditionally independent multivariable generalized linear models which can be

displayed as a directed acyclic graph. Thus, ABN modeling is analogous to a multivariate GLM

with multiple dependent variables.

Multidrug resistance counts (i.e., the number of antimicrobial agents to which a bacterial

isolate is resistant) are often analyzed as categorical variables, and very often categories must be

combined due to sparse cells. In such cases, logistic regression can be used to analyze the

different established dichotomies comparing each of the categories to a selected baseline

category (166, 167). This method results in loss of information as a result of collapsing some

categories of the response variable with a considerable loss of statistical power (166). Another

option is to use multinomial logistic regression and to build a series of logistic regression

equations together; however, the assumptions required of such a model are often unmet. Varga

et al., 2009 (167) used Poisson regression for the analysis of multiple antimicrobial resistances.

They defined the dependent variable as the number of antimicrobials to which an individual

isolate was resistant, potentially ranging from zero to the maximum number of antimicrobials

tested. However, their methods did not consider the generally ordinal nature of the MDR counts.

Ordered logistic regression analysis takes this natural ordering into account to examine the effect

of different risk factors on MDR count. When the proportional odds assumptions (i.e., the equality of the log-odds across the different cut points (categories) of the outcome variable) are met, a cumulative logit model (proportional odds model) can be used for the analysis of such ordered data (168).

In a proportional ordered logistic regression, the log-odds, and thus the odds ratio, are assumed to be constant across the order categories of the outcome and assumed only to differ by the levels of explanatory variable. However, the intercepts are allowed to vary across the categories of the outcome variable thus giving a series of parallel lines with constant slope but with different intercepts. It is exceedingly rare that these parallel line assumptions are met and so a more generalized ordered logit (gologit) model instead is used to relax the proportionality assumptions (169). In this model, both the intercepts and the slope (β's) are allowed to vary across the categories of the outcome variable. When the ordinal dependent outcome variable has more than 2 categories, the gologit model is equivalent to a series of binary logistic regressions where the categories of the dependent variable are dichotomized at each cut off.

In partial proportional odds model, a special case of gologit model, some of the explanatory variable coefficients will constant for all values of the categories of the outcome, while others will be allowed to vary. The Brant test provides both a global test of whether any variable violates the parallel lines assumptions, as well as tests the assumption for each explanatory variable separately (169). The gologit model compares all the categories greater than the current category to those less than or equal to the current category ($>$ vs. $\leq$ categories). Hence, positive coefficients indicate that higher values of the explanatory variable are associated with higher category levels of the outcome variable (in this case MDR count) than the level of category under consideration; on the other hand, the negative coefficients indicate that higher

values of the explanatory variable increase the likelihood of being in the current or else a lower category (169). When interpreting results for each panel, an expressed category of the outcome variable and all the lower coded categories serve as a reference group.

Analysis of MIC outcomes from susceptibility testing may proceed with outcomes treated as binary data (after categorizing based on a breakpoint into either susceptible or resistant); this is commonly done, though as explained above has certain limitations. First, there is loss of information associated with the binary categorization. Second, regression models do not converge when some of the resistance or susceptible cells defined by the treatment factors are sparse (i.e., there are few observations in each cell) or where empty cells exist (when all isolates are either susceptible or resistant). Third, over time there may be a shift in the MIC distribution within either or both of the resistant or susceptible populations that would not be evident or measured in the change in the proportion of susceptible or resistant bacteria. To this end, very few studies have employed different statistical approaches than the traditional binary classification based analysis.

As one example, Wagner et al., 2008 (22) considered MIC values as ordinal variables and modeled susceptibility data using a proportional odds model which evaluates the relative odds of increasing or decreasing one MIC level, as opposed to the logistic model which models the relative odds of resistance (versus susceptibility). However, as stated above under the analysis of MDR count data, the proportionality assumption for the MIC categories for each risk factor should be tested; notably, the authors did not report this test of assumption. Furthermore, there is a problem with handling left or right censored observations with MIC data arising from limited dilution plates. The authors above assigned the next lower or higher values for the MICs (that were less than or greater than testable limits) to overcome the problem of left and right

censoring. In another study (63), the MIC values were $\log_2$ transformed and modeled with a linear regression. Like the previous study, the MIC was arbitrarily assigned the next higher two fold dilution if bacteria grew at the highest concentration of the antimicrobial tested, in order to overcome the problem of right censoring. The MIC values take only limited values of doubling dilutions on a $\log_2$ scale; therefore, they truly are not continuous data suited to linear regression modeling.

Therefore, to overcome the issues of censoring which are typical of the MIC data in order to utilize the full information available from susceptibility testing, survival analysis has recently been adapted and introduced for the analysis of antimicrobial resistance data (170) and subsequently further refined and used by others (144). Survival analysis is a collection of statistical methods for the analysis of data where the outcome variable is expressed as time until an event occurs (171, 172). In the context of MIC data, the outcome of interest (time to event) is replaced by concentration until bacterial growth inhibition occurs (i.e., the MIC determination) (170). If bacterial growth is observed at a particular concentration, it is recorded as surviving at that concentration and if it fails to grow it is recorded as failure (an event of interest for the survival analysis). MIC data are left censored when bacteria failed to grow at the lowest measured concentration of antimicrobial agent tested and the MIC is reported as $\leq$ to the lowest concentration of the antimicrobial tested. Right censoring occurs when bacteria grow at the highest concentration of antimicrobial agent used and thus are reported as $>$ the highest concentration of the antimicrobial tested.

The MIC data can be graphically expressed, using Kaplan-Meier curves, as failure, hazard, or survivor function. The survival function *S(t)* represents the probability that a bacterial strain survives at least as high as a specified drug concentration, given that it grew at the

previous concentration. The hazard function *h(t)* is a rate that gives the instantaneous bacterial growth inhibition per unit concentration change, as that change approaches zero, conditional on the bacterial strain survived up to the current antimicrobial concentration considered. While the hazard function relates to growth inhibition, survivor function relates to surviving the antimicrobial concentration being considered given survival up to a certain MIC concentration. In survival analysis the effect measure used to evaluate impact of treatments (or other factors) on the MIC of bacterial isolates is the hazard ratio (HR) (171). An HR > 1 can be expressed as decreased survival, decreased resistance, increased susceptibility or increased failure (inhibited to grow).

The log-rank test is a nonparametric test that follows a chi-squared distribution and can be also used to compare the overall survival experiences of bacterial isolates obtained from treatment or control groups (171). A semi-parametric (with no assumed distribution of the outcome), Cox proportional hazards (PH) model (171), similar to typical survival analysis can be used to model the effect of treatment on the survival of bacterial isolates at increasing concentrations of antimicrobial agent (144, 170). Shared frailty can be used in the PH models to account for repeated measures within groups (such as pens or farms) and within samples (such as testing multiple bacterial isolates per sample). Cox PH model is robust even though the baseline hazard is not specified, giving close results to parametric models. Estimation of the Cox PH model uses a partial likelihood function rather than a complete likelihood function such as results for parametric-based estimation. This is because the likelihood formula considers probabilities only for those isolates which fail to grow (i.e., with the event of interest) and does not consider probabilities for those isolates which are censored (171); however, the survival concentration just prior to censorship is in fact used. Like the proportional odds model for ordinal MDR data

described above, Cox PH model assumes that the HR is constant over the MIC concentration ranges used, and that the concentration can have any value over the range of MIC values which effectively means it assumes that the MIC data are continuously coded.

Parametric regression models (with specified distributions based on the outcome) such as exponential, Weibull, lognormal, logistic and Gamma have also been used in addition to Cox PH model for the comparison of both agar disk diffusion and the micro-broth dilution results (144). The MIC results typically take only discrete concentrations with many isolates recorded with the same MIC values. The obvious problem of ties can be overcome by using an exact method as used by (144) or other methods such as Breslow, Efron and other exact marginal and partial likelihoods. Because of the inherently discrete nature of the data and the presence of many ties in the MIC data, discrete time (i.e. discrete concentration) survival (159) analysis is more appropriate for the analysis of MIC data. Discrete concentration survival analysis using a logistic regression model was employed for the study of antimicrobial resistance of *Enterococcus faecium* from Dutch broilers (170). The logistic model for hazards estimates log odds ratios as opposed to the HR of the Cox PH model; however, they are interpreted as a ratio of hazards (170). Discrete time survival analysis is generally employed on events that are recorded in discrete time and occurring at a relatively few time points; notably, this is exactly typical of MIC data. Discrete time survival models are specified in terms of discrete time hazard i.e. the conditional probability of the event occurring at a time point given that it has not already occurred. In order to make the concentration steps equidistant (i.e., like time in days) it is often necessary to log transform the concentrations to a base of 2, and to shift the values so that zero represents the value just below the lowest recorded concentration. Discrete time survival models become models for dichotomous responses after expanding the data to subject-time data (159).

Epidemiology of tetracycline, ceftiofur and copper resistance in *E. coli* from pigs

The widespread use of tetracyclines in humans, animals and agriculture has led to the widespread occurrence of tetracycline resistance, both in the commensal and pathogenic bacteria (16). The carriage of multiple *tet* genes of different classes is commonly found in Gram positive bacteria but is relatively uncommon in facultative Gram negative enteric bacteria (28).

The first tetracycline resistance was reported in 1953 (a few years after its first clinical use) in *Shigella dysenteriae* in Japan. The first multidrug resistant *Shigella* resistant to tetracycline, streptomycin and chloramphenicol was subsequently isolated in 1955 from Japan and reported at a prevalence of 0.02%. In 1960, MDR *Shigella* prevalence increased to 10%. In *S. flexneri* strains isolated between 1988 and 1993, 60% of the isolates were MDR (28). The magnitude of MDR, both in frequency and phenotype, has been continuously increasing both among Gram positive and Gram negative bacteria (28). Tetracycline resistance, commonly seen as part of a MDR phenotype, has become a global problem both in humans and animals.

In a prospective experimental study conducted in 1974, using 150 tetracycline supplemented (200 ppm) chickens and 150 control chicken, Levy et al., 1976 (135) observed that eventually all *E. coli* isolates became tetracycline resistant. In addition to tetracycline, over 70% of *E. coli* isolates were also resistant to more than two antibiotics, including ampicillin, sulfonamides and streptomycin. Similarly, they observed the eventual transmission of tetracycline resistant bacteria from chickens to farm personnel. The authors experimentally established the link between the use of in-feed antimicrobials and the occurrence of antimicrobial resistant bacteria in humans. In another report (173), the same authors also confirmed the spread of tetracycline resistance among *E. coli* through transferable plasmids.

The prevalence of tetracycline resistant generic *E. coli* isolated from pork chops at retail markets in the U.S., and tested by the FDA arm of NARMS (19), have indicated a fairly constant and established level from 2002-2010 with a mean prevalence of 50% and ranging from 44% in 2010 to 56% in 2004. The level of multidrug resistance, defined as resistance to ≥ 3 antimicrobial classes, has also been relatively constant with mean prevalence of 17% and ranging from 15% in 2009 to 21% in 2004 (Fig. 2.1).

In a retrospective study performed on a historical collection of *E. coli* isolated from 1950-2002 from animal and human sources, a significant upward trend in tetracycline resistance was observed (21). Tetracycline resistance was observed in 72% of pig isolates (n = 285) obtained during this time period. About 54% of these *E. coli* isolates were also resistant to ≥ 3 different antimicrobial classes. The authors also reported that tetracycline resistance commonly occurred as part of an MDR phenotype concurrently with other antimicrobials such as streptomycin, sulfonamides, ampicillin and chloramphenicol.

In the study conducted in finishing pigs to evaluate the effects of chlortetracycline dosage regimens on tetracycline resistance in *E. coli*, tetracycline resistance was 98% both when CTC was given continuously and as pulsed, when compared to 95% in the no antimicrobial group (22). This study indicated very high background tetracycline prevalence in the control pigs; therefore, the dosage regimens (continuous or pulsed supplementations of CTC) did not result in any significant measurable effects on the prevalence of tetracycline resistant *E. coli*. However, an on farm field trial conducted by Funk et al., 2006 (23) to investigate the effect of sub-therapeutic use of chlortetracycline on antimicrobial resistance in the fecal flora of swine, showed that aerobic Gram negative fecal isolates from the group which received sub-therapeutic CTC exhibited higher odds of being resistant to ampicillin (OR = 1.35), ceftriaxone (OR = 2.4)

and tetracycline (OR = 7.2) compared to isolates from the control group. This occurred, despite the observation that tetracycline resistance was very common among isolates from both the treatment and control groups. This study illustrates that the use of sub-therapeutic CTC can result in the co-selection of third generation cephalosporin resistance (ceftriaxone) among the enteric bacteria of pigs measured on phenotypic outcomes. However, since the study did not determine the actual resistance genes involved, nor their genomic locations, the genetic mechanism of co-selection was not established. Another major limitation of this study was that it was a cross sectional study where pigs were sampled only once at the end of their finishing cycle; thus, it was not possible to discern temporal relations. Another issue was that although a matched case-control study design was used, it was not accounted properly for in the analysis; indeed, this also might have affected the observed associations.

In a cross sectional study conducted among integrated multisite group cohorts of humans and animals (24) to investigate various risk factors associated with phenotypic antimicrobial resistance of *E. coli* isolates, the prevalence of tetracycline resistance was significantly ($P <$ 0.05) higher in the swine fecal *E. coli* population than in those from the associated human cohorts. Swine isolates were also more multidrug resistant than the human isolates. In a subsequent 3-year longitudinal study (25) at the same facility, following the baseline report, also indicated that swine *E. coli* isolates were more likely to be resistant to tetracycline (OR = 18.8; *P* < 0.001) and ceftiofur (OR = 5.6; *P* < 0.001) when compared to isolates from humans.

In another study, 47 purposively sampled farrow-to-finish swine farms in Canada were used to assess the association between on farm antimicrobial use, other management factors (26), and *E. coli* resistance to antimicrobials. In this study, 81% of the *E. coli* population was resistant to tetracycline. Tetracycline resistance was significantly associated with the age of the pigs

(higher in the younger weanling pigs as compared to finisher pigs: OR = 3.5; $P < 0.0001$) and with ceftiofur use on the farm (OR = 6.1; $P = 0.0$), indicating potential co-selection. In another report (27) these same authors found that antimicrobial resistance to a specific antimicrobial agent was more frequent on farms that used in-feed medication as compared to those did not, and a higher level of resistance prevalence was observed in the weaned pigs than in the finisher pigs. This study might have suffered from selection and information biases since the design included purposive selection of farms and a questionnaire was used to assess antimicrobial use and other management factors. Such biases could have resulted in overestimated or underestimated impacts of the risk factors on the level of antimicrobial resistance.

In a cross sectional risk factor study conducted to investigate the association between antimicrobial use and AMR in *E. coli*, Varga et al., 2009 (167) found that in-feed use of chlortetracycline in pig farms was significantly associated with increased risk of resistance to ampicillin and tetracycline. Importantly, they also found that the intercept in their tetracycline model was a positive number, suggesting that tetracycline resistance was not solely explained by concurrent tetracycline or other antimicrobial use in the farms. This positive intercept in the absence of the risk factors investigated also could be attributed to the high prevalence of tetracycline resistance in farms which did not use in-feed tetracycline; that is, the background levels of resistance that can be attributed to historical and cumulative use of antibiotics over the past 60 or so years. These authors also found that whereas in-feed use of any antimicrobial was associated with an increased risk of generic *E. coli* being resistant to multiple antimicrobials, the use of injectable trimethoprim-sulfamethoxazole and oxytetracycline were actually negatively associated with multidrug resistance. Clearly, the study has certain limitations. First, pig farms included in this study were not randomly selected. Since the study was a cross sectional design,

it is impossible to ascertain that the antimicrobial use caused the observed association with AMR. Information on the antimicrobial use practices was collected by questionnaire such that the study might have suffered from information (recall) bias.

In another study, Vieira et al., 2009 (174) investigated the association between on-farm tetracycline use patterns and the probability of detecting tetracycline resistant *E. coli* from healthy slaughter pigs in Denmark. They reported 34% prevalence of tetracycline resistant *E. coli* isolated from healthy pigs at slaughter. They found that the probability of detecting tetracycline resistant *E. coli* decreased as the interval between treatments increased, while it tended to increase with any increase in the treatment incidence rate. Interestingly, smaller herd size was associated with an increased probability of detecting tetracycline resistant *E. coli*, while larger herd size was associated with decreased probability of detecting resistance.

Ceftiofur, along with cefotaxime, and ceftriaxone (among others) is a 3rd generation cephalosporin. A crystalline free acid (CFA) formulation of ceftiofur is administered to swine intramuscularly at postauricular site at a dose of 5 mg/kg for the treatment of respiratory infections. Transferable resistance to 3rd generation cephalosporins is generally due to AmpC type beta lactamase hyper production, extended spectrum beta lactamases (ESBLs), and to a lesser extent due to metallo beta lactamases (MBLs). The cephamycinase encoding gene bla_{CMY-2} was formed as a result of AmpC beta lactamase hyper-production that became encoded by high copy number plasmids suitable for horizontal gene transfer. On certain plasmid types, the bla_{CMY-2} gene has been reported concomitantly with resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (65). The bla_{CMY-2}, bla_{PSE-1}, bla_{TEM-1} genes have been reported from *Salmonella enterica* isolated from U.S. food animals (92). Lutz et al., 2011 (58) reported 63% (1193/1899) ceftriaxone resistant *E. coli* prevalence from swine fecal

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samples.   Using PCR, 86.8% (n = 159) of the ceftriaxone resistant isolates harbored bla_{CMY-2}

gene. Similarly, 9.1% of S*almonella* isolates (n = 714) obtained from same swine fecal samples

were found to be resistant to ceftriaxone and were shown to contain bla_{CMY-2}.

For copper susceptibility, population-based studies involving wild type strains of *E. coli*

or other Gram negative enteric bacteria are scarce, and they have usually been based on banked

isolates from diagnostic laboratories. For example, Williams et al., 1993 (36) characterized 33

historical collections of enteric isolates for copper resistance (*E. coli*, *Salmonella* spp. and

Citrobacter freundii isolates) obtained from Australian and United Kingdom piggeries and

observed high mean levels of resistance of up to 18 mM of copper . Historical collections of

Gram positive (*Enterococcus* and *Staphylococcus* species) and Gram negative enteric bacteria

(*Salmonella* and *E. coli*) originating from broilers, cattle, and pigs in Denmark were tested for

copper susceptibility (156). Results indicated that whereas the enterococci formed two distinct

populations with respect to the copper MIC distribution, the other bacterial species formed a

unimodal population. Based on this finding, the authors concluded that meaningful acquired

copper resistance was found only in enterococci and was therefore absent among the

staphylococci and enteric Gram negative bacterial species tested. The MIC of all the *Salmonella*

isolates (n = 156) tested ranged from 20-28 mM while *E. coli* isolates (n = 202) exhibited MIC

ranges of 16-20 mM. Interestingly, there was a distinct difference in the median MIC

distribution of the three groups of bacteria. The enteric Gram negative bacteria had the highest

median MIC (24 mM for *Salmonella* and 20 mM for *E. coli*), followed by the enterococci (16

mM), while the staphylococcal species exhibited the lowest median MIC (2 mM for *S. aureus*

and 8 mM for *S. hyicus*) (Fig. 2.2). This suggests that enteric Gram negative bacteria

(*Salmonella* species and *E. coli*) are intrinsically more tolerant to copper, thus providing them a

competitive advantage over other bacterial species in the intestinal tract and other environments where copper concentrations may be elevated.

Mitigating antimicrobial resistance

Despite an apparently and arguably low risk associated with the use of antibiotics in animal production, it is critically important to apply all potential measures that can mitigate against and help contain the selection, propagation, and dissemination of AMR (9). Appropriate use of antimicrobial agents, both in humans and animals, combined with the discovery of novel antimicrobials, are likely to be the most effective means of combating the issue of antibiotic resistance (132).

Prudent use (also referred to as judicious use, or antimicrobial stewardship) of antimicrobial agents in veterinary medicine comprises all practical measures and recommendations that are intended to benefit both animal and public health while also preserving and maintaining therapeutic efficacy of the antimicrobials (5, 175, 176). These measures are intended to prevent or reduce the transfer of resistant bacteria (and resistance determinants) within animal populations and to humans (175). Among the ten key principles described by the World Veterinary Association are: that antimicrobial agents should not be used to mask poor hygienic practices, there is a need for quality assurance, following label directions is important, keeping records on the use of specific antimicrobial agent is essential and feeds directly into the monitoring and surveillance of antimicrobial resistance (9). Refinement, reduction and replacement of therapeutic prescriptions were all recommended for judicious uses of antibiotics for animal welfare (9). The use of antibiotics can be reduced if they are used only where their use is justified by evidence: such as limiting their use for prevention and control in nursery pigs, since research has shown that antimicrobials are most effective at this phase of

production. The use of antibiotics can further be refined by following a microbial safety relevant withdrawal period, which could minimize resistance dissemination (9). Replacement of antibiotics as growth promoters (AGPs) with possible non-antibiotic alternatives is an area of active research and development (9).

In the United States, the Interagency Task Force on Antimicrobial Resistance, composed of multiple agencies and co-chaired by Centers for Disease Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA) and the National Institutes of Health (NIH), was formed in 1999 to collaboratively combat AMR by focusing on surveillance, prevention and control, research, and drug development (177). The CDC (2) has recommended four core actions that should be taken to fight AMR. The first action involves infection prevention and control of the spread of resistant bacteria in the hospital, community, agriculture and environment. This reduces the amount of antibiotics needed to be used, thus slowing down the emergence and spread of AMR. Measures that could be taken include vaccination and improving the environmental hygiene under which animals are produced and thus helping to maintain and improve food safety. The second action is tracking resistant bacteria. The National Antimicrobial Resistance Monitoring System for enteric bacteria (NARMS) was established in 1996 as a collaboration among the CDC, FDA and the U.S. Department of Agriculture (USDA) with the objective of tracking changes in the levels of antimicrobial resistance of enteric bacteria (*Salmonella*, *Campylobacter*, *Shigella*, *E. coli* and Enterococci) from humans (CDC), retail meats (FDA – added in 2002) and food animals (USDA). The external subcommittee of the FDA's Science Advisory Board (178) evaluated the first 10 years of NARMS data and analyses, noted the likelihood of sampling bias and recommended the adoption of random selection of samples and specific farm data collection that would better enable the investigation of any link

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between farm use of antibiotics and the development and spread of AMR in human pathogens. Sampling of human clinical isolates, along with animal diagnostic laboratory isolates, both suffer from prior treatment bias. These biases limit the external validity of the results at the national level (85). Since 2006, the animal arm of NARMS discontinued the use of isolates from veterinary diagnostic laboratories, but continued that of obtaining animal isolates at slaughter (85). Retail samples and isolates that feed into NARMS are derived from the Foodborne Diseases Active Surveillance Network (FoodNet), operating under the emerging infectious disease program of the CDC. FoodNet was established in 1995 as a collaborative program among CDC, 10 state health departments, the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) and the FDA. It tracks trends for infections commonly transmitted through food and also provides clinical and epidemiologic data on some of the NARMS human isolates.

The third action involves improving the use of the existing antibiotics. Arguably greater amounts of antibiotics are used in the U.S. for food producing animals than for people (179). About 50% of antibiotic prescriptions in humans are estimated to be either unnecessary or else are misused (2). The CDC recommends the judicious use of antibiotics both in humans and animals since the use of antibiotics in both settings contributes to the emergence, persistence and spread of antibiotic resistant bacteria. Circumstantial evidence has suggested that the use of antibiotics in food producing animals can have public health impacts through four chains of events: use of antibiotics on the farm selects for antibiotic resistant bacteria while killing susceptible bacteria, these resistant bacteria can be transmitted from food producing animals to humans through the food supply, resistant bacteria can then cause infections in humans, and these infections can result in adverse health consequences for humans when antibiotic therapy is

indicated (2). Thus, because of the link between antibiotic use in food producing animals and the occurrence of antibiotic resistant infections in humans, it has been recommended that antibiotics should be used in food producing animals only under direct veterinary oversight (2, 180).

According to recently released documents, the FDA currently recommends that medically important antibiotics (including critically important, highly important and important antibiotics) should only be used under veterinary oversight for the treatment of infections and should not be used as AGPs (180). The FDA considers the use of medically important antibiotics for growth promotion purposes as injudicious (32). With the provisions of the two guidance documents for industry (#209 and 213) FDA, intends to institute voluntary measures to limit the use of medically important antibiotics in food producing animals and invoke stricter veterinary oversight in the use of these drugs. Most of the in-feed antimicrobials in the U.S. also are currently approved for over the counter use in food producing animals for the purposes of treatment, control, prevention of diseases and for growth promotion uses (32).

The fourth action recommended by CDC is to promote the development of new antibiotics and the development of new diagnostic tests for resistant bacteria (early detection and control). Since AMR is part of a natural evolutionary process, though it can be significantly slowed down, it cannot be eliminated (2). That said, the development of new antimicrobial agents has been significantly declining when compared to the rapidity with which AMR is occurring (2).

Establishing a direct relationship between the quantity of antibiotics used and development and levels of resistance is difficult due, in part, to a lack of specific antibiotic usage data at the farm level (181). Few ecological studies have found correlations between the amount of antimicrobials used and the concurrent frequency of antimicrobial resistance (25, 167, 174,

182, 183). Once emerged, antimicrobial resistance often persists even in the absence of direct antimicrobial selection pressure, thus minimizing the impact of antimicrobial removal strategies (4, 133, 181). The effect of removing antibiotic use on antimicrobial resistance depends on the duration that a specific antimicrobial has been marketed before such removals take place, as well as on the underlying mechanisms of resistance that were selected for. For relatively new antibiotics, removal of the antibiotic use can result in a significant reduction of resistance; on the other hand, for relatively older drugs, it often does not result in a significant reduction. For example, voluntary withdrawal of ceftiofur from *in ovo* use in broiler hatcheries in Canada resulted in a transient decline in the prevalence of ceftiofur resistant *Salmonella* Heidelberg and *E. coli* which then increased upon reintroduction of ceftiofur use (184). The ban on AGP uses of glycopeptides and macrolides from pigs in Denmark resulted in a significant reduction of resistant *E. faecium* to these classes of antibiotics (45). On the other hand, tetracycline resistance has remained relatively high despite cessation of the use of chlortetracycline as an AGP in swine feed for 13 years (185). Despite the ban on chloramphenicol use in US livestock production since 1980, chloramphenicol resistance has persisted, likely through co-selection with tetracycline, sulfonamides and other antimicrobials, though florfenicol is now available on the market (21).

Summary

Antimicrobials have been widely used in animal production to treat infectious diseases at therapeutic doses, for prevention and control of infections at therapeutic doses, and at subtherapeutic doses to enhance growth. Since many of the antimicrobials used in animals are also used in human medicine, there has been great concern that the use of antimicrobials in animals (particularly as antibiotic growth promoters (AGPs)) is responsible for much of the

antimicrobial resistance problem observed in human pathogens today.   Various studies and reports have indicated a qualitative linkage between the use of antimicrobials in animals and the occurrence of antimicrobial resistant bacteria in humans. Since the 1969 Swann report (186) in the United Kingdom (U.K.), that ultimately resulted in the U.K. ban of penicillin and tetracycline use as AGPs, similar measures have been undertaken in other European countries; these eventually resulted in the complete ban of the use of AGPs in animals in EU countries in 2006. In the United States, the use of in-feed antibiotics in animal production has been scrutinized and the Food and Drug Administration is taking important steps in limiting and regulating the use of medically important antibiotics.

Limiting or completely banning non-therapeutic use of antibiotics in animal production could paradoxically lead to reduced productivity, increased costs of production and increased foodborne diseases. Besides biosecurity measures, in order to maintain productivity and cost of animal production at optimum conditions under an AGP-free production system, the search for alternatives to antibiotics is currently an active research priority. The use of heavy metals is one of many such alternatives. The use of heavy metals in agriculture predated the discovery of antibiotics. However, there are concerns that heavy metals such as copper and zinc co-select for antibiotic resistance. Studies have shown that copper supplementation is associated with macrolide and glycopeptide resistance among enterococci in swine production. Furthermore, the use of zinc in swine production has been found to be associated with increased presence of methicillin resistant *Staphylococcus aureus* (MRSA). However, experimental studies evaluating the impact of elevated copper supplementation in pigs on antimicrobial resistance in Gram negative bacteria are lacking.

Experimental studies conducted in beef cattle have indicated an association between chlortetracycline use and the development of ceftiofur resistance in *E. coli*. Conversely, the use of ceftiofur in beef cattle has been associated with increased tetracycline resistance in *E. coli*. In pigs, several observational studies have demonstrated that the use of CTC at farms is associated with increased resistance to tetracycline and cephalosporins. However, experimental studies investigating the effects of in-feed CTC on ceftiofur resistance and other antibiotics commonly used in swine production are scarce. Most AMR studies have utilized traditional methods of bacterial culture followed by sensitivity testing, and to some extent detection of the resistance genes. Very few studies have used broader micro-ecological approaches for the study of AMR by examining the fecal metagenome.

The three studies described in the following pages are aimed at evaluating the impact of in-feed CTC and elevated levels of copper supplementation in weaned pigs on AMR in the gut microflora. The first study was designed to evaluate CTC and copper supplementation, either alone or in combination, on phenotypic AMR of Gram negative enteric bacteria using *E. coli* as an indicator organism. The second study was conducted to investigate the impacts of CTC and copper supplementation on the distribution and prevalence of tetracycline (*tet*A, *tet*B, *tet*C and *tet*E), ceftiofur (bla_{CMY-2}) and copper (*pco*D) resistance genes in *E. coli* isolates by using PCR. The last study is a micro-ecological study that was aimed at evaluating the impacts of CTC and copper supplementation on AMR in the gut microbiome of pigs by detecting the prevalence of 14 *tet* genes and quantifying gene copies of *tet*A, *tet*B, bla_{CMY-2} and *pco*D genes directly from total fecal community DNA. The dissertation closes with summaries and conclusions of these three efforts.
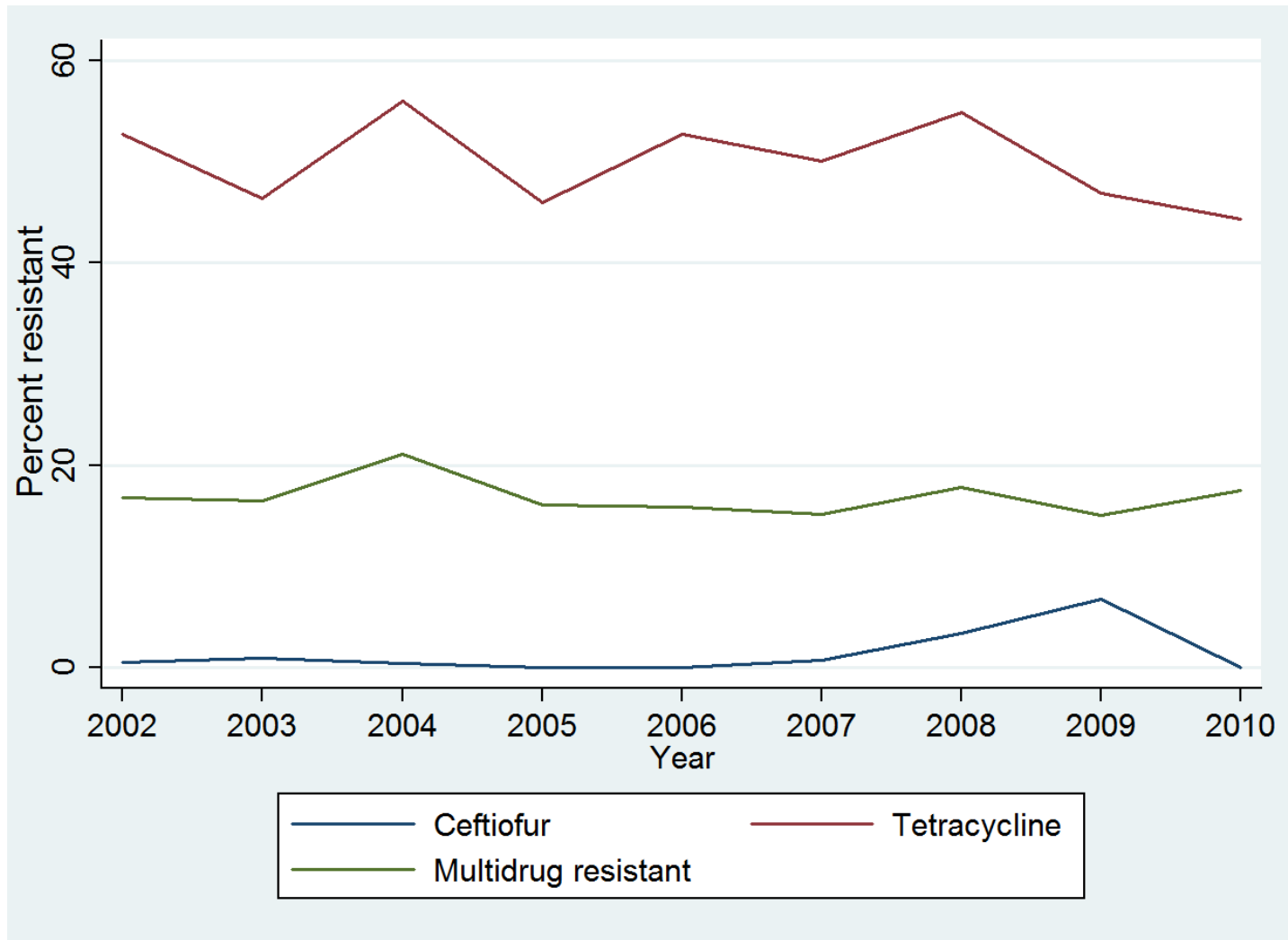
Figure 2.1. Line graph showing the reported prevalence of resistance to ceftiofur and tetracycline, and multidrug resistance of *E. coli* isolated from pork chops at retail market from 2002-2010 in the U.S..

Adapted from the FDA 2010 NARMS report (19)
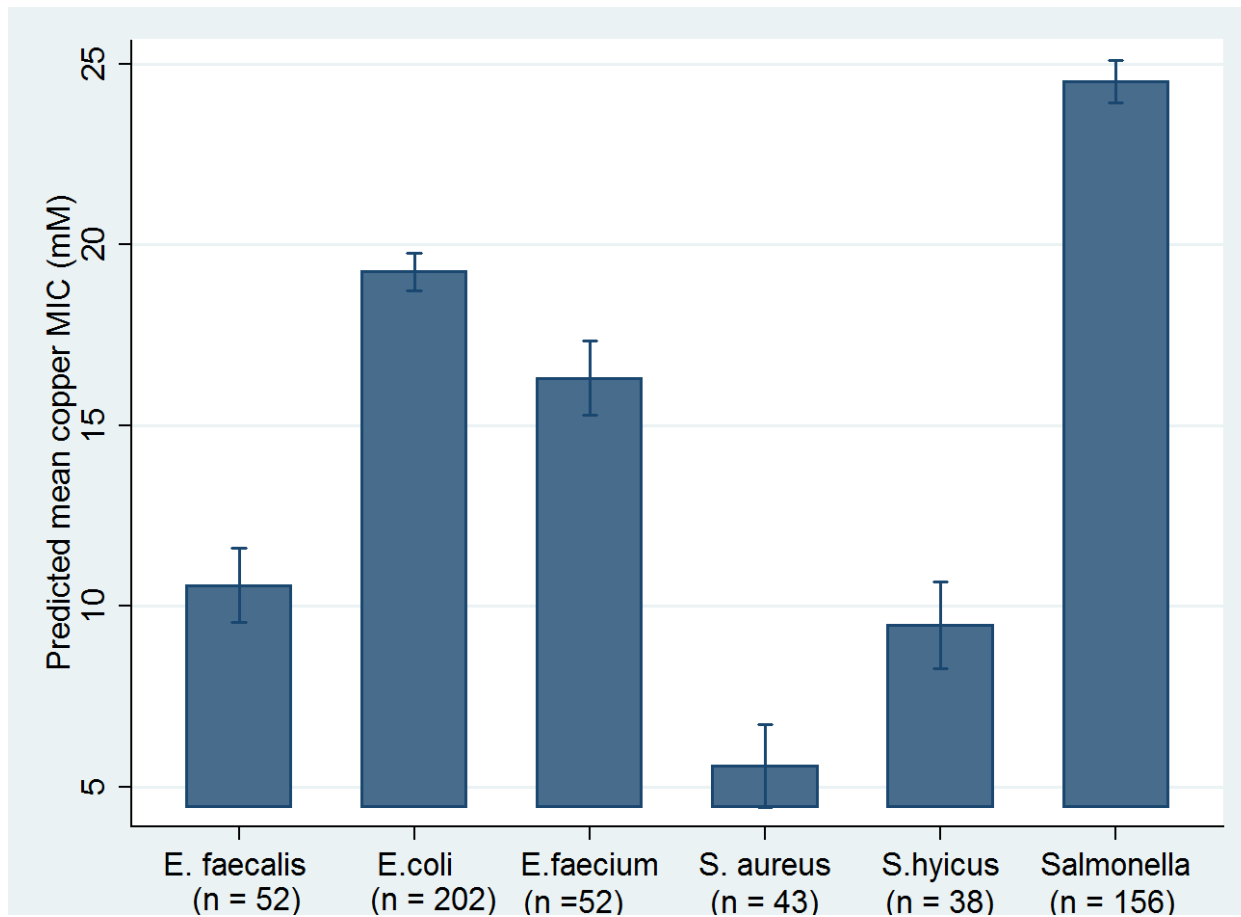
Figure 2.2. Mean minimum inhibitory concentrations (MIC) of copper for a variety of Gram positive and Gram negative isolates banked at a European diagnostic laboratory.

Adapted from Aarestrup et al., 2004 (156).

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Table 2.1. Antibacterial feed additives approved for swine growth promotion in the United States

Antibiotics	Antibiotic class	Synthetic antimicrobials	Antimicrobial class
Apramycin	Aminoglycoside	Arsanilic acid	Arsenical
Bacitracin methylene disalicylate	Polypeptide	Carbadox	Quinoxaline
Bacitracin zinc	Polypeptide	Roxarsone	Arsenical
Bambermycins	Glycolipid	Sulfamethazine	Sulfonamide
Chlortetracycline	Tetracycline	Sulfathiazole	Sulfonamide
Lincomycin	Lincosamide		
Neomycin	Aminoglycoside		
Oxytetracycline	Tetracycline		
Penicillin	Beta-lactam		
Tiamulin	Pleuromutilin		
Tylosin	Macrolide		
Virginiamycin	Streptogramin		

Adapted from Shryock and Page, 2006 (9); Cromwell, 2002 (40)

Table 2.2. List of WHO critically important human antimicrobials

Antimicrobial class	Antimicrobial(s)	Veterinary use only
Aminoglycosides	amikacin, gentamicin, kanamycin, neomycin, streptomycin	apramycin, framycetin
Carbapenems and other penems	doripenem, ertapenem, faropenem, imipenem, meropenem	
Cephalosporins (3rd and 4th generations)	cefepime, cefoperazone, cefotaxime, ceftriaxone	cefovecin, ceftiofur
Cyclic esters	fosfomycin	
Fluoro and other quinolones	ciprofloxacin, nalidixic acid, norfloxacin, ofloxacin	danofloxacin, difloxacin, enrofloxacin, ibafloxacin, marbofloxacin, orbifloxacin
Glycopeptides	dalbavancin, oritavancin, teicoplanin, telavancin, vancomycin	avoparcin
Glycylcyclines	tigecycline	
Lipopeptides	daptomycin	
Macrolides and ketolides	azithromycin, clarithromycin, erythromycin, midecamycin, roxithromycin, spiramycin, telithromycin	gamithromycin, kitasamycin, tildipirosin, tilmicosin, tulathromycin, tylosin,
Monobactams	aztreonam, carumonam	
Oxazolidinones	linezolid	
Penicillins (natural aminopenicillins and antipseudomonal)	amoxicillin, ampicillin, in, penicillin G, penicillin V , ticarcillin	penethamate hydroiodide
Polymyxins	colistin, polymyxin B	
Rifamycins	rifabutin, rifampicin, rifaximin, rifapentine, rifamycin	
Tuberculosis and other mycobacterial drugs	ethambutol, isoniazid, terizidone, tiocarlide	

Adapted from WHO, 2012 (84)

Table 2.3. WHO listing of highly important and important human antimicrobials

Antimicrobials class	Antimicrobial(s)	Veterinary use only
Highly important antimicrobials		
Amdinopenicillins	mecillinam, pivmecillinam	
Amphenicols	chloramphenicol, thiamphenicol	florfenicol
Cephalosporins (1st and 2nd generation) and cephamycins	cephalexin, cefalotin, cefoxitin, cefprozil, cefradine, cefroxadine, ceftezole, cefuroxime, flomoxef, loracarbef	cefalonium
Lincosamides	clindamycin, lincomycin	pirlimycin
Penicillins (antistaphylococcal)	cloxacilllin, dicloxacillin, flucloxacillin, oxacillin, nafcillin	
Pleuromutilins	retapamulin	
Pseudomonic acids	mupirocin	
Riminofenazines	clofazimine	
Steroid antibacterials	fusidic acid	
Streptogramins	quinupristin/dalfopristin pristinamycin	virginiamycin
Sulfonamides	sulfadiazine, sulfadimethoxine, sulfadimidine, sulfisoxazole, sulfamethizole, sulfamethoxazole, sulfapyridine, sulfathiazole, tetroxoprim, trimethoprim	ormosulfathiazole, phthalylsulfathiazole
Sulfones	dapsone, aldesulfone	
Tetracyclines	chlortetracycline, clomocycline, demeclocycline, doxycycline, lymecycline, metacycline, minocycline, penimepicycline, oxytetracycline, tetracycline	
Important antimicrobials		
Aminocyclitols	spectinomycin	
Cyclic polypeptides	bacitracin	
Nitrofurantoins	furazolidone, nitrofurantoin, nifurtoinol, nitrofural	furaltadone
Nitroimidazoles	metronidazole, tinidazole, ornidazole	

Table 2.4. OIE list of veterinary critically important antimicrobials

Antimicrobial class	Antimicrobial(s)
Aminocyclitol	spectinomycin
Aminoglycosides	streptomycin, kanamycin, neomycin, apramycin, gentamicin, tobramycin, amikacin
Cephalosporins	
1st generation	cefacetrile, cefalexin, cefalotin, cefapyrin, cefazolin, cefalonium
2nd generation	cefuroxime
3rd generation	cefoperazone, ceftiofur, ceftriaxone
4th generation	cefquinome
Macrolides	
Azalide	tulathromycin
Macrolides C14	erythromycin
Macrolides C16	josamycin, kitasamycin, spiramycin, tilmicosin, tylosin, mirosamycin, terdecamycin
Penicillins	
Natural Penicillins	benzylpenicillin, penethamate hydroxide, penicillin procaine
Amdinopenicillins	mecillinam
Aminopenicillins	amoxicillin, ampicillin, hetacillin
Aminopenicillins+betalactamase inhibitor	amoxicillin/clavulanic acid
Carboxypenicillins	ticarcillin, tobicillin
Ureidopenicillin	aspoxicillin
Phenoxypenicillins	phenoxymethylpenicillin, phenethicillin
Antistaphylococcal penicillins	cloxacillin, dicloxacillin, nafcillin, oxacillin
Phenicols	florphenicol, thiamphenicol
Quinolones	
1st generation	flumequin, miloxacin, nalidixic acid, oxolinic acid
2nd generation (Fluoroquinolones)	ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin
Sulfonamides	sulfadiazine, sulfadimethoxine, sulfadimidine, sulfafurazole, sulfamethazine, sulfadimethoxazole
Sulfonamides+Diaminopyrimidines	sulfamethoxypyridazine, trimethoprim+sulfonamide
Diaminopyrimidines	baquiloprim, trimethoprim
Tetracyclines	chlortetracycline, doxycycline, oxytetracycline, tetracycline

Table 2.5. OIE veterinary highly important and important antimicrobials

Antimicrobial classes	Antimicrobial(s)
Veterinary highly important antimicrobials	
Ansamycin-Rifamycins	rifampicin, rifaximin
Fosfomycin	fosfomycin
Ionophores	lasalocid, maduramycin, monensin, narasin, salinomycin, semduramicin
Lincosamides	pirlimycin, lincomycin
Pleuromutilins	tiamulin, valnemulin
Polypeptides	enramycin, gramicidin, bacitracin
Cyclic polypeptides	colistin, polymixin
Veterinary important antimicrobials	
Bicyclomycin	bicozamycin
Fusidic acid	fusidic acid
Novobiocin	novobiocin
Orthosomycins	avilamycin
Quinolones	carbadox
Streptogramins	virginiamycin

Table 2.6. List of tetracycline resistance genes registered in online databases as of 2013

Genes	Gene bank accession #	Genes	Gene bank accession #
	Efflux pump coding genes (30)		
*tet*A	AF534183, AJ419171, X75761, AF542061, J517790, AY196695, X00006, L27758, AJ313332	*tet*31	AJ250203
*tet*B	J01830, AF223162, V00611, AL513383, AJ277653, AF326777, AP000342	*tet*33	AJ420072
*tet*C	AB023657, AY043298, AY28550, X01654, AY046276, Y19114, AF055345, K00005, NC_003213, NC_003123, NC_003124, NC_002109, AY043299	*tet*35	AF353562
*tet*D	X65876 , L06798, D16172, AF467074, AF467077	*tet*38	AY825285
*tet*E	L06940	*tet*39	AY743590
*tet*G	AF071555, S52437, AF133140, AF133139, AJ276217	*tet*40	AM419751
*tet*H	U00792, AJ245947, Y15510, Y16103,	*tet*41	AY264780
*tet*J	AF038993	*tet*42	EU523697
*tet*K	M16217, U38656, S67449, J01764, U38428	*tet*43	GQ244501
*tet*L	M11036, X08034, D00006, X51366, X60828, D12567**,** U17153, AY081910, M29725, M235948	*tet*45	JF837330, GU584222, GU584217, JF837331
*tet*A(P)	AB001076, L20800	*tet*A(46)	HQ652506
*tet*V	AF030344	*tet*B(46)	HQ652506
*tet*Y	AF070999	*otr*B	AF079900
*tet*Z	AF121000	*otr*C	AY509111
*tet*30	AF090987	*tcr*3	D38215
	Ribosomal protection protein coding genes (12)		
*tet*M	U08812, X75073, M85225, M21136, M85225, X90939, X92947, X04388, U58985, X56353	*tet*W	AJ222769, AY049983, AY196919, AJ427422, AY196917, AJ427421, AY196918
*tet*O	M18896, Y07780, M20925	*tet*32	DQ647324, EF626941, EF626942, EF626943, EU722333
*tet*B(P)	L20800	*tet*36	AJ514254
*tet*Q	Z21523, U73497, X58717, L33696	*tet*44	FN594949, NZ_ABDU01000081
*tet*S	L09756, X92946	*otr*A	X53401
*tet*T	L42544	*tet*	M74049
	Inactivating enzymes coding genes (3)		
*tet*X	M37699, EU918344, AB097942	*tet*37	AF540889
*tet*34	AB061440		
	Unknown gene function (1)		
*tet*U	U01917		

Chapter 3 - Effects of chlortetracycline and copper supplementation on phenotypic antimicrobial resistance of fecal *Escherichia coli* from weaned pigs

Abstract

Chlortetracycline and copper are commonly used as in-feed antimicrobial growth promoters in U.S. pig production systems. The objective of this study was to evaluate the effects of CTC and copper supplementation in weaned pigs on phenotypic antimicrobial resistance among *Escherichia coli* isolated from feces. One hundred and sixty weaned pigs were randomized to 32 pens (5 pigs per pen) which were then randomly allocated to control, copper, chlortetracycline (CTC), or copper plus CTC treatment groups. Phenotypic susceptibilities of 1,152 *E. coli* isolates (288 per group) to antimicrobials were determined by broth microdilution. Data were analyzed using multilevel mixed effects logistic regression, discrete-time survival analysis, or generalized ordered logistic regression, as appropriate. All isolates were susceptible to amikacin, nalidixic acid, and ciprofloxacin. Tetracycline resistance was significantly ($P <$ 0.001) higher in the CTC-supplemented group (99.0% [95% CI = 98-100%]) compared to the control group (95% [95% CI = 94-97%]) with no significant effect of treatment period. The prevalence of resistance to most of the antibiotics decreased over the 6 weekly sampling periods in all treatment groups, likely reflecting the impact of age. About 91% of the *E. coli* isolates were resistant to ≥3 antimicrobial classes. CTC- supplementation was significantly associated with increased susceptibility of *E. coli* to copper when compared to the control group (HR = 7 [95% CI = 2.5–19.5]; $P < 0.001$). In conclusion, *E. coli* isolates from the nursery pigs exhibited high levels of antibiotic resistance, with diverse and multidrug resistant phenotypic profiles. A

high level of copper supplementation was associated with decreased prevalence of certain high-level multidrug resistance phenotypes; however, such associations require further investigation under varied field conditions.

Introduction

In swine production, antimicrobials are used, usually in feed or water, for prophylactic, therapeutic, or growth promotion purposes (187). Chlortetracycline (CTC) is the most widely used antimicrobial growth promoter (AGP), either alone or in combination with other drugs (e.g., with sulfamethazine, sulfathiazole, or penicillin G), in U.S. swine production (17, 18). Tetracycline resistance is the most widespread and commonly reported phenotype among both Gram positive and Gram negative bacteria (20). Tetracycline resistance occurs primarily due to acquisition of tetracycline resistance (*tet*) or oxytetracycline resistance (*otr*) genes and is mainly mediated either through active efflux of the molecule or through ribosomal protection (31).

Copper is an essential micro-mineral, commonly included in swine feed at National Research Council (NRC) recommended levels of 5-6 ppm (39). At increased concentrations well beyond its basic requirements, it has been shown to promote the growth of pigs (79). For bacteria, copper is an essential nutrient; however, like most micronutrients it is toxic in excess concentrations (56). Bacteria have developed mechanisms to regulate intracellular copper concentrations in the face of varying ambient concentrations. In addition to chromosomally mediated copper handling mechanisms, copper resistance in *E. coli* is mediated through a plasmid-borne copper (*pco*) resistance cluster consisting of seven genes: *pco*ABCDRSE (56).

As a result of escalating debate over whether using AGPs in animals results in a public health risk, AGPs were banned in the European Union in 2006 on the basis of the "precautionary principle" (75). More recently, the United States Food and Drug Administration (FDA) has

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deemed AGP use as "imprudent" and issued a guidance document to industry to voluntarily remove such label claims from their products (32). To compensate for any production losses due to AGP bans, non-antibiotic alternatives have become a major focus of attention (35).

Copper is one of the most commonly used heavy metals for growth promotion in swine production. There is a growing concern that heavy metals, such as copper, help to maintain a pool of antibiotic resistance through co-selection and cross-resistance (50). Transferable copper resistance (*tcr*B) in *Enterococcus* species has previously been found to be associated with macrolide resistance (*erm*B) and *tet*M genes (42-44, 156). Although the effects of metals on Gram positive enteric bacteria have been extensively studied in several farm animal species (41, 42, 44), associations between copper supplementation and antimicrobial resistance (and tetracycline resistance in particular) in *E. coli* have not yet been examined under properly controlled experimental conditions reflecting field conditions. Therefore, we conducted an experimental study to investigate the effects of copper and CTC supplementation in pigs on phenotypic susceptibilities of *E. coli* to antibiotics and copper.

Materials and methods

Experimental design and E. coli isolation

The study included a full factorial 2-way cluster randomized design using 160 weaned piglets that were randomized to 32 pens (5 per pen). The treatment groups consisted of control (basal diet with NRC recommended copper concentration at 16.5 mg/kg of feed), copper (fed copper sulfate at an elevated concentration of 125 mg/kg of feed), CTC (basal diet plus CTC at 550 mg/kg of feed), or copper plus CTC (basal diet plus elevated copper and CTC as above). The treatment groups were randomly assigned to pens (8 pens/treatment) while blocking by barn

(n = 2) and accounting for the geographical distribution of pens within barn to avoid fecal cross-contamination between pens. After two weeks of adaptation to the basal diets and local environment, piglets were fed experimental diets for 21 days followed by a washout (i.e., no CTC or copper) period of two weeks before being sent to grower operations. The study was performed in the segregated early weaning facility at Kansas State University, and animal handling and experimental designs were approved by the University Institutional Animal Care and use Committee (IACUC# 2773).

A total of 576 fecal samples (144 samples/treatment group) were collected weekly from three randomly selected piglets per pen over a period of 6 weeks. Fecal samples were collected per rectum, placed into individual WhirlPak bags (Nasco, Ft. Atkinson, WI), and transported on ice to the laboratory. For isolation of *E. coli*, approximately 1 gram of feces was mixed in 9 ml of buffered peptone water, and 0.1 ml of the suspension was spread-plated on MacConkey agar (Difco™, Becton Dickinson Co., Sparks, MD) and incubated at 37^0C for 24 h. Two distinct lactose-fermenting colonies per plate, characteristic of *E. coli*, 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 in protect beads (Cryocare®, Key Scientific Products, Round Rock, TX) for later analysis. A total of 1,152 isolates (288 per group) were tested for their phenotypic antimicrobial susceptibilities.

Antimicrobial susceptibility testing

For each isolate, minimum inhibitory concentration (MIC) for 15 antibiotics and copper was determined by a broth microdilution method following the clinical laboratory standards institute (CLSI) veterinary antimicrobial susceptibility testing guidelines (147). Antimicrobial susceptibility testing was performed with the National Antimicrobial Resistance Monitoring

System (NARMS) custom-made plates developed for Gram negative bacteria (Trek catalog #

CMV1AGNF and CMV2AGNF) using the Sensititre system (Trek Diagnostic Systems,

Cleveland, OH). 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. *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. Based on MIC values, the *E. coli* isolates were categorized into

susceptible (included intermediates) or resistant according to CLSI MIC interpretative criteria for

Enterobacteriaceae (147), except for streptomycin, for which NARMS consensus breakpoints

were used (19). The antimicrobial concentration ranges and clinical breakpoints are depicted in

Table 3.1.

Susceptibility to copper, in the form of copper sulfate (CuSO4), 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. CuSO4 concentrations used were 0, 12, 16, 20, 24, 28, and

32 mM concentrations. Because the addition of copper sulfate to the medium causes a

significant drop in pH (54), the medium was adjusted to a pH of 7.0 by adding NaOH prior to

inoculation. To ensure legitimate direct comparison of our results with those from other

published studies relying on an agar dilution method (156), the agar dilution method was

performed on 10 isolates selected from each MIC dilution to compare with the broth

microdilution method we used. The results from both methods were comparable (all were matched to dilution, with the exception of a very few at one dilution on either side).

Statistical analysis

Data were analyzed using STATA version 12.1 (Stata Corp LP, College Station, TX), and $P < 0.05$ was considered significant. A full-factorial model evaluating treatment (copper treatment, CTC treatment and time) and their two- and three-way interaction effects was used throughout the analysis. Sampling days were categorized into treatment periods as: baseline (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.

Multivariable multilevel mixed effects logistic regression was used to model the fixed effects of treatments and treatment period (and their interactions) with random effects accounting for repeats within pen and fecal sample on phenotypic resistance. This approach was also used to model the effects of treatment and treatment period on multidrug resistance (MDR: binary classified as resistance to ≥ 3 classes of antimicrobials) (19). A generalized ordered logistic regression model was used to examine the effects of treatments and treatment period on the number of antimicrobial agents and classes (Table 3.1) to which a single isolate was resistant, accounting for the ordinal nature of the outcomes. Clustering effects by pen were adjusted by requesting a clustered robust standard error. Copper MIC data were analyzed using discrete time survival analysis (159, 170) with fixed effects of treatment and treatment period and random effects of fecal sample and pen. "Time-to-event" of a typical survival analysis was replaced by

"concentration-to-event", with the "event" being growth inhibition. The log-rank test was used

to compare the survival experiences among the treatment groups and treatment periods.

Results

Antimicrobial susceptibilities

The prevalence of antimicrobial resistance (AMR) and the MIC of 15 antibiotics for *E.

coli* tested across all samples are illustrated in Table 3.2. The MIC values for ampicillin,

streptomycin, sulfisoxazole, and tetracycline all were right-censored beyond the highest

concentration tested for greater than 60% of the isolates. All isolates were susceptible to

amikacin (note: present only on CMV1AGNF; n = 802 isolates) and the quinolones

(ciprofloxacin and nalidixic acid); as a result, these three antimicrobials were excluded from

subsequent multivariable analyses. The prevalence of azithromycin resistance did not differ by

copper ($P = 0.122$) or CTC ($P = 0.826$) treatment. Because not all isolates were tested for

azithromycin (n = 350 isolates), it too was also excluded from multivariable analysis.

Individual binary resistance outcomes, cross-tabulated by treatment group and treatment

period, are provided in Table 3.3. Resistance to beta-lactams decreased significantly ($P < 0.05$)

over time in all three of the treated groups, with no change in the control group. Kanamycin and

tetracycline resistances during the treatment period were significantly ($P < 0.05$) higher in the

CTC-supplemented group compared with controls. 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 (Figure

3.1).

A total of 101 unique phenotypic profiles was observed. Only three isolates (0.35%) were pan-susceptible to all 15 antibiotics tested on the NARMS panel, while 7 isolates (0.6%) were susceptible to an observed maximum of 12 antibiotics. The predominant phenotype (22.5%; n = 1,152) was of multidrug resistance to 10 drugs, followed by a phenotype with resistance to 8 different antibiotics (12%; n = 1,152). In contrast to these highly MDR phenotypes, isolates exhibiting resistance solely to tetracycline were limited to just under 6% of the total isolates (n = 1,152) (Table 3.4).

Multivariate analysis of E. coli resistance to multiple AMR outcomes

Six antimicrobial resistance binary outcomes including amikacin/azithromycin, kanamycin, trimethoprim/sulfamethoxazole, ciprofloxacin, nalidixic acid, and tetracycline were excluded from the multivariate analysis because the model failed to converge due to sparsely populated categories. Copper supplementation was significantly associated with lowered resistance to ampicillin ($P = 0.007$), amoxicillin/clavulanic acid ($P = 0.001$), ceftriaxone ($P = 0.049$), and cefoxitin ($P < 0.001$) among isolates obtained in the post-treatment period when compared with the baseline isolates; however, these differences were not seen during the treatment period itself, nor among the remaining five antimicrobials. Copper plus CTC supplementation was significantly associated with increased resistance to amoxicillin/clavulanic acid ($P = 0.001$), cefoxitin ($P < 0.001$), and ceftiofur ($P = 0.013$) among post-treatment period isolates compared with day 0 isolates; once again, these associations were not significantly different during the treatment period when compared with either pretreatment or post-treatment period (Table 3.3). Pairwise correlations (associations) among all possible sets involving the nine antibiotics that were included in the multivariate analysis are shown in Table 3.5. As

expected, the beta-lactam resistances were highly correlated (90–98%); furthermore, the cephalosporin resistances were especially highly correlated with one another (94–98%).

Multidrug resistance

Defining multidrug resistance (MDR) as the number of antibiotics across all isolates, the median MDR count was 8 (95% CI = 8-8). The frequency distribution of MDR is shown in Fig. 3.2. The isolates were most commonly resistant to 10 antibiotics, both across all the samples and when subdivided by treatment groups (with the exception of the CTC group, in which an MDR count of 8 prevailed). The distribution of the number of antibiotic resistances appeared somewhat bimodal, with peaks at 3–4 and again at 8–10. Across all the sampling days, but subdivided by treatment group, the median count of MDR was 7 (95% CI = 7–8), 8 (95% CI = 8–8), 8 (95% CI = 7–8) and 8 (95% CI = 8–8) in the control, CTC, copper, and copper plus CTC groups, respectively. Across all treatment groups, the median MDR was 10 (95% CI = 9–10), 8 (95% CI = 8–8) and 8 (95% CI = 7–8) for pretreatment, during treatment, and post-treatment isolates, respectively. No statistically significant (P = 0.2194) treatment effect was observed on the median MDR count (Kruskal-Wallis χ^2 = 4.2 with 3 degrees of freedom [df]); however, median MDR count was significantly (P = 0.0001) higher among pretreatment isolates (Kruskal-Wallis χ^2 = 36.0 with 2 df). Results of the generalized ordered logit model also showed that the MDR count decreased significantly (P < 0.001) across the treatment periods. Copper supplementation tended to be associated with increased MDR when comparing MDR counts of >6 to ≤6 (OR= 1.7; 95% CI = 1.02–2.7; P = 0.041), with no observed CTC effect (P > 0.05).

Multidrug resistance count examining the number of different antimicrobial classes (0–8 classes) to which a single *E. coli* isolate was resistant was also examined. Across all sampling days, the median count of MDR class count was 6 (95% CI = 5–6), 6 (95% CI = 5–6), 5 (95% CI

= 5–6) and 6 (95% CI = 6–7) in the control, CTC, copper, and copper plus CTC groups,

respectively. Across all treatment groups, the median MDR class count was 7 (95% CI = 6–7), 6

(95% CI = 5–6) and 6 (95% CI = 5–6) for pretreatment, during treatment, and post treatment

isolates, respectively. No statistically ($P = 0.1308$) significant treatment effect was observed on

the median MDR class count (Kruskal-Wallis $\chi^2 = 5.3$ with 3 df), but the median MDR class

count was significantly ($P = 0.0001$) higher in the pretreatment isolates regardless of treatment

effect (Kruskal-Wallis $\chi^2 = 24.6$ with 2 df). Generalized ordered logistic regression analysis also

showed that MDR class significantly ($P < 0.05$) declined over time, with no apparent treatment

effect ($P > 0.05$).

Defining MDR as an isolate exhibiting resistance to ≥3 classes of antimicrobials

(thereafter, a binary classification), 91% (95% CI = 89–92%) of all the isolates (n = 1,152) were

MDR (Fig. 3.3A). Univariate analysis (LR$\chi^2 = 8.7$; 3 df) suggested, and Fig. 3.3B shows, that *E.

coli* isolates in the CTC supplemented group were more likely to be MDR ($P = 0.033$) when

compared with the control group (94% [95% CI = 91–97%] versus 88% [95% CI = 84–91%])

with no significant ($P = 0.363$) difference among the treatment periods (LR$\chi^2 = 2.0$; 2 df).

Examined using multivariable multilevel mixed effects logistic regression analysis, CTC

supplementation significantly ($P = 0.040$) increased MDR probability among the *E. coli* isolates

obtained during the treatment period.

Determination of copper susceptibility

Across all isolates, median MIC was 20 mM, ranging from 12–32 mM (essentially, the

limits of our assay). Figure 3.4A shows copper MIC frequency distribution by treatment groups.

The majority of the isolates had an MIC value of 20 mM; this specific MIC value ranged from

57% in the control group to 71% in the CTC supplemented group. The MIC50 and MIC90 of

the *E. coli* were 20 mM and 28 mM across all the isolates, respectively. Using the log rank test, *E. coli* from the control group generally exhibited higher survival function (thus, were more resistant) ($P = 0.027$, 3 df) when compared with the treatment groups. 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 or increased failure rates ($P = 0.0018$) compared with *E. coli* obtained from the no-CTC group. By discrete time survival analysis, CTC by period interaction showed a significant effect on 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 compared with isolates from the control group. Figure 3.4B shows the survival curves for each of the treatment groups plotted against copper concentration.

Using the log rank test, copper MIC of the *E. coli* isolates was significantly associated with decreased resistance to gentamicin ($P = 0.0092$), kanamycin ($P < 0.0001$), sulfisoxazole ($P < 0.0001$) and trimethoprim/sulfamethoxazole ($P = 0.0011$). The susceptibility of *E. coli* isolates to increasing concentrations of copper by four binary antibiotic outcomes is given in Fig.3.5. Using the Cox proportional hazards regression model, gentamicin resistance (HR = 1.1 [95% HR = 0.97-1.2]) was no longer significantly associated with copper MIC. However, isolates resistant to kanamycin (HR = 1.2 [95% CI = 1.03-1.4]), sulfisoxazole (HR = 1.3 [95% CI = 1.1-1.5) and trimethoprim/sulfamethoxazole (HR = 1.3 [95% CI = 0.99-1.78) had increased susceptibility to copper. The kanamycin containing phenotypic profile sulfisoxazole-kanamycin-streptomycin-tetracycline was significantly ($P = 0.008$) associated with a higher copper MIC. Among 83 of

98

the *E. coli* isolates with this unique profile, 82 isolates had copper MIC of ≥20 mM. Of these 83

isolates, 79.5% and 13.3% had copper MIC values of 20 and 24 mM respectively.

Discussion

Susceptibility to antimicrobials

Generally speaking, prior to the start of treatment, the AMR levels among the *E. coli*

isolated from the study pigs were already extremely high; this makes it very difficult to evaluate

the effect of CTC supplementation on AMR in pigs of this age group. At first glance, this might

appear to be related to the prior use of antimicrobial agents in the breeder farm that supplied the

study piglets, or else to a persistent, diverse, and resistant *E. coli* population present in the

environment of the segregated early weaning facility where this experiment was conducted.

Otherwise it could be reflective of the widespread dissemination of AMR associated with the use

of antibiotics for long period of time since their discovery. Similar results have been reported,

however, across agricultural production species and systems that point to a strong and

generalized association between isolates derived from neonatal animals and MDR phenotypes as

a result of more frequent antimicrobial therapy and/or prophylaxis in neonates than older animals

(188, 189).

We did not observe resistance to amikacin, ciprofloxacin, and nalidixic acid, similar to

other studies in porcine *E. coli* (19, 24, 25, 185). Among aminoglycosides, resistance to

amikacin is rare due to its resistance to enzymatic inactivation (90). Ciprofloxacin and nalidixic

acid are not approved in swine, but enrofloxacin has some limited labeled use in swine. The low

azithromycin resistance (6.3%, n = 350) observed could be also due to lack of direct exposure to

azithromycin since it is not approved for use in swine in the U.S. However, there are some

macrolides that have been approved, while still others are contraindicated due to toxicity. The fact that copper supplementation was significantly ($P = 0.01$; OR = 0.08 [95% CI = 0.01–0.67]) associated with a decrease in azithromycin resistance suggests that copper supplementation might exert an antagonistic effect on azithromycin resistance elements, thus offering a potential replacement, at least for macrolide AGPs such as tylosin, in swine production.

The most common resistance phenotypes were observed to older antimicrobials: tetracycline (97%), sulfisoxazole (82%), ampicillin (75%), and streptomycin (71%). These drugs have been used extensively in animals as AGPs, alone and in combination with other products, which can facilitate co-selection through genetic linkages. The percentage (36%; n = 1,152) 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 (63). About 89% of these isolates also were co-resistant to ceftiofur, as is similar to reports from cattle *E. coli* (62, 63). Because chloramphenicol use in food animals has been prohibited in the U.S. since the 1980s, chloramphenicol resistance is likely maintained among *E. coli* through co-selection with other antibiotics in the ACSSuT group. Because CTC supplementation significantly increased phenotypic tetracycline resistance, and ceftiofur and tetracycline resistances were strongly associated ($P < 0.001$; OR= 5.1[95% CI = 2.4-11.2]), we speculate that direct selection for tetracycline resistance co-selects for ceftiofur-resistant *E. coli*. On the other hand, ceftiofur use could also select for tetracycline resistance (62); however, this cephalosporin product was not used on study pigs in this facility during the trial period.

With the exceptions of tetracycline and sulfisoxazole, resistance to the remaining antibiotics decreased over time when CTC and copper were supplemented—either alone or in

combination—compared with the control group and beyond what would be expected by aging. It has been reported elsewhere that AMR decreases as a result of aging; this occurs equally in conventional and antibiotic-free swine herds (185, 189). Interestingly, reductions in kanamycin and chloramphenicol resistance during the treatment period were transient; they returned to pre-exposure levels after the treatments were removed. The level of kanamycin resistance reported here (23.5%) is similar to that reported elsewhere in *E. coli* isolated from swine (24, 25). Acquired resistance to kanamycin, through R-plasmids, is relatively common among *E. coli* and other Gram negative rods. A single plasmid can confer cross- or co-resistance to other aminoglycosides and to other drug classes (90). Thus, in the absence of direct antibiotic pressure, kanamycin resistance could be maintained as a result of co-selection; that is, because it was found in association with sulfisoxazole, tetracycline, streptomycin, chloramphenicol, amoxicillin/clavulanic acid, ceftriaxone, cefoxitin, and ceftiofur in the present study.

Using a binary coding system (i.e., susceptible or resistant), for each of the 15 antibiotics tested, a theoretical maximum of 2^{15} (32,768) possible phenotypic combinations (profiles) exists. However, as reported elsewhere (190), the actual observed phenotypic combinations are limited by genetic linkages and the prevalence of the individual resistances. We observed a total of 101 unique phenotypic resistance profiles across all the isolates from our study. There were 60, 45, 55, and 43 unique resistance profiles observed from control, copper, CTC, and copper plus CTC groups, respectively. This indicates that resistance profiles in the control group are relatively more diverse; and in the presence of copper (alone or in combination with CTC) resistance profiles tended to be less diverse. Most of these profiles are common to all treatment groups; only one pan-susceptible isolate was observed from the control group, whereas the remaining two were from the copper plus CTC group. Resistance profiles that were unique to each

treatment group were sparse. It is important to note that all of the phenotypic correlations between pairs of antibiotics (Table 3.5) are in a positive direction, and although all are significantly different from zero ($P < 0.05$), the width of the confidence limits varies more than can be explained simply from the prevalence position on the binomial distribution.

The *E. coli* isolates generally were highly multidrug resistant. On the basis of the count of the number of antimicrobials (ignoring antibiotic class, for now) to which isolates were resistant, the isolates showed a moderately bimodal distribution, usually at 4 and 10, in all treatment groups (Fig. 3.2). This bimodal distribution was also evidenced elsewhere in *E. coli* derived from feedlot cattle (62). However, unlike typically reported right-skewed distributions in populations at equilibrium (i.e., not under antimicrobial selection pressures), the *E. coli* population in our study exhibited a somewhat left-skewed distribution, thus indicating that *E. coli* populations from our study pigs were highly MDR. About 25% (n = 1,152) of the *E. coli* isolates were resistant to 10 different antimicrobial agents.

On the basis of NARMS classification criteria (19), 15 antimicrobials were collapsed into 8 classes (Table 3.1); of these, the highest proportion (34.4%) of the *E. coli* isolates (n = 1,152) was resistant to 7 different classes (Fig 3.3). The tendency to be resistant to many antimicrobial agents and classes exhibited a decreasing temporal trend when examined both by descriptive and analytic techniques; this, in turn is highly likely to be due to an aging effect as has been previously reported elsewhere (185, 189). Ceftiofur and tetracycline resistances were associated with MDR class count. The median number of antibiotics to which ceftiofur-resistant isolates (n = 746) were resistant was 9, compared with a median of 4 for a typical ceftiofur-susceptible isolate (n = 406). Similarly, median MDR based on the number of antibiotic resistance was 8 (95% CI = 8-8) and 3 (95% CI = 2–3) among tetracycline-resistant (n = 1,119) and susceptible (n

= 33) *E. coli*, respectively. The median number of antibiotics to which both tetracycline- and ceftiofur-resistant isolates (n = 737) were resistant was also 9, compared with a median of 2 antimicrobials for isolates (n = 24) susceptible to both drugs. On the basis of binary definition (resistance to ≥3 classes), a great majority (91%) of *E. coli* isolates (n = 1,152) were also MDR.

Copper resistance

With respect to the copper MIC distribution of isolates, the *E. coli* isolates tended to form a unimodal population in all the treatment groups. Collapsed across all isolates, 93% of the isolates had an MIC of ≥20 mM, which suggests little to no acquired resistance mediated through the *pco* gene among the isolates (156). Population-based studies on copper susceptibilities in *E. coli* are limited; most studies are based on banked isolates, and these often are from diagnostic laboratory submissions. Williams et al., 1993 (36) reported the ready growth of *E. coli* isolates at 18 mM of CuSO4 from an Australian pig farm. If this cutoff value was considered, 93% of the isolates in the present study, with an MIC of ≥20 mM, would have been categorized as resistant. Since there is no consensus breakpoint for copper susceptibility among *E. coli*, we used survival analysis to analyze the full MIC range. The MIC distribution of the *E. coli* isolates we studied is almost identical to that reported from livestock-associated *E. coli* in Denmark (156), and our *E. coli* population was generally able to tolerate high concentrations of CuSO4 similar to that study. Paradoxically, our study isolates from the control group were able to tolerate higher concentrations of $CuSO_4$ than the treated groups, including those with supplemented copper in the feed, with as of yet no verifiable biological explanation. It seems plausible that the population of *E. coli* selected by the two treatments and combination was prioritized on antibiotic resistance phenotype much more than on copper MIC since the MIC distribution is so narrow when contrasted with that of *Enterococcus* species for example (41-44).

The survival experience differed significantly ($P = 0.027$) among the groups at an MIC value of 20 mM, as seen in Fig. 3.4B. Similar to our finding, Aarestrup and Hasman (156) reported that *E. coli* and *Salmonella* tolerated higher concentrations of copper than other bacteria, which could provide them a survival advantage in a high-copper environment. Copper has been used for growth promotion purposes in swine production for over 60 years (36), and bacteria can also be exposed to metal contamination in the environment (50), though this is less likely in modern confinement operations. Unlike in Europe, the level of copper used in swine feed in the U.S. is not regulated and is sometimes higher, ranging typically from 125 to 250 ppm (46). This suggests that the pig gut flora, and *E. coli* in particular, could be well adapted to high levels of copper due to constant exposure to copper through feed or from the swine production environment.

In conclusion, the *E. coli* population derived from post-weaned pigs that we studied exhibited resistance to most antimicrobials, with a few notable exceptions such as to fluoroquinolones. These isolates were highly multidrug resistant, exhibiting a diverse range of resistance profiles. As expected, CTC supplementation was associated with increased tetracycline resistance beyond the already high levels of tetracycline resistance at baseline. It appeared that higher levels of copper supplementation as were used in this experiment decrease multidrug resistance counts; however, this needs to be further investigated under these and other field conditions and, ideally, using additional culture and non-culture-dependent genotypic endpoints.
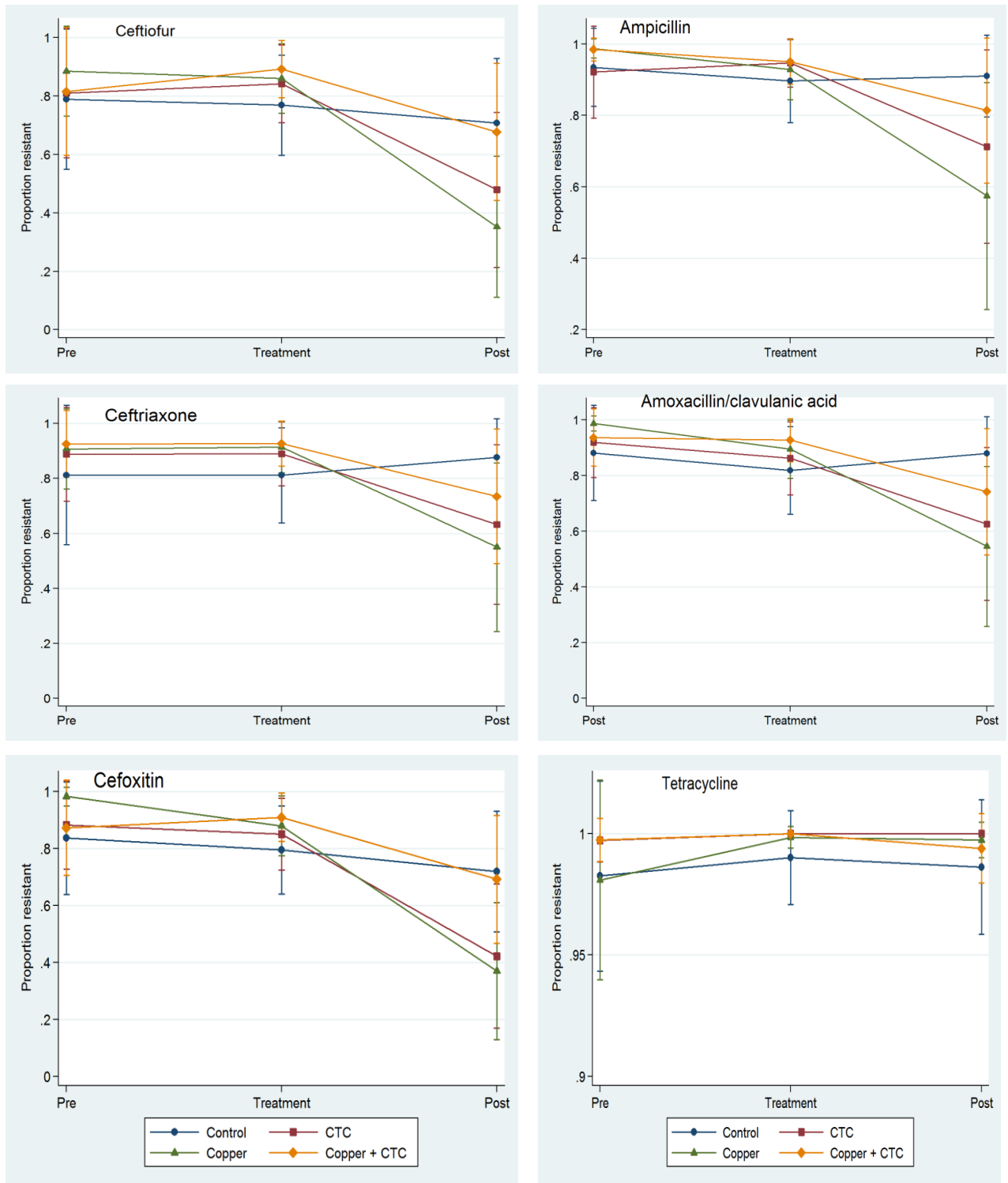
Figure 3.1. Model-adjusted resistance prevalence of 1,152 *E. coli* isolated from feces of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither to the antibiotics tested.

Figure 3.1 (Continued)
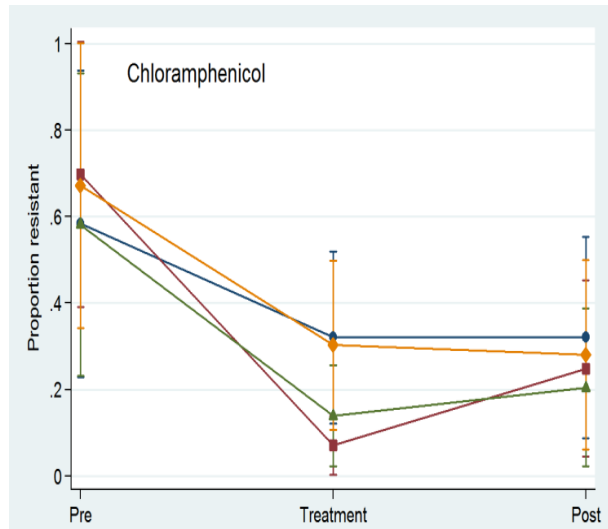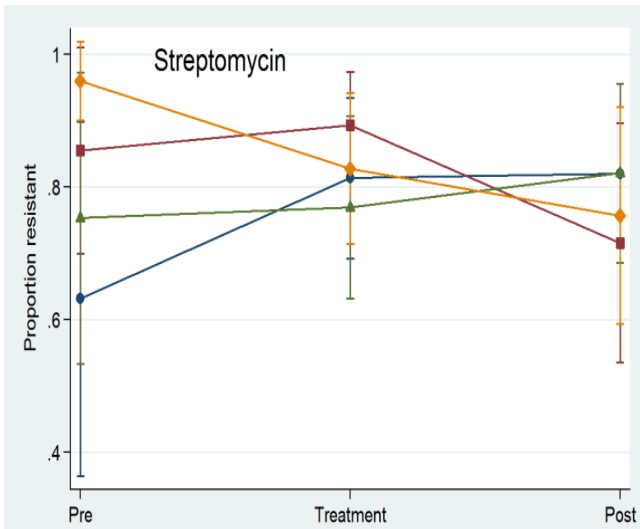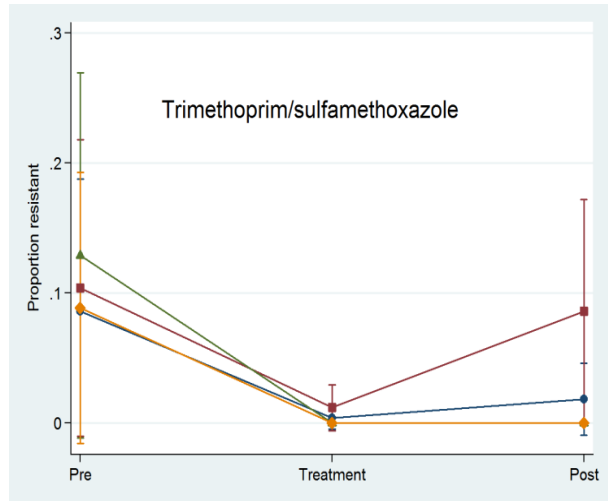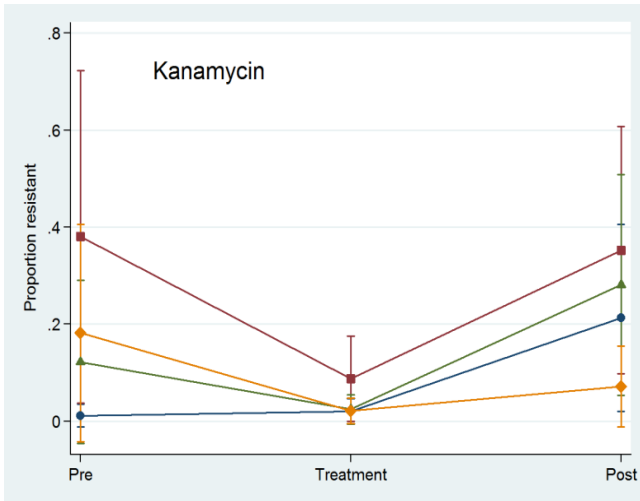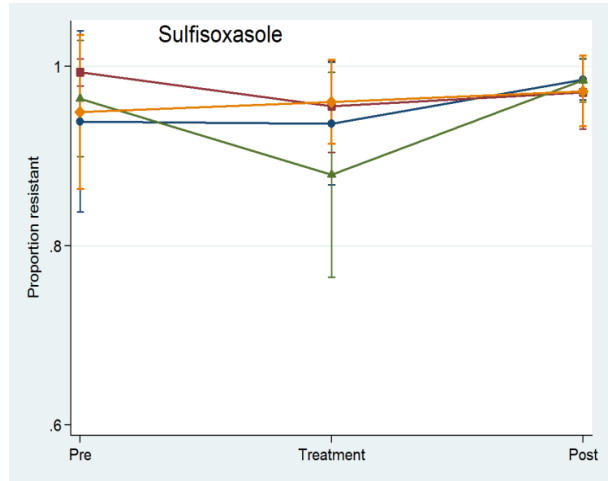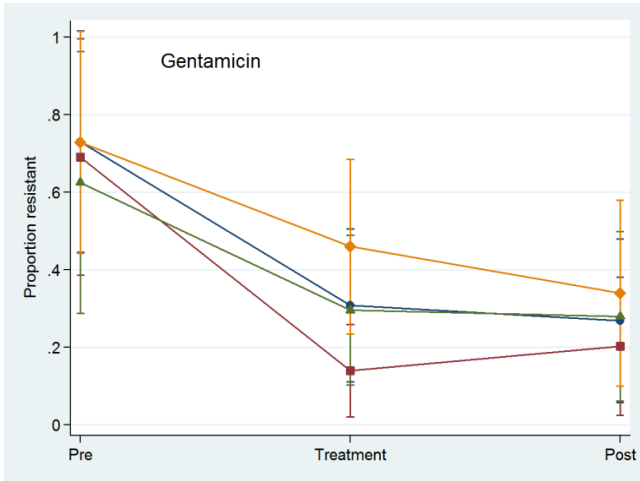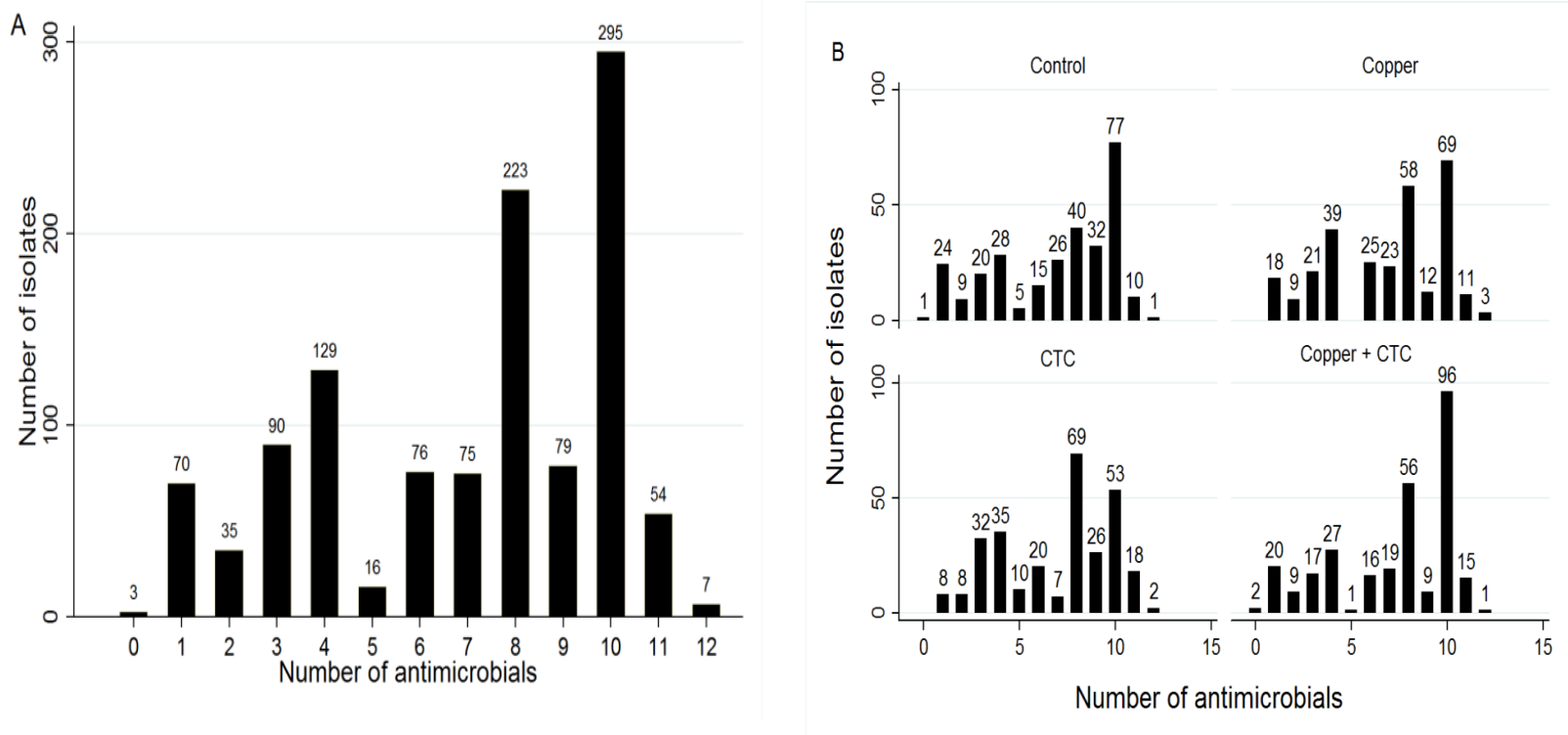
Figure 3.2. Multidrug resistance count distribution of fecal *E. coli* isolates obtained from weaned pigs experimentally supplemented with chlortetracycline (CTC), copper, both or neither.

Frequency distribution of phenotypic resistance shown on the basis of the number of antimicrobial agents across all isolates (n = 1,152) (A), and by treatment group (n = 288/group) (B).
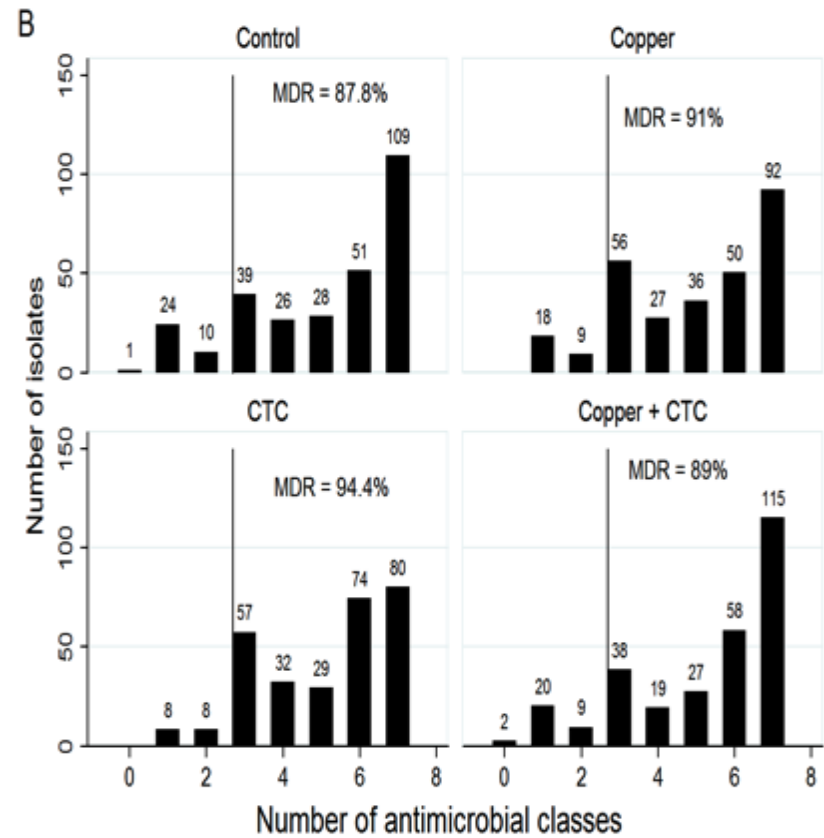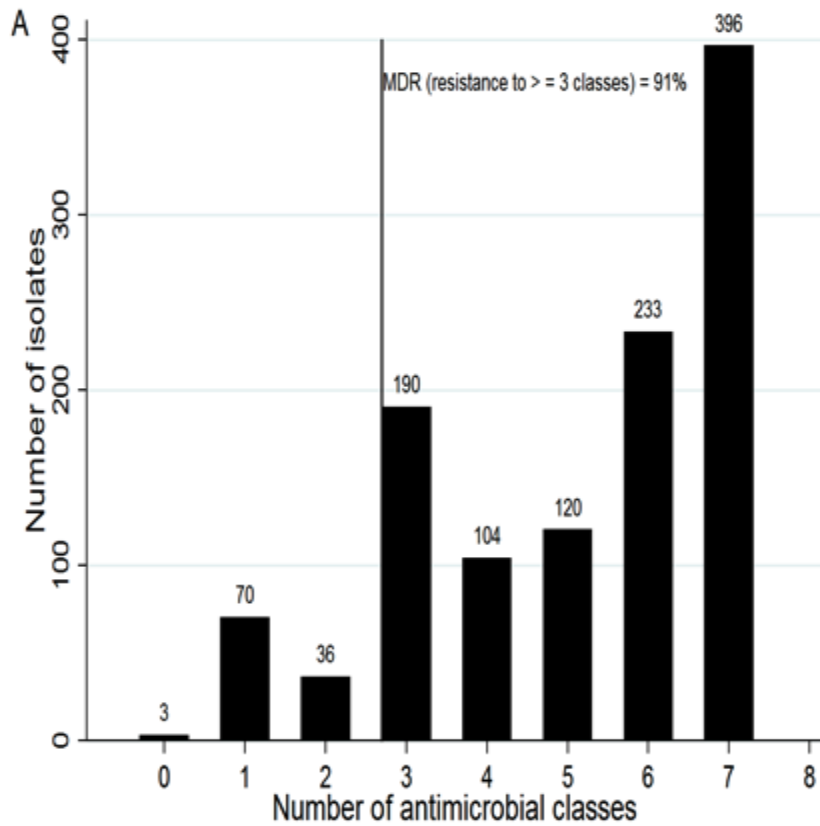
Figure 3.3. Multidrug resistance class count distribution of fecal *E. coli* isolates obtained from feces of weaned pigs experimentally supplemented with chlortetracycline (CTC), copper, both or neither.

Frequency distribution of phenotypic resistance on the basis of the number of antimicrobial classes across all isolates (n = 1,152) (A), and by treatment group (n = 288/ group) (B). Vertical bars at ≥3 represent cutoff value for MDR classification.
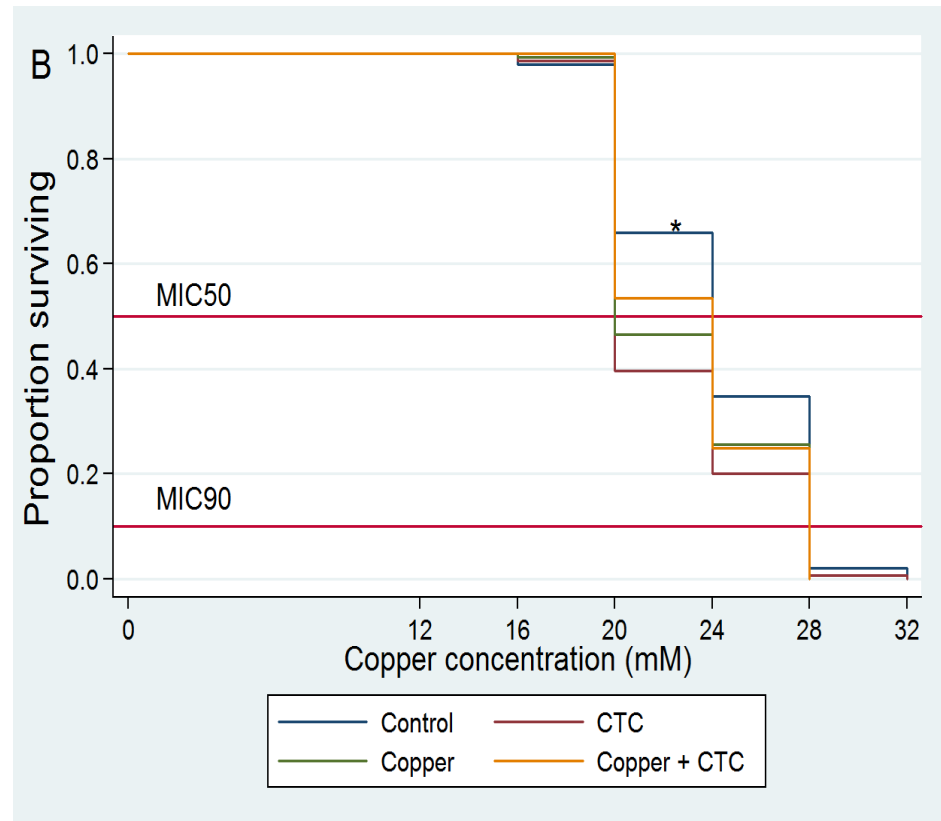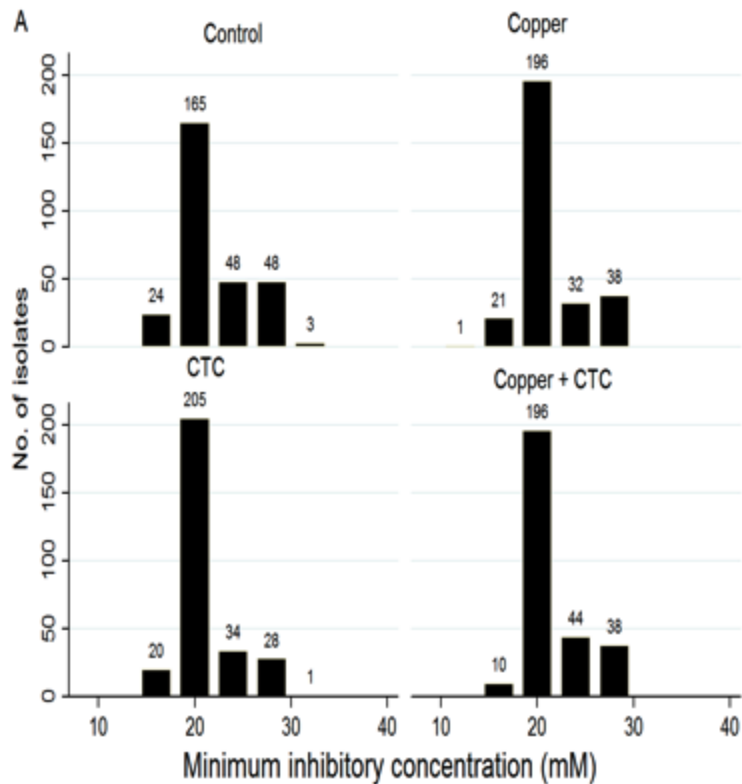
Figure 3.4. Copper susceptibilities of 1,152 *E. coli* isolates obtained from feces of weaned pigs that received chlortetracycline (CTC), copper, both or neither.

(A) Distribution of the minimum inhibitory concentration (MIC) of *E. coli* isolates by treatment group (n = 288/group). Numbers on the bar graph represent the number of isolates with a particular MIC value.

(B) Survival plot against increasing mM concentration of copper only for isolates obtained during the treatment period. *isolates from the CTC group were more susceptible.
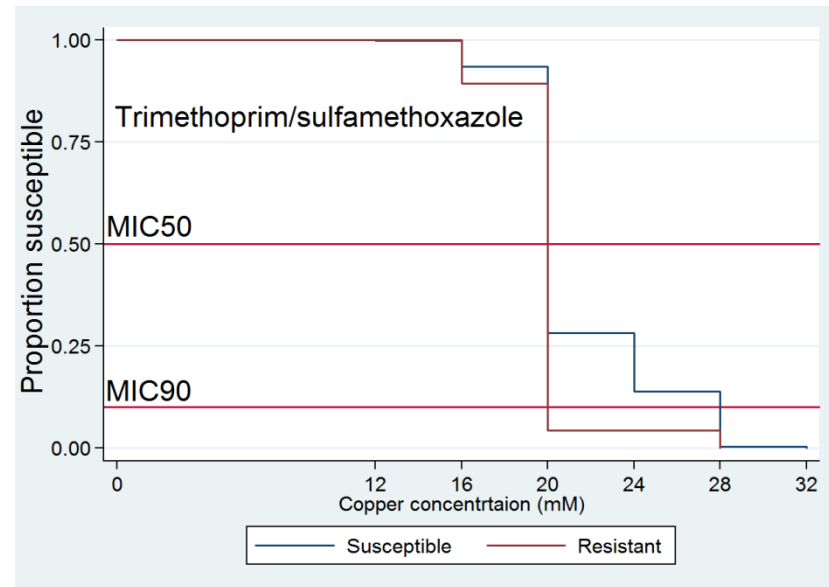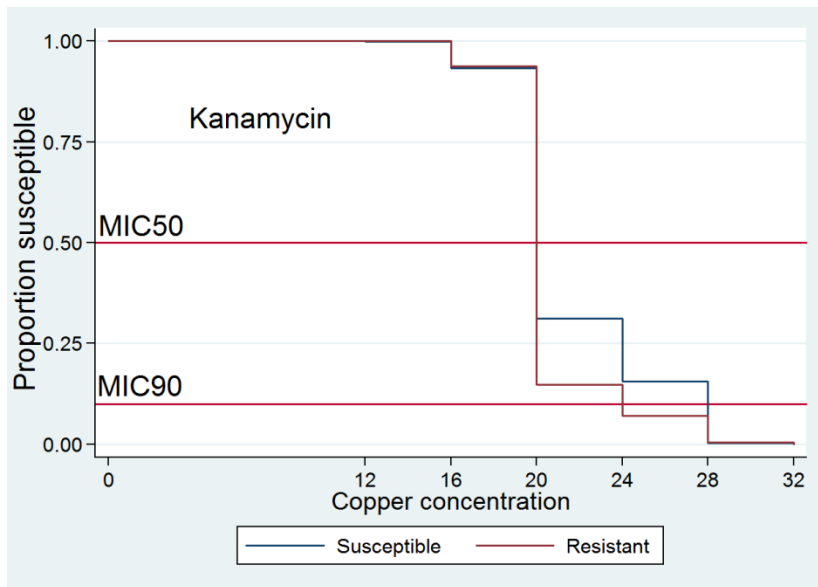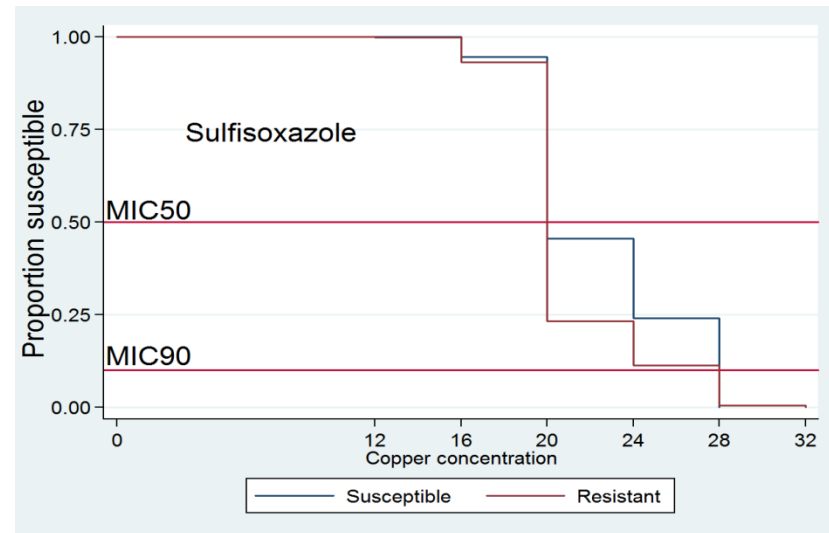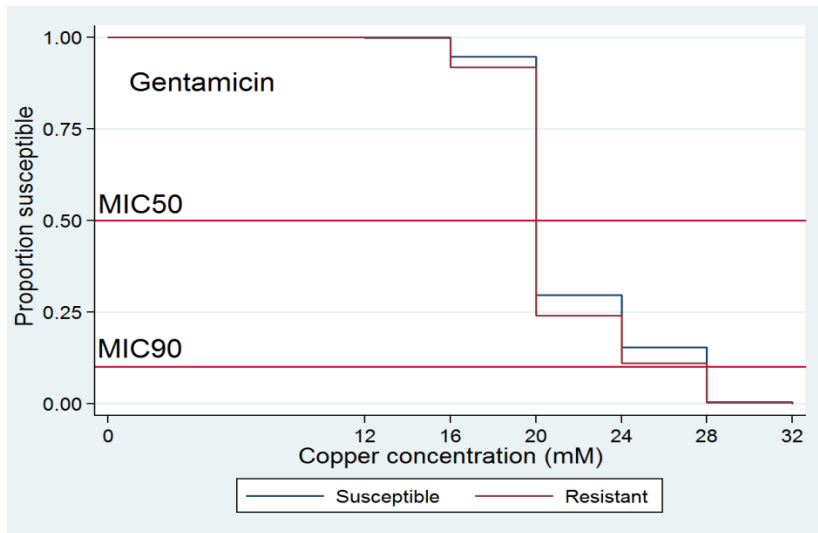
Figure 3.5. Kaplan-Meier survival function illustrating cumulative susceptibilities of antibiotic resistant and susceptible *E. coli* (n = 1,152) isolates to increasing concentrations of copper.

Table 3.1. Antibiotic concentration ranges and resistance break points used for susceptibility testing of *E. coli* (n = 1,152) isolated from fecal samples of weaned pigs experimentally fed chlortetracycline, copper, both or neither

Antimicrobial class (FDA, 2012 (19))	Antimicrobial agent	Abbreviations	WHO classification (WHO, 2012 (84))	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 (147).

[b] National Antimicrobial Resistance Monitoring System breakpoint was used (19).

Table 3.2. Minimum inhibitory concentration (MIC) distribution (squashtogram) of *E. coli* (n = 1,152) isolated from fecal samples of weaned pigs supplemented with chlortetracycline, copper, both or neither

Antimicrobial[a]	% Resistant	95% CI[c]	Distribution of MICs in ug/ml (%)[d]															
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
AMI[b]	0.0	0.0, 0.5						0.3	19.2	60.5	19.6	0.5						
AZI[b]	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 3.1.

[b] Amikacin (n = 802) and azithromycin (n = 350) were tested with NARMS CMV1AGNF and CMV2AGNF panels respectively.

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

[d] The unshaded areas indicate the range of dilutions tested for each antimicrobial. The vertical bars indicate the 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 3.3. 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

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	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	
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.

Table 3.4. 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	Phenotypic profile[a]	Treatment group				Overall (n =1,152)
		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

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	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.

Table 3.5. **Model-defined pairwise correlations (with 95% CI) between phenotypic AMR of *E. coli* (n = 1,152), 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	1							
	(0.87, 0.97)								
AXO	0.95	0.97	1						
	(0.9, 0.97)	(0.95, 0.99)							
CHL	0.54	0.56	0.55	1					
	(0.45, 0.62)	(0.46, 0.64)	(0.46, 0.63)						
FIS	0.28	0.28	0.30	0.61	1				
	(0.10, 0.43)	(0.11, 0.44)	(0.12, 0.47)	(0.51, 0.70)					
FOX	0.90	0.94	0.94	0.49	0.26	1			
	(0.83, 0.94)	(0.92, 0.96)	(0.91, 0.96)	(0.37, 0.59)	(0.08, 0.44)				
GEN	0.57	0.59	0.61	0.75	0.41	0.5	1		
	(0.47, 0.66)	(0.49, 0.68)	(0.51, 0.69)	(0.66, 0.81)	(0.24, 0.56)	(0.38, 0.61)			
STR	0.59	0.59	0.59	0.69	0.66	0.52	0.72	1	
	(0.51, 0.66)	(0.52, 0.65)	(0.51, 0.67)	(0.61, 0.76)	(0.54, 0.76)	(0.42, 0.62)	(0.64, 0.79)		
TIO	0.94	0.96	0.98	0.49	0.28	0.95	0.52	0.53	1
	(0.89, 0.97)	(0.92, 0.98)	(0.97, 0.99)	(0.38, 0.59)	(0.09, 0.44)	(0.93, 0.97)	(0.39, 0.63)	(0.43, 0.63)	

[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.

Chapter 4 - Effects of chlortetracycline and copper supplementations in the diet of weaned pigs on the prevalence of antimicrobial resistance genes in *Escherichia coli* isolated from feces

Abstract

Chlortetracycline (CTC) and copper are commonly used growth promoters in U.S. pig production. We investigated the impact of CTC, copper or their combined supplementations in weaned pigs on the prevalence of tetracycline (*tet*A, *tet*B, *tet*C and *tet*E), copper (*pco*D) and ceftiofur (*bla*$_{CMY-2}$) resistance genes in fecal *Escherichia coli*. A total of 1,152 isolates obtained from 32 pens (5 pigs/pen) which were randomized to control, copper, CTC, or copper plus CTC treatment groups were tested for the *tet* genes, *bla*$_{CMY-2}$ and *pco*D genes by PCR. The prevalence of *tet*A, *pco*D and *bla*$_{CMY-2}$ genes decreased over the treatment periods regardless of treatment groups; while that of *tet*B had increased. Although CTC and copper supplementations were associated with increased *tet*B prevalence, their combination was paradoxically associated with reduced *tet*B prevalence. *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 other. The *tet*A and *bla*$_{CMY-2}$ genes were positively associated with multidrug resistance (MDR) counts, but *tet*B and *pco*D were negatively associated with MDR counts. The expansion of *E. coli* population harboring *tet*B over *tet*A associated with CTC supplementation most likely indicates gene substitution at the bacterial population level. The high prevalence (72%) of the *bla*$_{CMY-2}$ gene suggests that ceftiofur resistance is readily maintained in the absence of ceftiofur use in these pig cohorts, most likely through genetic linkage to *tet*A. The roles of copper supplementation in pig production and *pco-*

mediated copper resistance in *E. coli* need to be further explored because the strong negative association observed with *tet*A and *bla*$_{CMY-2}$ points to potential opportunities to select for a more innocuous tetracycline resistance profile.

Introduction

Antimicrobial resistance (AMR) is one of the greatest public health threats in the world (3) and is largely driven by the use and overuse of antimicrobial agents in humans and animals (191). The selective pressure posed by use of antibiotics including antibiotic growth promoters (AGPs) in food animal production, could foster amplification and dissemination of antibiotic-resistant bacteria and their resistance determinants (70, 75, 192). Evidence of qualitative links between AGP use of antimicrobials in food animals and the emergence of antibiotic-resistant bacteria is mounting (11, 168, 193). Tetracyclines have commonly been used in humans and agriculture (31) for more than six decades. Chlortetracycline (CTC) and oxytetracycline are the most commonly used AGPs in U.S. swine production (17). Tetracycline resistance is inducible (28) and is mainly the result of tetracycline resistance (*tet*) genes acquired through horizontal gene transfer (31). Tetracycline resistance mechanisms include: 1) the efflux of tetracycline from the cell, 2) ribosomal protection from the action of tetracyclines, or 3) enzymatic degradation of tetracyclines (31). A regularly updated online database reports the discovery of 30 efflux, 12 ribosomal, 3 enzymatic, and 1 unknown *tet* or *otr* genes[3].

In swine, copper is an essential micronutrient required for normal physiological activities and is typically included in the diet as part of the trace mineral supplement at the National Research Council recommended dose (5-6 ppm) (39). In the U.S. at higher dietary levels (100-

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[3] http://faculty.washington.edu.marilynr/

250 ppm), it is also used for growth promotion purposes (39, 76). Because copper is toxic to bacteria at high concentrations, *E. coli* and other Gram negative bacteria have developed tightly regulated mechanisms coded by genes on the chromosome or plasmids (49, 55, 122) to maintain copper homeostasis. A copper resistance determinant (*pco*) identified on the pRJ1004 plasmid of *E. coli* (54) consists of seven genes, *pco*ABCDRSE, arranged in two operons, *pco*ABCD and *pco*RS, and a separate *pco*E gene (55). The *pco*ABCD operon is regulated by its own two-component regulatory genes: *pco*RS and the expression of all the proteins (PcoABCD) are required to confer copper resistance (55). PcoD is an inner membrane protein that pumps PcoC-associated copper into the cytoplasm to be oxidized by multicopper oxidase, PcoA (55).

Ceftiofur is an extended-spectrum third-generation cephalosporin that was first approved by the U.S. Food and Drug Administration (FDA) in 1988 for the treatment of bovine and swine respiratory diseases (57). Its continued use in animals has been scrutinized as a result of cross-resistance with ceftriaxone, a closely related antibiotic that is a first-line treatment of salmonellosis in children (58). Ceftiofur resistance is mediated primarily by a widely disseminated plasmid-borne AmpC type β-lactamase, the bla_{CMY-2} cephamycinase gene (59, 60). *E. coli* can act as important reservoir of AmpC bla_{CMY} genes and can be transferred to other, more pathogenic bacteria such as *Salmonella enterica* (61). Previous phenotypic study has shown that subtherapeutic use of CTC resulted in the co-selection of third generation cephalosporin resistance (ceftriaxone) in enteric bacteria of pigs (23). Furthermore a complete sequence of the IncA/C plasmids originated from cattle showed the presence of bla_{CMY-2} gene along with other multiple antimicrobial resistance genes such as *tet*A (64).

Chlortetracycline and copper are commonly used for growth promotion in U.S. swine production system. It has been shown that copper supplementation was associated with

increased prevalence of transferable copper resistance (*tcr*B) gene in enterococci in pigs (43, 44).

However the impact of individual or combined supplementations of CTC and copper in weaned

pigs on antimicrobial and copper resistance genes in *E. coli* has not been studied. The objective

of this study was to investigate the impact of CTC, copper or their combined supplementations in

weaned pigs on the prevalence of *tet* genes (*tet*A, *tet*B, *tet*C and *tet*E), *pco*D and *bla*$_{CMY-2}$ genes

in fecal *E. coli*.

Materials and methods

Experimental design and E. coli isolation

A total of 160 weaned pigs that were obtained from a commercial pig farm were

randomized to 32 pens (with five pigs per pen) blocked on body weight. After two weeks of

adaptation the pens were randomized to one of the four treatment groups in a two-by-two full

factorial balanced design. The treatment groups consisted of control (basal diet with required

copper concentration at 16.5 mg/kg of feed), copper (copper sulfate fed at an elevated

concentration of 125 mg/kg of feed), CTC (basal diet plus CTC at 550 mg/kg of feed), or copper

plus CTC at above dosages. Fecal samples were collected weekly for 6 weeks from three pigs

out of five pigs housed in each of 32 pens. A total of 1,152 *E. coli* strains isolated from 576 fecal

samples (two isolates per sample) obtained from 160 weaned pigs were used in the study.

PCR detection of resistance genes

E. coli isolates were tested for *tet* (A, B, C, E), *bla*$_{CMY-2}$ and *pco*D genes. DNA was

extracted by heating a 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 4.1. 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 (194) was adapted using a pre-optimized commercially available multiplex PCR master mix kit (Qiagen) according to the manufacturer's instructions. First, 500 µl of primer mix was prepared by adding 10 µl of each of the primers from 100 µm stock concentration and nuclease free water to give a concentration of 2 µm of each primer. The final 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 sec, annealing at 60°C for 90 sec, and extension at 72°C for 90 sec, 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 PCR thermal profile consisted of initial activation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. For bla_{CMY-2} (154), 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 sec, annealing at 55°C for 45 sec, and extension at 72°C for 30 sec, with final extension at 72°C for 5 min.

Statistical analysis

All data analysis was performed using STATA version 12.1 (StataCorp LP, College Station, TX), and $P < 0.05$ was considered significant. A full factorial model to evaluate the effects of copper, CTC, and their interaction on the prevalence of resistance genes was used throughout the analysis. Sampling days were further categorized into treatment periods as: baseline (day 0), treatment period (days 7, 14, and 21), and post-treatment period (days 28 and 35). For descriptive statistics, the binary outcomes (presence/absence) for each of the genes within an isolate were aggregated and expressed as proportions with exact 95% binomial confidence intervals. Initially a likelihood ratio chi-square test was used to compare the unadjusted effects of treatment and treatment period. *tet*C and *tet*E were not considered beyond descriptive statistics due to their sparse presence.

Multilevel mixed-effects logistic regression model was used to model the fixed effects of treatments and treatment periods (and their interactions) along with random effects for repeats within pen and within individual fecal samples on each of the individual binary genotypic resistances. The model was a three level hierarchical model consisting of isolate within sample and sample within pen. The fixed effects of resistance genes (and their interactions) on the binary classified phenotypic MDR (resistance to ≥ 3 antimicrobial classes), according to NARMS classification (19), was also assessed by multilevel mixed-effects logistic regression, including random effects for fecal samples and pen. Multivariate (162) and bivariate probit regression models were used to simultaneously model the effects of treatment and treatment period on multiple binary outcomes (presence or absence of the four resistance genes) and on the prevalence of just the *tet*A and *tet*B genes, respectively, and to adjust for the biological dependencies that can result from genetic linkages between different antimicrobial resistance

genes. Cluster-adjusted robust standard errors (SE) were used to adjust for the pen effect. A generalized ordered logistic regression model was used to further assess the associations of the resistance genes (*tet*A, *tet*B, *bla*$_{CMY-2}$, and *pco*D) with MDR counts, which were defined on the bases of the number of antimicrobial agents and classes to which isolates were resistant. In all the regression models, first a full model with the main effects and all possible interactions was built and model selection was conducted in a backward manual selection method. When interaction terms were not significant, only the main effects were evaluated. Bonferroni test was used to adjust for multiple comparisons.

Results

Prevalence of resistance genes

Out of 14 different *tet* genes originally targeted by four multiplex PCR assays, the vast majority of genes detected from the first 576 *E. coli* isolates tested (data not shown) were *tet*A and *tet*B, and to a lesser extent *tet*C and *tet*E. Subsequently, we elected to target only these four *tet* genes (A, B, C, and E) in a multiplex PCR for the remaining 576 isolates. Across all treatments, unadjusted prevalence of the resistance genes was as follows: *tet*A (66% [95% CI = 64–69%]), *tet*B (48% [95% CI = 45–51%]), *tet*C 2.1% (95% CI = 1.3–3.1%), *tet*E 1.6% (95% CI = 0.9–2.5%), *pco*D (16% [95% CI = 14–18%]) and *bla*$_{CMY-2}$ (72% [95% CI = 69–75%]). As can be seen from the 95% CI, the prevalence of *bla*$_{CMY-2}$ was significantly greater than that of *tet*A, which was in turn, significantly greater than that of *tet*B followed by *pco*D. The prevalence of each of the resistance genes cross-tabulated by treatment group and treatment period is summarized in Table 4.2. 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 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.

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 in the control pigs. *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 other (Table 4.3).

Model-adjusted prevalence from multilevel mixed-effects logistic regression analysis for

each of the resistance genes is plotted across treatment periods in Fig 4.1. Prevalence of *tet*A

decreased through subsequent treatment periods: 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]) 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 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)

compared with the control groups. *E. coli* isolates in the post-treatment period exhibited higher

prevalence of *tet*B (*P* = 0.024), which was 2.5 (95% CI = 1.13–5.5) 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). In all resistance

genes, treatment by day interaction was not significant (*P* > 0.05).

Figure 4.2 shows the marginal plot of the adjusted prevalence of *tet*A and *tet*B genes

across the treatment period as obtained from the bivariate probit analysis. Detection of *tet*A

alone decreased across the treatment periods in all treatment groups except in the copper plus

CTC group, where it increased during the treatment period. On the other hand, *tet*B-only

prevalence increased across the treatment periods in all treatment groups. Among isolates in

which both *tet* genes were found, the pattern of decreasing prevalence through time was more

similar to that of *tet*A than of *tet*B (Fig. 4.2). The prevalence of both genes occurring together

decreased significantly during treatment period (OR = 0.2; 95% CI = 0.1–0.4) and in the post-

treatment period (OR = 0.5; 95% CI = 0.3–0.9) compared to the baseline with no treatment

effects (*P* > 0.05). Only a few isolates (n = 48) harbored neither *tet*A nor *tet*B throughout, and

this finding did not vary by treatment or treatment period (*P* > 0.05; Fig. 4.2).

From the bivariate probit regression output, overall detection of *tet*A, adjusted for *tet*B, decreased significantly both during treatment (OR = 0.65[95% CI = 0.48–0.87]; *P* = 0.004) and post-treatment (OR = 0.55 [95% CI = 0.38–0.78]; *P* = 0.001) periods compared with day 0 where, as expected, no significant treatment differences (*P* > 0.05) were found. However, both copper (OR = 1.4 [95% CI = 1.0–1.9]; *P* = 0.046) and CTC (OR = 1.5 [95% CI = 1.1–2.04]; *P* = 0.010) supplementations were significantly associated with increased *tet*B prevalence relative to the control group across all treatment periods adjusted for *tet*A. The combined supplementation of copper and CTC was significantly associated with decreased detection of *tet*B alone (OR = 0.52 [95% CI = 0.33–0.82]; *P* = 0.004) compared with the control group. Isolates obtained during the post-treatment period had significantly (*P* = 0.025) higher levels of *tet*B alone compared with day 0 isolates (OR = 1.4 [95% CI = 1.04–1.82]) across all isolates. Treatment by sampling day interaction was not significant in the bivariate regression model (*P* > 0.05).

Resistance gene profiles

Genotypic profiles for four of the resistance genes (excluding *tet*C and *tet*E) detected among *E. coli* are shown in Fig 4.3. None of the four targeted genes was detected in 2% of the isolates studied (*n* = 1,152). 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.

Tetracycline resistance gene profiles

Table 4.4 shows the distribution of the four *tet* (A, B, C, and E) genes detected. At least one of the four *tet* genes was detected in 97% (1,117 of 1,152) of the *E. coli* isolates. Among

these (n = 1,117), 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* = 1,117) with at least one *tet* gene and 96% of all the isolates tested (*n* = 1,152). 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 targeted were detected, 13 (37%) were phenotypically resistant to tetracycline. Among phenotypically tetracycline-resistant *E. coli* (n = 1,119), 99% were positive for one or more of the four *tet* genes tested. A duplex PCR targeting only *tet*A and *tet*B could explain 98% of the phenotypic tetracycline resistance (*n* = 1,119).

Associations between resistance genotypes and multidrug resistance

On the basis of the number of antimicrobials (theoretically, from 0–15) to which individual *E. coli* 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). Analyses of the generalized ordered logistic regression indicated that the presence of *tet*A (proportional OR = 3.3 [95% CI = 2.1–5.0]; $P < 0.001$) and bla_{CMY-2} (prop OR = 22.2 [95% CI = 14.9–33.1]; $P < 0.001$) were significantly associated with a higher MDR count; however, *tet*B was not significantly ($P = 0.410$) associated with MDR count (proportional OR = 1.2 [95% CI = 0.8–1.7]). The association between *pco*D gene and MDR count was inconsistent across counts. Up to a MDR count of 5 antimicrobials, the presence of the *pco*D gene was negatively associated with MDR count ($P <$

0.05). For resistance counts ranging from 6–7 antibiotics, there was no significant ($P > 0.05$) association between pcoD and MDR count. Examined differently, by dichotomizing at an MDR count of 10, the presence of the pcoD gene was significantly ($P = 0.003$) and positively associated with *E. coli* isolates that were resistant to more than 10 antibiotics (OR = 3.5 [95% CI = 1.5–7.8]) compared with resistance counts of 10 antibiotics or less.

Based on the number of different antimicrobial classes isolates were resistant to, generalized ordered logistic regression showed that both *tet*A and bla_{CMY-2} genes were significantly ($P < 0.001$) and positively associated with a higher MDR count. *tet*B was not significantly ($P = 0.462$) associated with higher MDR class counts. *E. coli* isolates carrying the pcoD gene were less likely ($P < 0.001$) to be classified as MDR when comparing >2 to ≤2 classes of antibiotics than isolates with no pcoD gene (propOR = 0.25; 95% CI = 0.11–0.5). Beyond an MDR count of >3 antimicrobial classes, however, there was no significant ($P > 0.05$) association between pcoD and MDR.

Among *E. coli* isolates ($n = 1,043$) that were MDR according to phenotypic binary classifications of NARMS based on resistance 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 more than two antimicrobial classes. On the other hand, the presence 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 pcoD genes together were significantly ($P < 0.001$) associated with reduced MDR classification of the isolates.

Discussion

Prevalence of resistance genes

The combined effects of copper and CTC (that is CTC by copper interaction) on the prevalence of *tet*A were synergistic during the treatment period in that *tet*A was significantly (P = 0.019) higher in the copper plus CTC–supplemented group compared to the control group. In contrast, the combined treatments (CTC by copper interaction) appeared to be antagonistic to *tet*B; that is, the prevalence of *tet*B was higher (P = 0.006) in the groups that received individual supplementations of copper or CTC. Importantly, when examining multiple genes that exhibit negative correlations with one another, and when each gene explains similar phenotypes in isolates, gene substitution is to be expected. In other words, when prevalence 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. Discerning the presence of this relationship is one of the advantages of genotypic analysis over the phenotypic testing.

Another salient feature of the two 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 suggests a form of gene substitution at the population (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 it has been observed elsewhere (188, 189) that 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

counts than *tet*B. Although the prevalence of *tet*A was higher in the CTC-supplemented group

(69% (95% CI = 65–72%) than in the non-CTC–supplemented group (64% (95% CI = 60–68%),

CTC did not significantly expand the *tet*A gene across all treatment periods. Overall, the

prevalence of *tet*B was 49% (95% CI = 45–54%) in the CTC-supplemented group and 47% (95%

CI = 42–51%) in the non-CTC–supplemented group. Adjusted for clustering effects by pen and

repeated sampling, however, CTC and copper supplementations significantly expanded the *tet*B

gene. In contrast to previous studies (117, 195) that reported higher prevalence of *tet*B over *tet*A

among porcine *E. coli*, our study indicated that the overall prevalence of *tet*A (66%: 95% CI =

64–69%) is significantly higher than that of *tet*B (48%: 95% CI = 45–51%). This difference is

most readily explained by age differences in the study populations in that Boerlin et al., 2005

(195) detected higher *tet*B in finisher pigs, which is in agreement with our observation that *tet*A

was gradually replaced by *tet*B as the pigs aged. Unlike our study, in which we tested all *E. coli*

isolates for genotypes, Bryan et al., 2004 (117) tested only those portions of *E. coli* that had a

tetracycline MIC value of ≥93 µg/ml. We also observed that *tet*B confers a higher level of

tetracycline resistance to *E. coli* isolates than other *tet* genes, typically at MIC values greater than

32 µg/ml of tetracycline.

     The *pco*D gene was selected in this study based on the fact that the gene products of the

*pco*ABCD operon are expressed on polycistronic message from the same promoter that results in

stoichiometric production of the four gene products (56) required for full copper resistance in *E.*

coli (55). This gene was also targeted previously by others (Dr. Henrik Hasman, National Food

Institute, Lyngby, Denmark: personal communication). The level of *pco*D detection did not

differ significantly ($P > 0.05$) by treatment group, suggesting that the *pco*D gene did not

contribute to copper resistance of the *E. coli* isolates in a meaningful way at this level of copper

supplementation. High level of copper in the diets of pigs in this study did not appear to affect

the prevalence of the gene (*pco*D), which has been reported (55, 122) to be important for

bacterial resistance among *E. coli*. This result is in direct contrast with results seen for Gram

positive enterococci in pigs and cattle when copper was supplemented at high levels, resulting in

expansion of the isolates that harbor the *tcr*B gene (42-44). A combination of both plasmid and

chromosomal genes are required for copper resistance in *E. coli*, such that resistance to high

copper concentrations can be expressed while maintaining the intracellular copper concentrations

required for normal physiological functions (36). The high doses of copper (125 to 250 ppm)

used in U.S. swine production (46), or exposure to same in the ambient environment, for over six

decades (36) may have led enteric Gram negative bacteria to become intrinsically adapted to

higher copper concentrations. This adaptability confers a survival advantage in the

gastrointestinal tract in the presence of high copper concentration (156).

The highest prevalence of *bla*$_{CMY-2}$ (compared with other genes) observed in the present

study, and its significant association with higher MDR counts in the absence of selective

pressure by ceftiofur use in our study population, suggests its maintenance through genetic

linkage with other antimicrobial resistance genes (particularly with *tet*A). Factors that contribute

to long-term maintenance of the *bla*$_{CMY-2}$ gene in the absence of ceftiofur use, however, are

unclear (58, 196). The *bla*$_{CMY-2}$ gene is commonly associated with large plasmids that can also

carry other antimicrobial resistance genes (197) such as *tet*A (64); thus, selection pressure

exerted by other antimicrobials such as tetracyclines could result in the dissemination of the

bla$_{CMY-2}$ gene (58). Funk et al., 2006 (23), for example, reported that AGP use of CTC in pigs

was positively associated with the occurrence of MDR (including resistance to ceftriaxone)

Gram negative bacteria.

Associations between phenotypic and genotypic resistance

The prevalences of *tet*A, *tet*B and *bla*CMY-2 were significantly associated with phenotypically tetracycline resistance while no significant association was observed for *pco*D gene. The prevalence of *tet*A (67% [95% CI = 65–70%), *tet*B (49% [46–52%]) and *bla*CMY-2 (73% [70–75%]) in the phenotypically tetracycline-resistant isolates (n = 1,119) were significantly higher than in the very few tetracycline-susceptible isolates (33 out of 1,152). On the other hand, the corresponding prevalences in the phenotypically tetracycline-susceptible isolates (n = 33) were 27% (95% CI = 13–46%) for *tet*A, 9% (95% CI = 1.9–24%) for *tet*B, and 45% (95% CI = 28–64%) for *bla*CMY-2; however, no significant difference was found between tetracycline-resistant and -susceptible isolates with respect to the prevalence of the *pcoD* gene (*P* = 0.808). This indicates the presence of co-selection between tetracycline and ceftiofur resistances as previously reported (23) and lack of such co-selection between tetracycline and copper resistances in the *E. coli* population studied. The detection of *tet* genes from phenotypically tetracycline susceptible isolates is an advantage of using direct genotypic examination of AMR since resistance genes might not be phenotypically expressed.

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%]). On the other hand, *tet*A (90% [86–93%] vs. 57% [95% CI = 53–60%]) and *bla*CMY-2 (85% [95% CI = 81–89%] vs. 67% [95% CI = 64–70%]) were significantly more likely in isolates with tetracycline MIC values ≤32 µg/ml than in isolates with MIC values >32 µg/ml. This, perhaps paradoxically, suggests that use of higher sustained levels of tetracycline could favor lower MDR count isolates harboring *tet*B vs. higher MDR count isolates with *tet*A and *bla*CMY-2. On the other hand, using lower levels of tetracycline, such as by

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feeding tetracyclines for growth promotion, could result in a greater expansion of the high MDR

count fraction, or the fraction of *E. coli* harboring a gene coding for resistance to a critical

antimicrobial such as third generation cephalosporins (ceftiofur).

Whether or not isolates were phenotypically resistant to ceftiofur, the prevalence

of both *tet*A and *bla*$_{CMY-2}$, as expected, were significantly higher in the ceftiofur-resistant isolates

(n = 746) than in the ceftiofur-susceptible isolates (n = 406) (79% [95% CI = 75–81%] vs. 44%

[95% CI = 39–49%] for *tet*A and 97% [95% CI = 95–98%] vs. 27% [95% CI = 22–31%] for

bla$_{CMY-2}$). For *tet*B and *pco*D, however, the relationships were opposite. The prevalence of *tet*B

and *pco*D were significantly higher in the ceftiofur-susceptible isolates than in ceftiofur-resistant

isolates (68% [63–72%] vs. 37% [34–41%] for *tet*B and (29% [95% CI = 24–33%] vs. 9% [7–

11%] for *pco*D). This result once again indicates that the strong association observed between

phenotypic ceftiofur and tetracycline resistances may be mediated mainly through *tet*A. We also

observed that *tet*A and *bla*$_{CMY-2}$ were positively associated, supporting the premise that these two

genes were likely co-localized on a single mobile genetic elements such as plasmids or

transposons (195). Previous reports found both genes harbored on the same IncA/C plasmid in

cattle (64).

The remarkably opposite features of *tet*A and *tet*B genotypic prevalence through time are

vividly illustrated by the fact that they exhibit a strong (75%) negative association among

themselves, most often avoiding joint carriage within the same isolate. This negative association

between *tet*A and *tet*B is also consistent with previous reports from porcine *E. coli* isolates (195).

It is possible that *tet*A and *tet*B are carried on plasmids of the same incompatibility group (195)

or that most of the *tet*B genes detected in our *E. coli* population are also carried on the

chromosome (114, 115). Carrying a duplicative resistance trait on a plasmid would yield little

fitness advantage. The finding that *tet*B was not significantly associated with MDR suggests this could be due to its chromosomal location.

Using a single multiplex PCR assay for *tet*A, *tet*B, *tet*C, and *tet*E showed 98% agreement between the detection of any of these genes and phenotypic tetracycline resistance. The agreement between *tet*A or *tet*B detection (or both) and tetracycline resistance was 98%; thus, it seemed possible to predict most of the phenotypically expressed tetracycline resistance in these weaned pigs by targeting only *tet*A and *tet*B. As previously reported (117, 195), tetracycline resistance in porcine *E. coli* is mainly conferred by *tet*A and *tet*B. From the *E. coli* isolates that were phenotypically ceftiofur resistance (n = 746), 97% of them were also positive for *bla*$_{CMY-2}$ gene suggesting that targeting *bla*$_{CMY-2}$ by PCR can predict most of phenotypic ceftiofur resistance in *E. coli* population.

The median copper MIC ($P < 0.05$) and the survivor functions (log rank test χ^2 (1df) = 1.2; $P = 0.2749$) of the *E. coli* isolates did not significantly differ by *pco*D gene carriage. This indicates that the *pco* gene did not significantly confer copper resistance in the population of *E. coli* studied. This finding is in contrast to *Enterococcus* spp., in which the presence of a single transferable copper resistance (*tcr*B) gene confers higher levels of resistance to copper, and MIC values are typically very low in its absence (42-44). It has been reported that *E. coli* and other Gram negative enteric bacteria such as *Salmonella* are generally more tolerant to high copper concentration, perhaps through chromosomally mediated copper resistance, that gives them a survival advantage over other bacteria in the gut of pigs (156).

Association between resistance genes and multidrug resistance

The most remarkable feature we observed was that higher phenotypic MDR counts (defined in terms of the number of antimicrobials to which *E. coli* isolates were resistant) were

typically associated with either bla_{CMY-2} or tetA. On the other hand, the presence of either of

tetB or pcoD was associated with lower MDR. Despite the fact that elevated copper

supplementations in our study did not appear to favor pcoD-containing isolates, that such a

possibility exists to select for lower levels of copper resistance and against critically important

antimicrobials is inviting. In the absence of the following specific resistance genes, the median

number of antimicrobials to which isolates were resistant was 4 (95% CI = 4–5) for tetA-

negative, 4 (95% CI = 3–4) for bla_{CMY-2}-negative, and 8 (95% CI = 8–8) for tetB-negative or

pcoD-negative isolates. In the presence of the genes, the corresponding median values were 8

(8–9) for tetA-positive, 9 (8–9) for bla_{CMY-2}-positive, 6 (6–7) for tetB-positive, and 4 (95% CI

=4–6) for pcoD-positive.

A higher MDR count was found when the bla_{CMY-2} gene was detected in combination

with tetA or tetB; in fact, 38% of the 1,152 isolates carried both tetA and bla_{CMY-2} and exhibited

a median phenotypic MDR count of 9. We observed the same scenario with a number of

different antimicrobial classes (max of 8) to which isolates were resistant; the median number of

antimicrobial classes to which isolates were resistant was 6 in the presence of tetA or bla_{CMY-2}

genes, but in their absence the median was 3. In the presence of tetB or pcoD genes, the median

was 5 and 4, respectively, and in their absence the median was 6. The presence of bla_{CMY-2} in

Salmonella has been reported to be associated with MDR (60). The IncA/C plasmids known to

carry bla_{CMY-2} can confer resistance to a diverse group of drugs and have wide host range (64,

198, 199); thus, it is possible that tetA and bla_{CMY-2} genes are co-localized and widespread on

these promiscuous plasmids. On the other hand, tetB could be mainly chromosomal, or perhaps

the plasmid carrying it does not harbor resistance to other drugs. The plasmid carrying the pcoD

gene may be rare and seems less likely to carry resistance determinants to other drugs; however, our study did not include plasmid analyses, so such associations remain statistical in nature.

Apart from *E. coli,* the *pco* gene can be found in other enteric Gram negative bacteria, such as *Salmonella* (36). The *pco* gene has been argued to be auxiliary to the chromosomally mediated copper homeostasis mechanisms; thus, phenotypically observed high tolerance in our *E. coli* population could be a result of intrinsic tolerance to copper exposure and, therefore, less dependent on the *pco* gene (56). In *E. coli*, plasmid-borne copper resistance is linked with the chromosomal system for full copper management (52, 56). Thus, plasmid and chromosomally mediated copper handling in *E. coli* should be viewed together as conferring copper resistance together (46).

In conclusion, CTC supplementation expanded the *E. coli* population harboring *tet*B over *tet*A, and gene substitution at the bacterial population level as pigs age likely explains the increase in *tet*B prevalence. The observed high prevalence of the *bla*$_{CMY-2}$ gene suggests that ceftiofur resistance is readily maintained in the absence of ceftiofur use in these pig cohorts, most likely through genetic linkage to *tet*A. The role of copper supplementation in swine production and *pco*-mediated copper resistance in *E. coli* need to be further explored because the strong negative association with *tet*A and *bla*$_{CMY-2}$ points to potential opportunities to select for a more innocuous tetracycline resistance profile.
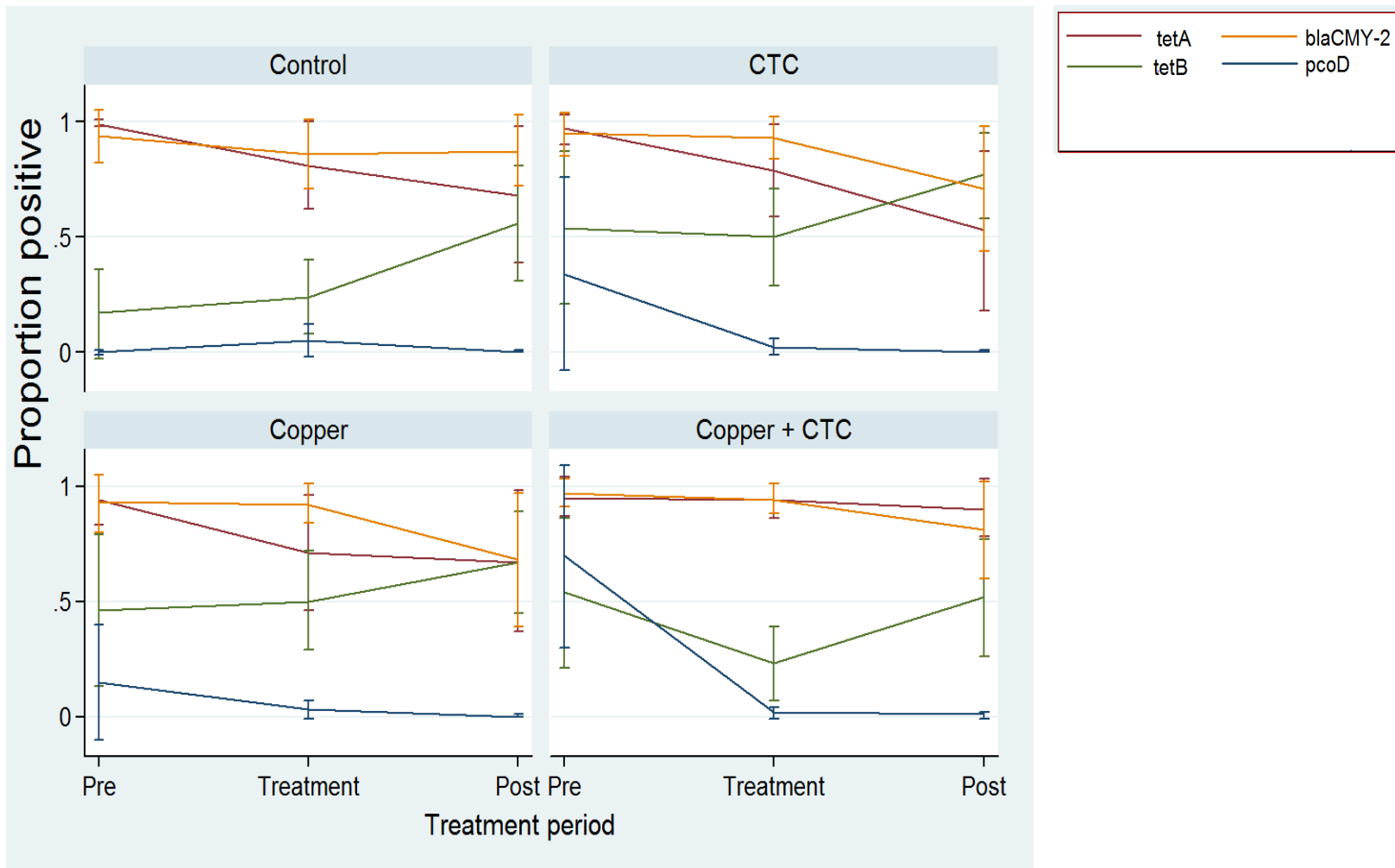
Figure 4.1. Adjusted predictions of the prevalence of resistance genes detected from *E. coli* (n = 1,152) obtained from fecal samples of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither.
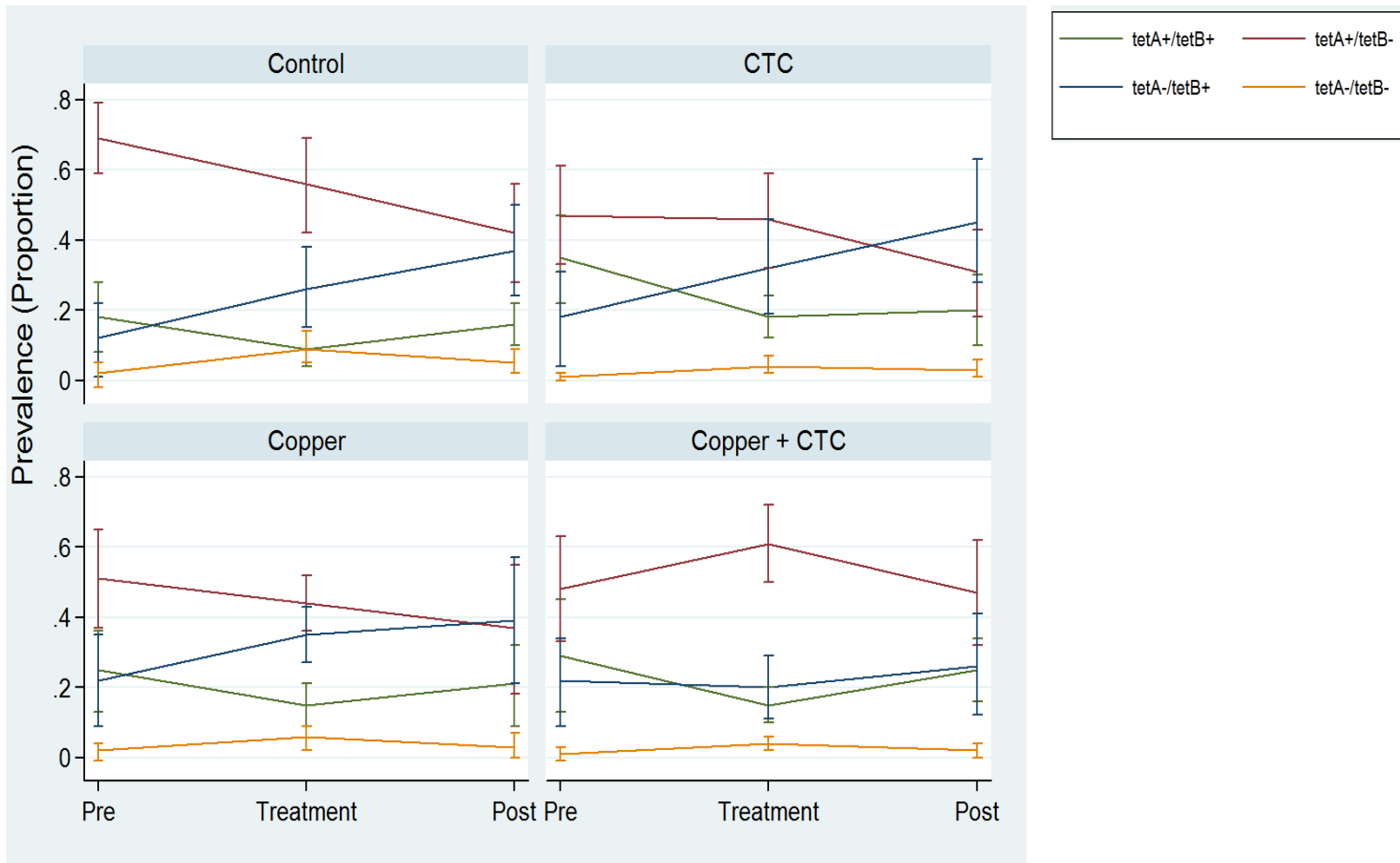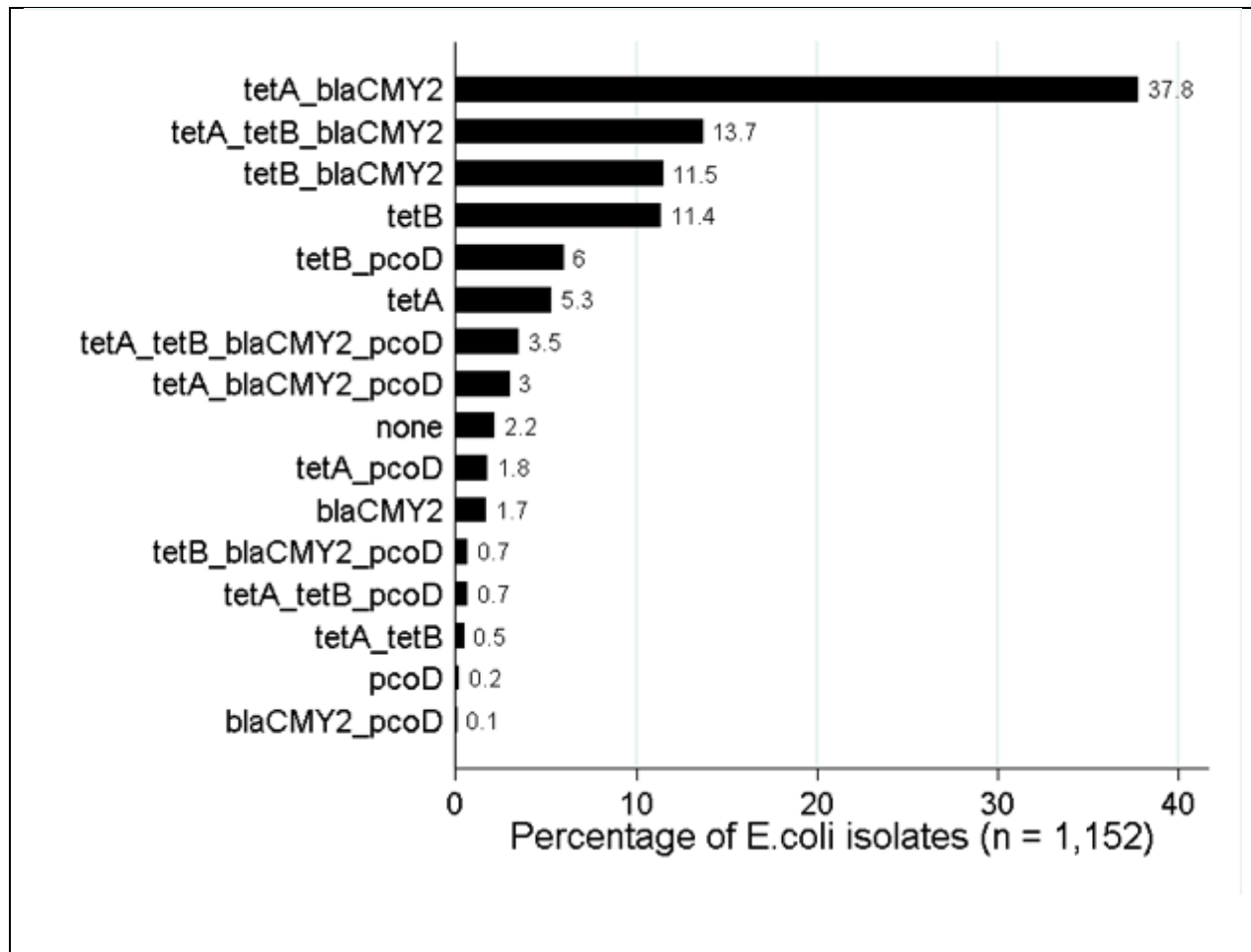
Figure 4.2. Bivariate analysis of *tet*A and *tet*B genes detected from *E. coli* (n = 1,152) obtained from feces of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither.

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Figure 4.3. Distribution of genotypic profiles for four resistance genes detected from *E. coli* isolates (n = 1,152) obtained from feces of weaned pigs supplemented with chlortetracycline, copper, both or neither.
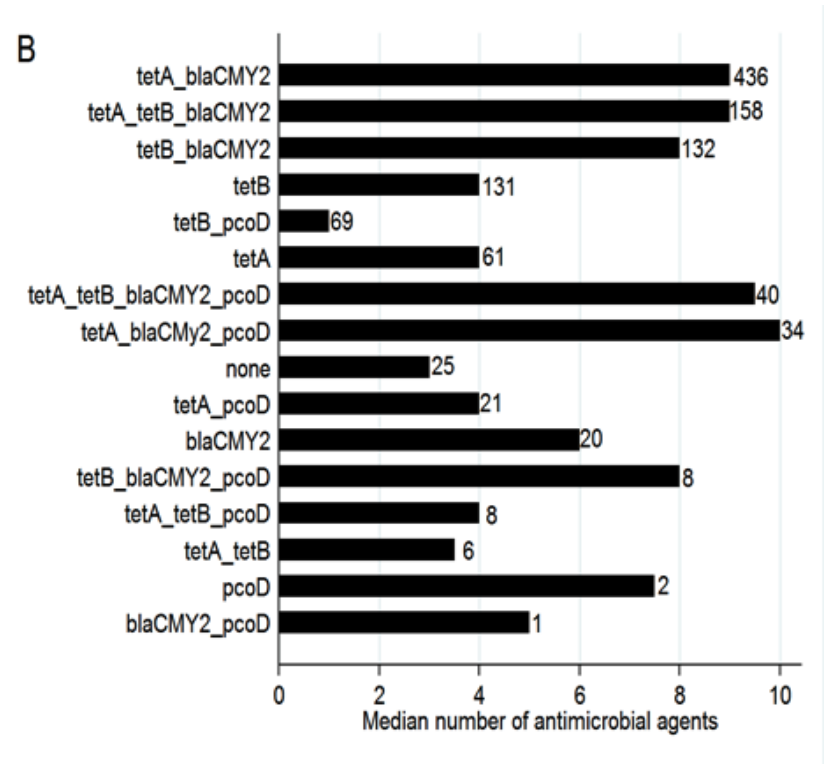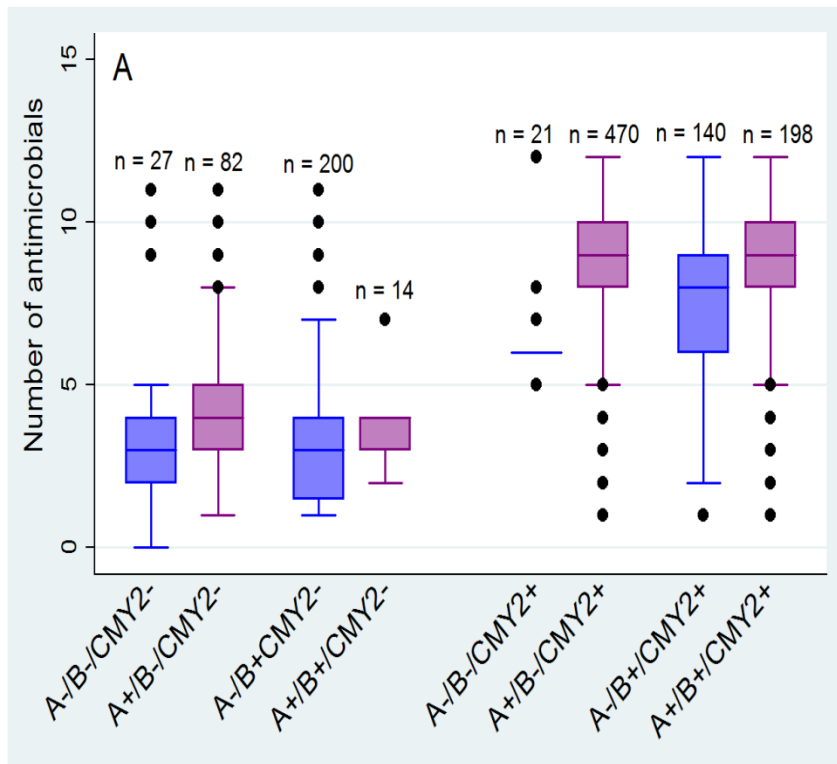
Figure 4.4. Median multidrug resistance count of *E. coli* (n = 1,152) on the basis of the number of antimicrobials to which resistance was exhibited.

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. genotypic profiles. Numbers on the bar graph represent the number of isolates with unique genotypic profiles.

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Table 4.1. Primer sequences, amplicon size and positive control strains used for PCR detection of antimicrobial resistance genes from fecal *E. coli* (n = 1,152) 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)	Reference	*E. coli* strains	Gene bank accession no.	Source
*tet*A	*tet*A-F	GCT ACA TCC TGC TTG CCT TC	210	(194)	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	(194)	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	(194)	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	(194)	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	(154)	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.

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Table 4.2. Prevalence (%) of resistance genes among fecal *E. coli* isolates (n = 1,152) 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.

Table 4.3. Model-defined pairwise correlations (with 95% CI) based on a multivariate probit model, among four resistance genes in fecal *E. coli* (n = 1,152) from weaned pigs fed diets supplemented with chlortetracycline, copper, both or neither

	*tet*A	*tet*B	bla_{CMY-2}	*pcoD*
*tet*A	1			
*tet*B	-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	

Table 4.4. Distribution of tetracycline resistance genes detected from fecal *E. coli* isolates obtained from weaned pigs experimentally supplemented with chlortetracycline, copper, both or neither

tet genes		Frequency	Percentage
*tet*A		544	47.2
*tet*B		329	28.6
*tet*C		1	0.1
*tet*E		12	1.0
*tet*A-*tet*B		202	17.5
*tet*A-*tet*C		5	0.4
*tet*A-*tet*E		3	0.3
tetB-*tet*C		8	0.7
*tet*B-*tet*E		3	0.3
*tet*A-*tet*B-*tet*C		10	0.9
	none	35	3.0
	Total	**1,152**	**100.0**

Chapter 5 - Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the feces of weaned pigs

Abstract

Use of antibiotics such as chlortetracycline (CTC) in food animals is fiercely debated as a cause of antimicrobial resistance (AMR) in human pathogens and alternatives to antibiotics such as heavy metals have been proposed. We used metagenome-based approaches to investigate the effects of CTC and copper supplementation on AMR in the gut microbial ecology of pigs. Total community DNA was extracted from 569 fecal samples collected over a 6-week period from groups of 5 pigs housed in 32 pens that were randomized to receive either control, CTC, copper, or copper plus CTC regimens. Qualitative and quantitative PCR were used to detect the presence of 14 tetracycline resistance (*tet*) genes and to quantify gene copies of *tet*A, *tet*B, bla_{CMY-2} (for ceftiofur resistance gene), and *pco*D (copper resistance genes), respectively. The prevalence of *tet*A and *tet*B detection decreased over the treatment periods, whereas the prevalence of *tet*C and *tetA(*P) increased. CTC and copper plus CTC supplementation increased both the prevalence and gene copy numbers of *tet*A, while decreasing both the prevalence and gene copies of *tet*B, when compared with the control group. Although the mean log10 gene copies of *tet*A, *tet*B, and bla_{CMY-2} decreased with time and as pigs aged, that of *pco*D initially increased during the first and second weeks of the treatment period and thereafter declined. In conclusion, *tet* genes were initially very diverse in the gut bacterial community of weaned pigs; thereafter, copper and CTC supplementation differentially impacted the prevalence and quantity of tetracycline, ceftiofur and copper resistance genes.

Introduction

Antimicrobial resistance (AMR) is one of the major public health challenges of the 21st century (1, 66, 200), and international collaboration is needed to combat this complex and multifactorial issue (67, 85). Public health concern about AMR is largely focused on the use of antimicrobial growth promoters (AGP) in livestock; however, prevention and control uses of antibiotics are also under considerable scrutiny (11). Chlortetracycline (CTC) and oxytetracycline are among the most commonly used antibiotics in pig production in the United States (17, 18). Tetracycline resistance is acquired mainly through mobile genetic elements (plasmids and transposons) (31) and represents the most commonly observed resistance among a wide range of bacteria (105, 201). Forty-six tetracycline resistance (*tet* and *otr*) genes are currently known[4].

Alternatives to antimicrobials are being sought because of the public health risk of AMR associated with the use of AGPs in animals; one option is to use metals such as copper and zinc (35). Copper is an essential trace element that is sometimes used as a growth promoter at a dose elevated beyond that which is required for basic metabolic needs (46). Bacteria exposed to metals have also developed the ability to resist high concentrations of metals, including copper (79). Among *E. coli*, in addition to chromosomally mediated copper homeostasis, a plasmid-borne copper resistance determinant (*pco*) has been suggested to confer resistance to copper. The *pco* determinant consists of seven genes arranged in two operons, *pco*ABCD and *pco*RS, with *pco*E as a separate gene (55, 122). The *pco*ABCD genes are normally expressed as a polycistronic message from the same promoter under a two-component *pco*RS regulatory

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[4] http://faculty.washington.edu.marilynr/

system, with stoichiometric production of the four gene products (56); importantly, expression of all four genes is required for full resistance (55). On the other hand, the *pco*E gene is not strictly required for full expression of copper resistance; however, it acts as a sponge to sequester toxic copper which provides additional time needed for the expression of the other *pco* genes (55).

Antimicrobial resistance has been studied largely using culture-based methods, which typically involve bacterial isolation followed by sensitivity testing, or sometimes by testing bacterial DNA for the presence of AMR genes (149). These isolate-based methods underestimate the true magnitude and also the dynamics of AMR because the majority of bacteria are non-cultivatable on culture media (202). Quantitative methods, based on the determination of gene copies from total community DNA, can give more accurate information on the impact of antimicrobial use on AMR load in any given bacterial ecology (149), such as the pig gut. Complete sequence analysis of IncA/C plasmids originating from cattle *E. coli* has shown that this plasmid typically carries two copies of the bla_{CMY-2} genes, along with other resistance genes including *tet*A (64). Copper supplementation favoring plasmid-borne *pco* genes also could select for antibiotic resistance such as tetracycline and ceftiofur (50). Therefore, we used a culture-independent total community DNA approach to investigate the impact of therapeutic doses of in-feed CTC and elevated levels of copper supplementation on antimicrobial and copper resistance genes among the gut bacteria of pigs. Our hypotheses were that the in-feed use of tetracycline (CTC) expands certain tetracycline resistance genes and that copper supplementation in pigs differentially co-selects for certain tetracycline and ceftiofur resistance genes.

Materials and methods

Experimental design and DNA extraction

The study included a total of 569 fecal samples collected over 6 weeks from 4 treatment groups (8 replicates of 5 pigs per pen; n = 160 weaned pigs total), starting at three weeks of age, that were fed either: only the basal diet (control), the basal diet supplemented with copper at 125 mg/kg of feed, the basal diet plus CTC at 550 mg/kg of feed of CTC, or else copper plus CTC at above dosages. The experimental design is schematically illustrated in Fig. 5.1. The study was performed in the segregated early weaning facility at Kansas State University, and animal use protocols were approved by the university institutional animal care and use committee (IACUC# 2773).

Total community DNA was extracted from 200 mg of the fecal samples using a QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The DNA concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and stored at -20^{0}C until used. *E. coli* strains positive for *tet* genes were obtained from the laboratory of Dr. Marilyn Roberts (Washington State University). Positive *E. coli* strain for *bla*$_{CMY-2}$ was originally obtained from University of Illinois (203) and positive *E. coli* strain with pRJ1004 for *pco*D was obtained from Dr. Henrik Hasman (the National Food Institute, Technical University of Denmark). DNA used as positive controls were extracted from these *E. coli* strains with the PureYield Plasmid Miniprep System (Promega Corporation, Madison, WI) following overnight culture in Luria-Bertani broth (Difco, BD, Sparks, MD) incubated at 37°C.

PCR detection of tetracycline resistance genes

A previously developed multiplex PCR protocol and primers (194) with a pre-optimized multiplex PCR master mix kit (QIAGEN, Valencia, CA) were used to detect 14 different *tet* genes, grouped into 4 multiplex PCR assays, from each fecal sample (Table 5.1). *E. coli* strains with *tet*-positive plasmids were obtained from the laboratory of Dr. Marilyn Roberts (Washington State University). A multiplex PCR reaction mixture of 50 µl consisted of 17 µl of nuclease-free water, 25 µl of master mix, 5 µl of the primer mix, and 3 µl of DNA template. A positive control mixture for each of the multiplex assays and a no-template control were included on each plate. Thermal cycling 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 with a final extension at 72°C for 10 min. PCR products were analyzed using capillary gel electrophoresis in the QIAxcel system (QIAGEN, Valencia, CA).

Quantification of resistance genes from fecal community DNA

We used Brilliant II SYBR green master mix (Agilent technologies, La Jolla, CA)–based qPCR for quantification of *tet*A, *tet*B, *bla*$_{CMY-2}$, and *pco*D genes from total fecal community DNA. All qPCR assays were performed in duplicate reactions in Mx3005P thermocycler (Stratagene Corporation, La Jolla, CA), and amplification data were analyzed with MXPro 4.1 (Stratagene Corporation, La Jolla, CA) software. The qPCR standard curves, defined as gene copy numbers versus cycle threshold (Ct) values, were generated using purified PCR products obtained from positive controls for each of the four genes. Ten-fold serial dilutions were made from known concentrations of purified PCR products and 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 gene copies per reaction were used to create a standard curve for *tet*A and *pco*D genes. For *tet*B and *bla*$_{CMY-2}$ genes, 10^4, 10^3, 10^2, 10^1, and 10^0 dilutions were used to generate the standard curves.

Standard curves were run in triplicate on every plate for all unknown samples. Non-template control (NTC), ATCC 25922 *E. coli* strain (negative control strain), along with template DNA from the positive control strains (positive control) were included in all qPCR reaction plates. The primers used for the quantification of the resistance genes are given in Table 5.2.

For the quantification of *tet*A, total qPCR reaction mixture of 20 μl consisted of 6.25 μl water, 10 μl master mix (Agilent Technologies, La Jolla, CA), 0.75 μl reference dye (30 nm), 0.5 μl each of the primers, and 2 μl template DNA. The thermal cycling program was 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 28 s. For *tet*B quantification, a total volume of 20 μl reaction consisted of 6.825 μl of water, 10 μl of master mix, 0.375 μl of reference dye, 0.4 μl of each of the primers, and 2 μl of template DNA. Thermal reaction conditions were 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Gene copies of the bla_{CMY-2} gene were quantified in a 20-μl mixture consisting of 5.25 μl of water, 10 μl of master mix, 0.75 μl of reference dye, 1 μl each of the primers, and 2 μl of template DNA. Thermal profiles consisted of 40 cycles of denaturation at 95°C for 10 s, annealing at 52°C for 30 s, and extension at 79°C for 17 s. For *pco*D gene quantification, 20-μl reaction volume comprised of 6.45 μl of water, 10 μl of master mix, 0.75 μl of reference dye, and 0.4 μl of the primers and 2 μl of DNA template. The qPCR thermal conditions were 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1min. In all qPCR assays, melting curve analysis was performed with a final dissociation step at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. All qPCR assays were initiated by a denaturation step of 95°C for 10 min to activate the HotStarTaq DNA polymerase enzyme.

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Statistical analysis

A full factorial analysis in STATA 12.1 (STATA Corporation, College Station, TX) was used to analyze the effects of treatments over the treatment periods. In all statistical analyses a *P*-value < 0.05 was considered significant for identifying differences across treatment groups or treatment periods and Bonferroni-adjusted significance tests were used for multiple comparisons. Model selection was based on manual elimination of non-significant terms starting with a full model (a model that included all 3- and 2-way interactions and the main effects of the two treatments and treatment period), and thereafter first removing the non-significant highest-order interaction, then 2-way interactions, and finally main effects terms, where applicable.

For analysis of the prevalence of *tet* genes, the weekly sampling dates were categorized into pretreatment (day 0), during treatment (days 7, 14, 21), and post-treatment periods (days 28 and 35). Binary outcomes (presence/absence) of the *tet* genes were initially presented as percentages along with the 95% exact binomial confidence intervals, and Fisher's exact test or the likelihood ratio test (LRT) were used, as appropriate, to contrast the prevalence of the genes by treatment group and treatment period. The median (with 95% CI) was used to compare the number of *tet* genes detected per fecal sample by treatment groups using Kruskal-Wallis test. Because all other genes had sparse cells, indicating either low prevalence or high saturation, further multivariable statistical analyses were performed only for *tet*A, *tet*B, and *tetA(*P) genes. Random effects logistic regression was used to analyze the fixed effects of treatment, treatment period, and their interactions by including pen as a random effect in the model. Bivariate probit regression with a robust clustered standard error accounting for pen effect was used to model the binary outcomes of *tet*A and *tet*B simultaneously to adjust for their biological dependencies. Ordinal logistic regression with a robust standard error to adjust for clustering by pen was fit to

evaluate the effect of treatment and treatment period on the number of different *tet* genes detected per fecal sample. Our unit of analysis was the binary outcome for each fecal sample (presence or absence of the *tet* genes) and our experimental unit was pen.

Quantitative data were analyzed using multilevel mixed-effects linear regression models to assess the effects of the two treatments and sampling day on three outcomes: 1) absolute (non-standardized) gene copy numbers per gram of feces; (2) standardized quantities of the genes normalized to the total DNA concentration in the original fecal samples; and (3) relative quantities of *tet*A to *tet*B per gram of feces. The ratio of *tet*A to *tet*B was derived by dividing *tet*A gene copies by *tet*B gene copies present in 1 g of feces. Genes were normalized by dividing gene copies per μl to the initial DNA concentration (expressed as ng/μl) and were subsequently $\log_{10}$ transformed. The normality of the $\log_{10}$ transformed gene copies per gram of wet feces was tested graphically by histogram and Q-Q plots. The model was a three-level hierarchical model in which individual gene copy readings (our unit of analysis) were nested within fecal sample (duplicate reactions per sample), which in turn was nested within pen (our experimental unit). Each reaction reading from qPCR was used as the unit of analysis. On each sampling day fecal samples were collected from three pigs among the five pen mates; therefore, pig was not included as a random effect in the model. The model included the random effects of duplicate reactions per sample and pen, and fixed effects of treatments, sampling day, and their interactions. Three reactions for *tet*A and 11 reactions for bla_{CMY-2} had no CT values. Only one of the duplicate readings had no CT value, which did not yield a negative result at the sample level. To deal with missing observations, these observations were assigned a value of zero, and for later analyses, we added half the value of the lowest nonzero value observed in the samples (following Boyer et al., 2013 (204)) to all observations. The estimated gene copy numbers in

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each reaction were back-calculated to gene copies per gram of wet feces for each sample, which was then transformed to $\log_{10}$ to achieve normality. Multivariate multiple linear regression models were used to simultaneously model the four resistance genes (*tet*A, *tet*B, *bla*$_{CMY-2}$ and *pco*D) together to account for their biological dependencies; that is, the genes were quantified from the same fecal sample arising from pigs under the same treatment and day effects. Pairwise Pearson correlations between resistance genes with Bonferroni-adjusted significance tests to adjust for multiple comparisons were calculated, and scatter plots were generated to display these relationships.

Results

Prevalence of tet genes

Three genes, *tet*D, *tet*K, and *tet*S, were not detected in any of the fecal samples, and *tet*E (from two control pens) and *tet*G (one sample from a control and copper pen) were each detected in only two fecal samples. The *tet*O, *tet*Q, and *tet*X genes were detected in 100% of the fecal samples, regardless of the treatment groups. The prevalence and distribution of the remaining six genes (*tet*A, *tet*B, *tet*C, *tet*L, *tet*M and *tetA(*P)), cross tabulated by treatment groups and treatment period, are shown in Table 5.3. Prevalence of *tet*B was marginally different (Bonferonni-adjusted $P = 0.04$) among the treatment groups during the treatment period. The prevalence of *tetA(*P) was significantly higher ($P = 0.003$) in the CTC plus copper supplemented group (86%; 95% CI = 76-93%) compared with the control group (60%; 95% CI = 48-71%) during the treatment period. While the prevalence of *tet*B significantly ($P < 0.001$) decreased over the subsequent treatment periods, that of *tet*(P) increased significantly ($P < 0.001$), reflecting a secular trend (Table 5.3). In the copper supplemented group, the prevalence of *tet*A

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in the post-treatment samples (63%; 95% CI = 22-51%) was significantly ($P = 0.011$) lower than in the pre-treatment samples (95%; 95% CI = 76-100%). Since regression models for most of the individual *tet* genes failed to converge, either as a result of scarcity or saturation, only *tet*A, *tet*B and *tet*A(P) were modeled beyond these descriptive analyses.

Multilevel mixed-effects logistic regression analysis for the prevalence of tetA, tetB and tetA(P)

Results of individual multilevel mixed-effects logistic regression analysis for *tet*A, *tet*B, and *tet*A(P) analyses are shown in Fig. 5.2. The single three way (CTC*copper* treatment period) and all two way interactions (CTC*copper, CTC* treatment period and copper* treatment period) were not significant ($P > 0.05$). Overall, CTC supplementation was significantly associated with increased detection of *tet*A (OR = 1.7 [95% CI = 1.1-2.8). The detection of both *tet*A and *tet*B decreased significantly ($P < 0.001$) through the treatment period. Interestingly, the level of detection of *tet*A(P) demonstrated a significantly increasing ($P < 0.05$) secular trend across subsequent treatment periods.

Across all treatment groups and sampling days, unadjusted prevalence of *tet*A (77% [95% CI = 74– 81%]) was significantly higher than that of *tet*B (57% [95% CI = 53–61%]). The prevalence of *tet*A was significantly ($P < 0.05$) higher than that of *tet*B in the post-treatment samples, except for in the CTC-supplemented group. The prevalence of *tet*A (83% [95% CI = 72–91%]) was significantly higher than that of *tet*B (46.5% [95% CI = 35–59%]) in the copper plus CTC–supplemented groups during the treatment period. Bivariate probit analysis of *tet*A and *tet*B showed that probability of detecting both genes together dropped significantly from pre-treatment levels through the treatment period. Adjusted for biological interdependences (and pen effect), a significant ($P < 0.001$) 3-way interaction was observed between treatment period,

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copper, and CTC supplementation for both genes. The combined supplementation of copper and CTC tended to increase *tet*A only detection, whereas it did not affect *tet*B only detection in fecal samples compared to the control group (Fig 5.3).

Analysis of multiple co-detected tet genes using ordinal logistic regression

The median number of *tet* genes detected per fecal sample was eight across all samples. The median number of *tet* genes detected per sample did not differ significantly ($P > 0.05$) among the treatment groups across all treatment periods. The number of genes detected per fecal sample ranged from 4–10 (control), 5–9 (copper), 6–9 (CTC), and 5–9 (copper plus CTC). The distributions of the number of *tet* genes detected per fecal sample is illustrated in Fig. 5.4. The frequency and distribution of the different *tet* genes combinations is listed in Table 5.4. Across all samples, 26 distinct *tet* gene combinations were observed. Control, CTC, and copper plus CTC groups each contained 13 unique genotypic profiles, whereas the copper group had 14 unique profiles. The top three most common genotypic profiles observed were of 8- and 9-gene combinations. Fecal samples obtained during the treatment period (proportional OR = 0.5 [95% CI = 0.3–0.7]) and post-treatment period (proportional OR = 0.5 [95% CI = 0.3–0.9) tended to have fewer numbers of *tet* genes, suggesting a decreasing trend in the diversity of *tet* genes in the fecal flora over time.

Quantification of resistance genes from fecal samples

Model-adjusted mean $\log_{10}$ copies per gram of feces for each of the 4 genes are depicted in Fig. 5.5. Mean $\log_{10}$ copies of *tet*A, *tet*B, and *bla*$_{CMY-2}$ decreased significantly ($P < 0.05$) over time; however, mean $\log_{10}$ copies of *pco*D gene increased significantly ($P < 0.001$) on days 7 and 14 and then returned to baseline following day 21. Mean *pco*D gene quantities were

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significantly higher in the control group than in other treatment groups on day 14, but *pco*D gene

copies in the copper plus CTC group were significantly higher than the control samples on days

21 and 35 (Fig. 5.5).

Multivariate analysis (analyzing *tet*A, *tet*B, *bla*$_{CMY-2}$ and *pco*D genes together) gave the

same conclusion as the individual gene results, with small differences seen in the coefficients

and the *P*-values. The independent variables (copper, CTC, day, and all possible interactions)

were significant as a group (F$_{23, 1112}$ = 9.89, *P* < 0.0001). Following the multivariate regression,

all pairwise correlations between residual values of the genes were positive except for *tet*A and

*pco*D. The Breusch-Pagan test of independence was significant (χ^2_6 = 590.544, *P* < 0.0001),

indicating, as expected, that the residuals of the four genes were not independent from each

other. Pairwise correlation analysis of the raw data for the four genes also indicated the presence

of a significant (*P* < 0.05) correlation, except between *tet*A and *pco*D genes (Fig. 5.6). The

highest (66%) correlation was observed between *tet*A and *bla*$_{CMY-2}$ when compared with the rest

of the pairwise correlations.

Results of resistance gene copies, adjusted to initial DNA concentration of the total

community DNA in the fecal samples, essentially gave the same results as the analysis based on

non-standardized copies of genes, with minor differences in the coefficients and P-values (Fig.

5.7).

Considering the relative ratios of *tet*A to *tet*B, the mean copies of *tet*A generally were

higher than *tet*B copies. The *tet*A to *tet*B ratio was significantly different from unity (equality)

on days 7 (*P* = 0.015) and 14 (*P* = 0.006) across all samples and treatment groups compared with

the baseline. Copper plus CTC supplementation drove *tet*A copies even higher on days 14 and

21 when compared to those of control and copper-only supplemented samples on the same respective days (Fig. 5.8).

Discussion

Antimicrobial resistance studies in pigs have focused largely on specific bacterial species rather than the entire gut microbiota and have mainly used culture-based approaches (25, 205, 206). Recently, culture independent approaches based on total community DNA have been used to study the ecology of antimicrobial resistance genes in cattle (149, 154, 207) and in pigs (208, 209). In this study, we investigated the effects of supplementation of CTC and copper over a 3-week period on tetracycline, ceftiofur, and copper resistance elements in the pig metagenome.

Tetracycline resistance genes

We investigated the impact of in-feed CTC and copper supplementations on the prevalence and distribution of 14 *tet* genes (out of potentially 46 genes) based on previous reports from cattle (207) and pig (208) metagenomes. We found a large diversity of *tet* genes in the pig gut, including in control pigs that were not directly exposed to CTC or copper. This result is consistent with previous reports (74, 209-212) indicating high background tetracycline resistance in pigs; this can make it difficult to compare and contrast the effects of treatment interventions. The predominant *tet* genes detected were *tet*O, *tet*Q, *tet*X, *tet*M, and *tet*L. The *tet*O, *tet*Q, and *tet*M genes encode for ribosomal protection proteins and are found in both Gram positive and Gram negative bacteria (28, 29). The *tet*L gene, which codes for an efflux protein, has been reported mainly from Gram positive bacteria and less frequently from Gram negative bacteria (28, 29). Unlike most of the efflux genes, *tet*L is found mainly on small transmissible plasmids that can integrate into the chromosome of some Gram positive bacteria (28, 29).

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Similar to our finding, Holman and Chenier (74) also reported *tet*O and *tet*Q from 100% of the fecal samples of pigs studied, regardless of treatment group. They also detected *tet*L and *tet*M among at least 50% of the fecal samples in each treatment group. Tet(X) is a flavin-dependent monooxygenase that inactivates 1st-, 2nd-, and 3rd-generation tetracyclines such as tigecycline (15, 120, 121).

In contrast to the 0% prevalence of *tet*D and *tet*S that we observed, Harvey et al., 2009 (207) reported 87% and 100% prevalence of these genes, respectively, in the feces of beef cattle. This result may be attributed to differences in the gut microbiome of cattle versus pigs. Similar to our finding, Harvey et al (207) did not detect *tet*K from the cattle fecal samples. In general, *tet*D and *tet*S genes have been reported exclusively from Gram negative and Gram positive bacteria, respectively, of both animal and human origin, whereas *tet*K is reported both from Gram positive (frequently) and Gram negative bacteria (less frequently) (28, 29). The *tet*E and *tet*G genes are reported almost exclusively from limited genera of Gram negative bacteria, including *Salmonella* (28, 29); thus, the low prevalence of such genera in these pigs could result in low detection of the genes.

The efflux genes *tet*A and *tet*B, found only in Gram negative enteric bacteria, are carried on large conjugative plasmids, which also can carry other antimicrobial as well as heavy metal resistance genes. This contributes to a widespread dissemination of multidrug-resistant enteric bacteria (28). The *tet*B gene has the widest host range among *tet* genes in Gram negative bacteria. We observed a decrease in the prevalence of both *tet*A and *tet*B in fecal community DNA across subsequent treatment periods, which could be attributed to an aging effect. On the other hand, we observed an upward trend across the same aging of the pigs in the prevalence of *tet*C and *tet*A(P) detection. This overall trend could be the result of a gradual shift in the

microbial population of the gut as pigs age and consume more solid feedstuffs (202). This is supported by the increase, or more stable occurrence, of the predominantly Gram positive *tet* genes (A(P), O, Q, L, and M) and a decrease in Gram negative *tet* genes (A and B). The *tet*C gene, which encodes for efflux pump proteins, has been reported from most genera of the *Enterobacteriacae* family. Copper supplementation significantly increased the prevalence of *tet*A(P) during the treatment period. *tet*A(P), efflux gene, was previously reported from *Clostridium* species, which initially expand and then contract in numbers as pigs age (202, 213). Ribosomal protection genes, including *tet*M-- which has the widest host range, are not commonly found among the Gram negative enteric bacteria, probably due to lower levels of resistance conferred by this mechanism when compared with the efflux pump genes. Ribosomal protection genes do not appear to provide a major survival advantage for Gram negative enteric bacteria, which are likely to be constantly exposed to tetracycline from the modern pig gut environment (29).

Interestingly, in the bivariate analysis of *tet*A and *tet*B, *tet*A only prevalence was significantly higher in the copper plus CTC–supplemented group during the treatment period compared with the control group. This result contrasted sharply with the prevalence of *tet*B-only detection which was significantly lowered compared to the control group. This clearly indicates that CTC supplementation, either alone or in combination with copper, selects for a broader bacterial population harboring *tet*A (Fig. 5.3).

Quantification of resistance genes

Quantitative measurements in longitudinal studies of antimicrobial resistance genes based on the entire microbiome are crucial to better monitor the dynamics of AMR (62, 154). To our knowledge, this is the first longitudinal study to quantitatively measure and directly compare the

abundance of two *tet* genes (*tet*A and *tet*B), a ceftiofur resistance gene (*bla*$_{CMY-2}$), and a Gram negative copper resistance determinant (*pco*D) among fecal samples obtained from pigs treated or untreated with copper or CTC. Log$_{10}$ mean copies of *tet*A, *tet*B, and *bla*$_{CMY-2}$ genes all decreased over time. In some measure of contrast, *pco*D gene quantities increased at first during treatment, followed by a gradual decrease through the remaining sampling days. All of these declines in resistance elements may be due to the aging effect of the pigs as previously reported elsewhere (188, 189). This declining trend in gene copy numbers also could be attributed to changes in the underlying bacterial population, with Gram positive bacteria gradually replacing Gram negative bacteria as pigs move from post-weaning to grower stages of production (202). The four resistance genes we targeted exclusively confer resistance to Gram negative bacteria, whereas the underlying bacterial population (as represented by the total community DNA) largely expands as a result of an increase in Gram positive bacteria.

Supplementation with copper alone had opposing effects on the abundance of the *tet* genes as it tended to decrease gene copies of t*et*A but increase the levels of *tet*B (Figs. 5.5, 5.7 and 5.8). Interestingly, we observed that copper and CTC supplementation significantly reduced the quantities of *bla*$_{CMY-2}$ on the 7th day after treatment; however, as with *tet*A, when CTC was supplemented alone *bla*$_{CMY-2}$ gene copies were higher than in the control group during the treatment period (Figs. 5.5 and 5.7). Paradoxically, the log$_{10}$ mean copies of *pco*D in the control group were significantly higher than the copper treatment groups on day 14 (Fig. 5.5). The *pco*D gene copies remained more or less constant through time in the entire treatment group. This finding remains unexplained; however, it may be that the advantages conferred by the copper resistance gene for Gram negative bacteria (*pco*) versus that for Gram positive bacteria (*tcr*B) are relatively of a lesser magnitude, as has been shown in MIC distributions in work by others (41,

42, 44). Thus, high doses of copper may select for copper resistant Gram positives like *Enterococcus* spp., where the *tcr*B gene confers very high levels of resistance, and away from *E. coli*, as an example. Rouch and Brown (56) earlier pointed out that *pco*-mediated copper resistance is an auxiliary mechanism that cooperates with the host bacterial cell copper management systems; in essence, it can modestly extend the range of environmental copper concentrations over which the bacterial cell can survive.

Generally, the gene copies of *tet*A were several orders of magnitude higher than those of *tet*B and *bla*$_{CMY-2}$, indicating that the overall microbial population possesses more *tet*A. This result was also reflected in the analysis of the ratio of *tet*A to *tet*B, in which *tet*A concentration was generally much higher than that of *tet*B. Yu et al., 2005 (214) also reported greater abundance of *tet*A from pig manure using qPCR absolute quantification. The high correlation between *tet*A and *bla*$_{CMY-2}$ we observed could indicate that these two genes are genetically linked as reported (64) and that ceftiofur resistance can be maintained through co-selection by CTC supplementation in pig feed, and in the absence of direct selective pressure from ceftiofur (181). We used a relative quantification method by normalizing resistance gene copies to the total DNA concentration (instead of a variable referent gene such as 16s rRNA (154)) in the fecal sample. This method was also used by Holman and Chenier (74) to investigate the impact of subtherapeutic doses of tylosin and CTC on *tet* and *erm* gene copies from pig fecal samples. In our analysis, both non-standardized and standardized methods produced the same conclusions. A total fecal community DNA approach does not differentiate whether bacterial DNA is from live or dead bacteria; although this is of little consequence for studying gut microflora, it could have an impact on studies that extend into the farm environment. The AMR gene pools can

serve as a constant source of horizontal gene transfer among bacterial population sharing the same ecology (70).

The major drawback of the total community approach is that it is impossible to attribute the resistance genes to particular bacteria. In essence, this makes such analysis classically ecological in nature, albeit in an opposite (micro) direction than the usual analyses, which are typically macro-ecological and well-documented in the epidemiological and even in the sociological literature. Thus, the approach is subjected to a unique form of bias known as the "ecological fallacy," in which an inference made at the aggregated level (in this study of genes interpreted at pen or sample level) should not be assumed to apply at the disaggregated level (bacteria level). Overall, such an approach gives an overall picture of the resistance genes in the gut at a micro-ecological level for evaluating the impact of growth promoters in agriculture. It is not a stand-alone approach; rather, it should be relied upon to supplement other approaches such as isolate-based analysis to avoid making inappropriate policy decisions on the basis of a single outcome measure.

Conclusions

This is the first experimental study to investigate the individual or combined effects of CTC and copper supplementation on the distribution of *tet* genes and quantities of tetracycline and cephalosporin resistance genes in the gut microbial ecology of pigs. Overall, *tet* genes were diverse in the total gut bacterial community of the study pigs; importantly, copper and CTC supplementation differentially expanded several of the resistance genes studied. This result suggests that such supplementation may favor gene expansion of certain target bacteria, perhaps at the expense of other gene targets or bacteria. Community DNA analyses provide an adjunct

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approach to evaluating effects on the microbial ecology of the gut that complements, but does

not replace, phenotypic and genotypic analysis of cultivatable bacteria.

Overall, 32 pens with 5 pigs per pen were randomized to pen-level treatments (n = 160 pigs)			
CONTROL group (8 pens) (n = 40 animals; 5 pigs per pen)	**CTC** group (8 pens) (n = 40 animals; 5 animals per pen)	**COPPER** group (8 pens) (n = 40 animals; 5 animals per pen)	**COPPER + CTC** group (8 pens) (n = 40 animals; 5 animals per pen)
Received basal diet (CuSO$_4$ at 16.5 mg/kg of feed)	Received basal diet + CTC (at 550 mg/kg of feed)	Received elevated copper (at 125 mg/kg of feed)	Received elevated copper plus CTC (at above doses)
Fecal samples collected from 3 animals per pen each week for 6 weeks (n = 144 total samples)	Fecal samples collected from 3 animals per pen each week for 6 weeks (n = 144 total samples)	Fecal samples collected from 3 animals per pen each week for 6 weeks (n = 144 total samples)	Fecal samples collected from 3 animals per pen each week for 6 weeks (n = 144 total samples)
Fecal samples available for analysis (n = 143)	Fecal samples available for analysis (n = 142)	Fecal samples available for analysis (n = 141)	Fecal samples available for analysis (n = 143)

Figure 5.1. Schematic representation of the trial design used to evaluate the effect of chlortetracycline (CTC), copper, neither or their combined supplementation in weaned pigs on the level of resistance genes detected in fecal community DNA.
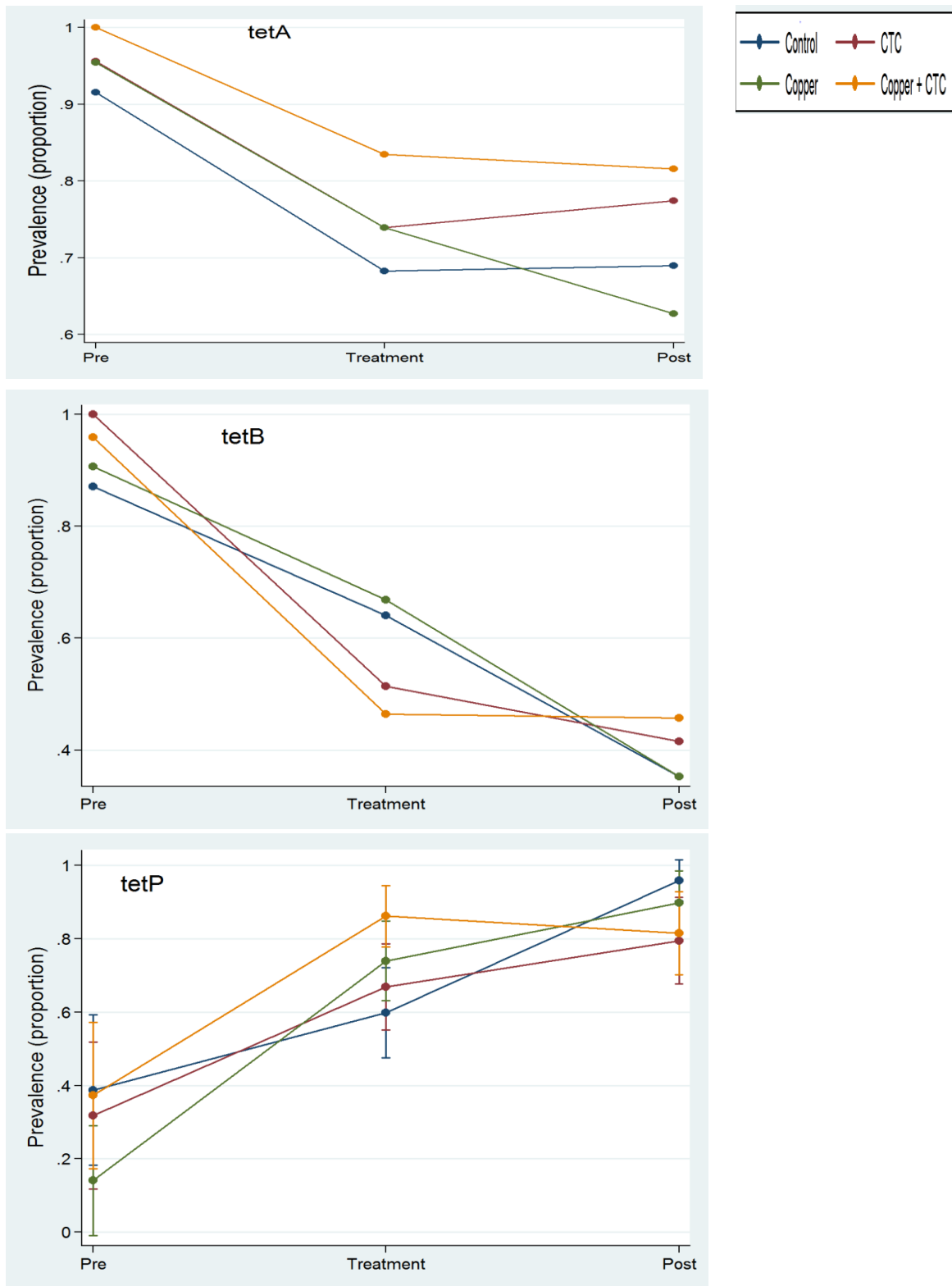
Figure 5.2. Model adjusted prevalences of *tet*A, *tet*B, and *tet*A(P) from the fecal community DNA of weaned pigs receiving chlortetracycline (CTC), copper, both or neither across three treatment periods.

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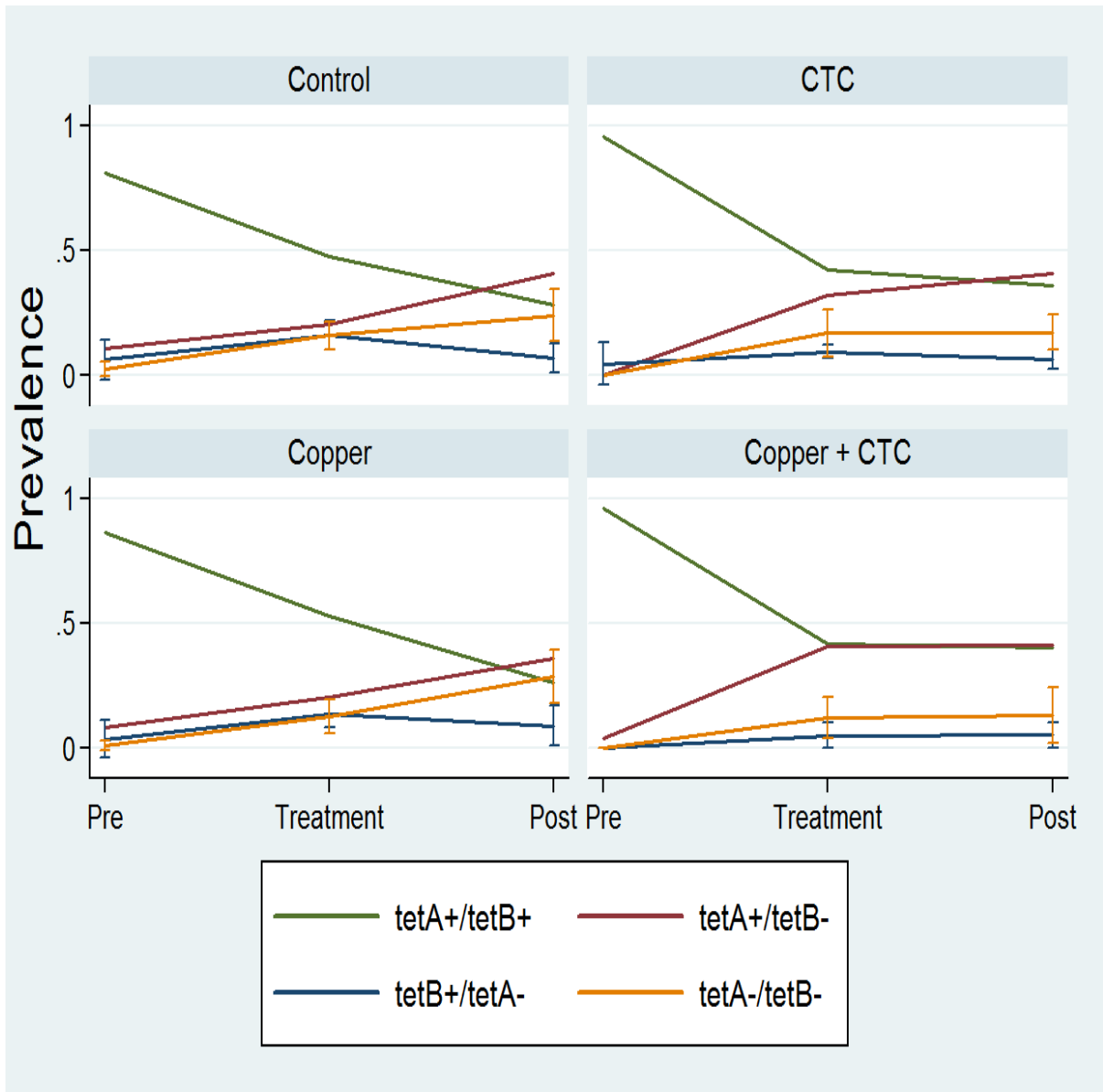
Figure 5.3. Bivariate distribution of *tet*A and *tet*B detection from fecal samples (n = 569) of pigs supplemented with chlortetracycline (CTC), copper or their combination by treatment period.
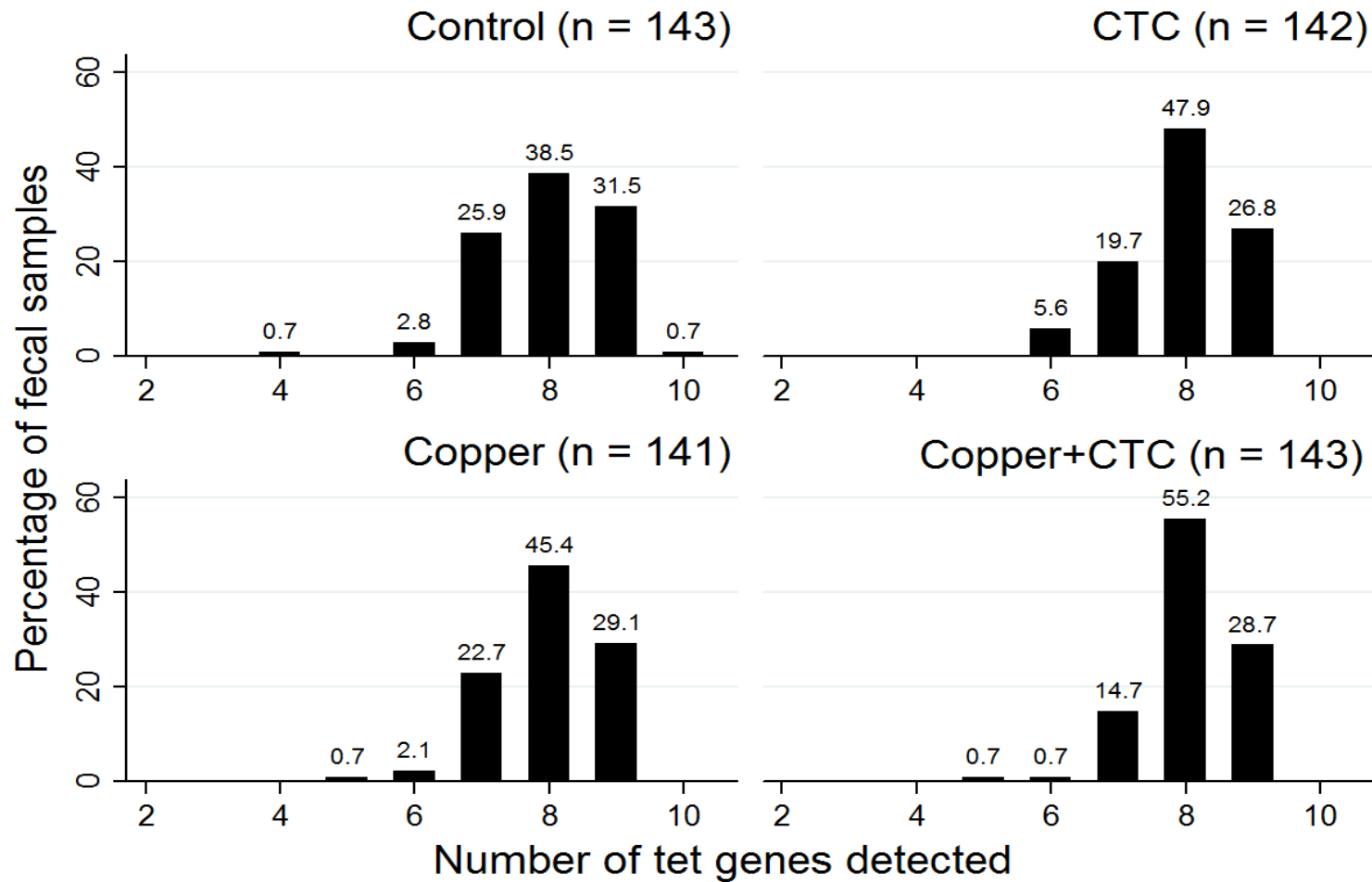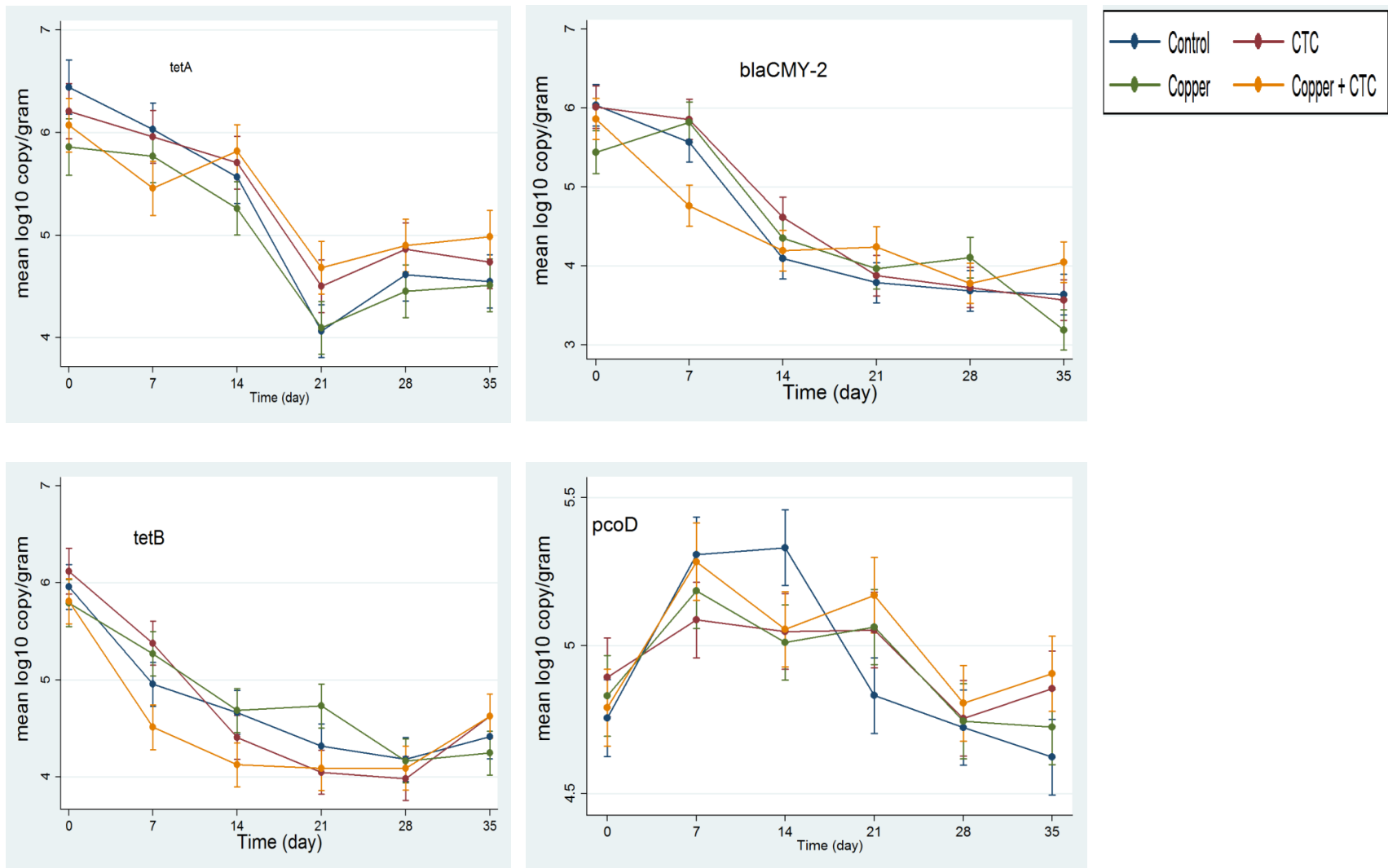
Figure 5.4. Bar graph showing the percentage of fecal samples plotted against the number of tetracycline resistance genes detected from a single fecal sample from pigs supplemented with chlortetracycline (CTC), copper, both or neither.

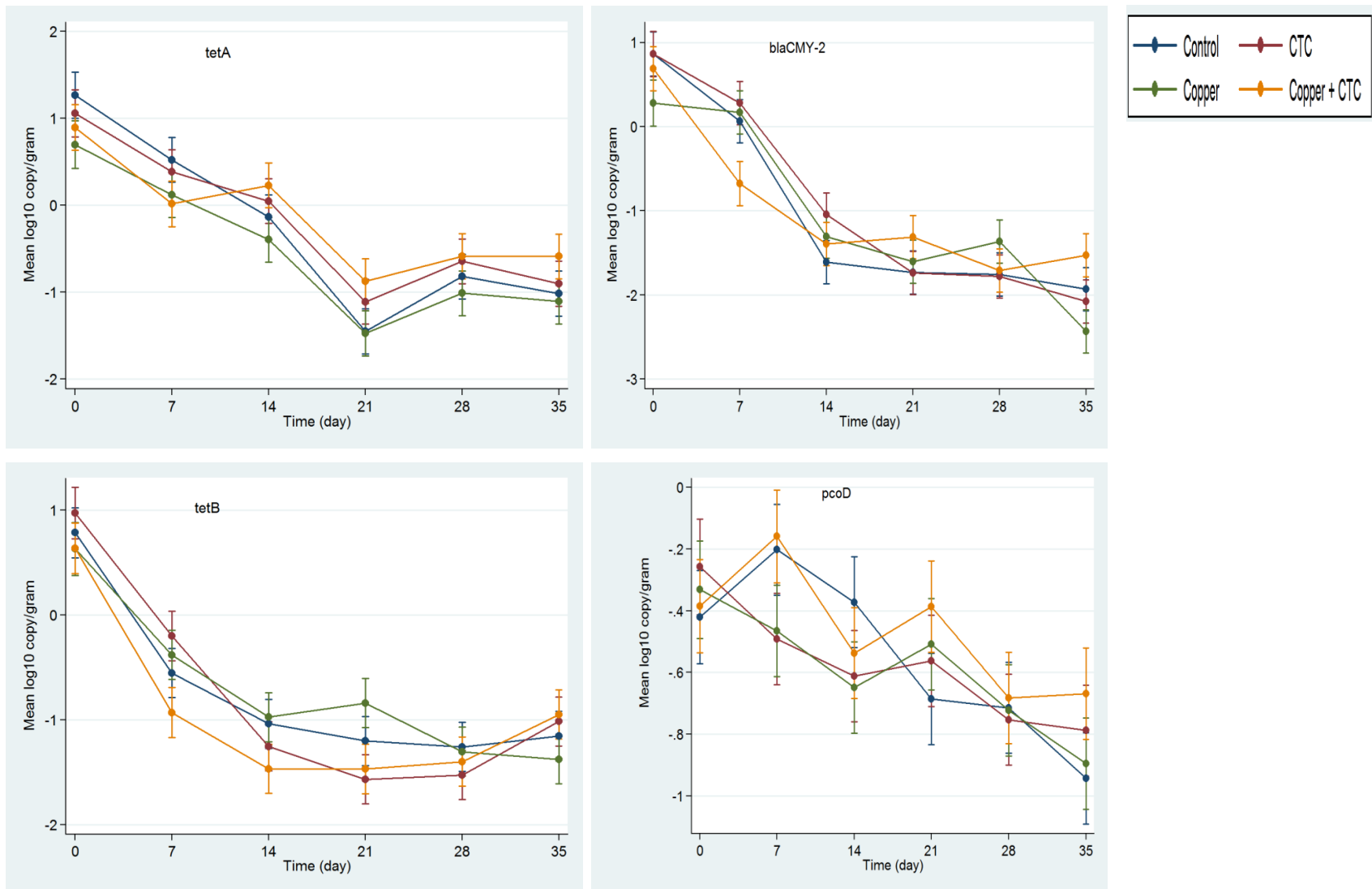**Figure 5.5. Non-standardized mean $\log_{10}$ copies of resistance genes from total community DNA obtained from fecal samples (n = 569) of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither over treatment period.**

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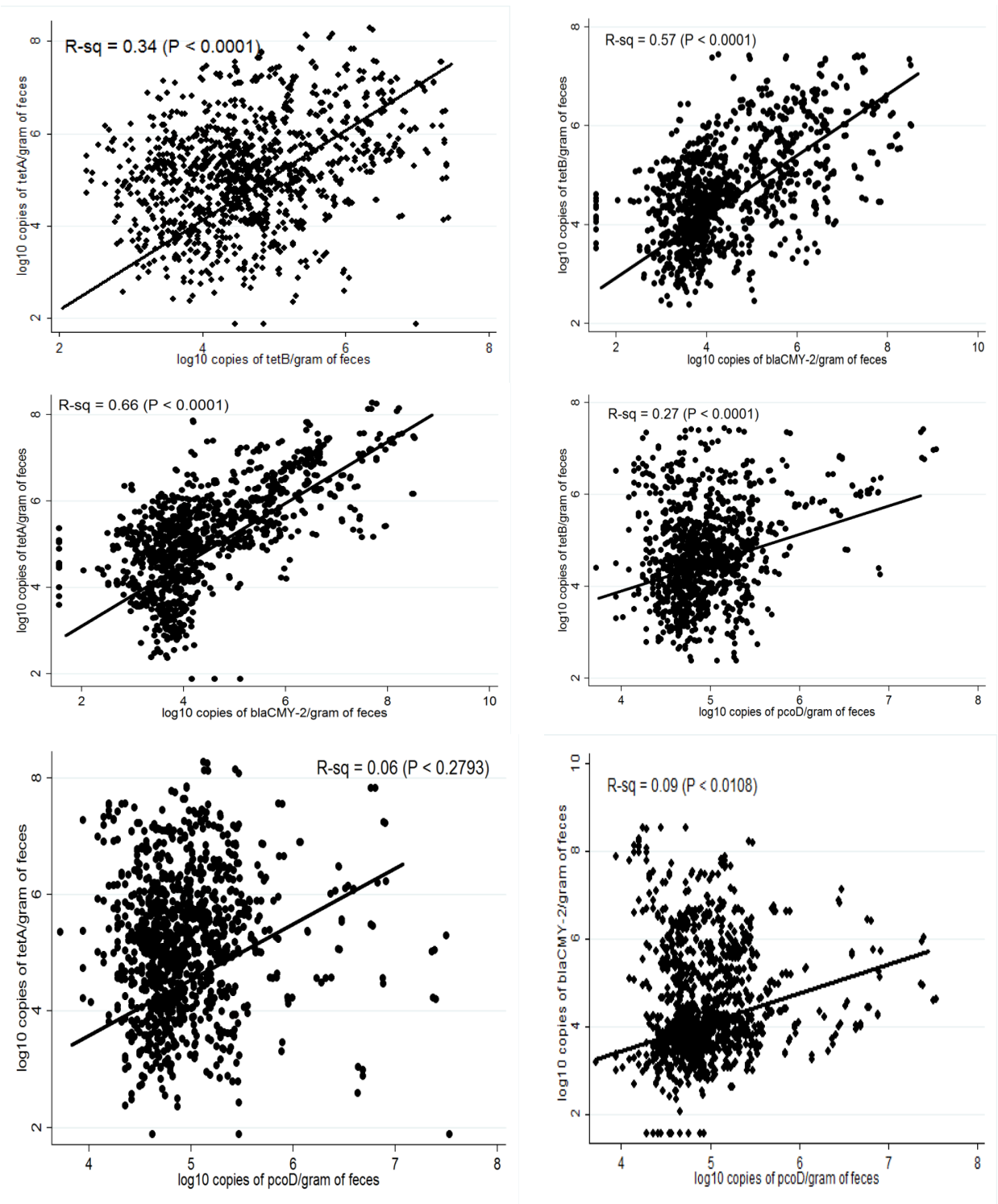
Figure 5.6. Pairwise correlations between resistance genes quantified from fecal samples of weaned pigs receiving copper, chlortetracycline, both or neither.

Figure 5.7. Mean log$_{10}$ copies of resistance genes standardized to initial total DNA concentration obtained from fecal samples (n = 569) of weaned pigs supplemented with chlortetracycline (CTC), copper, both or neither over treatment period.
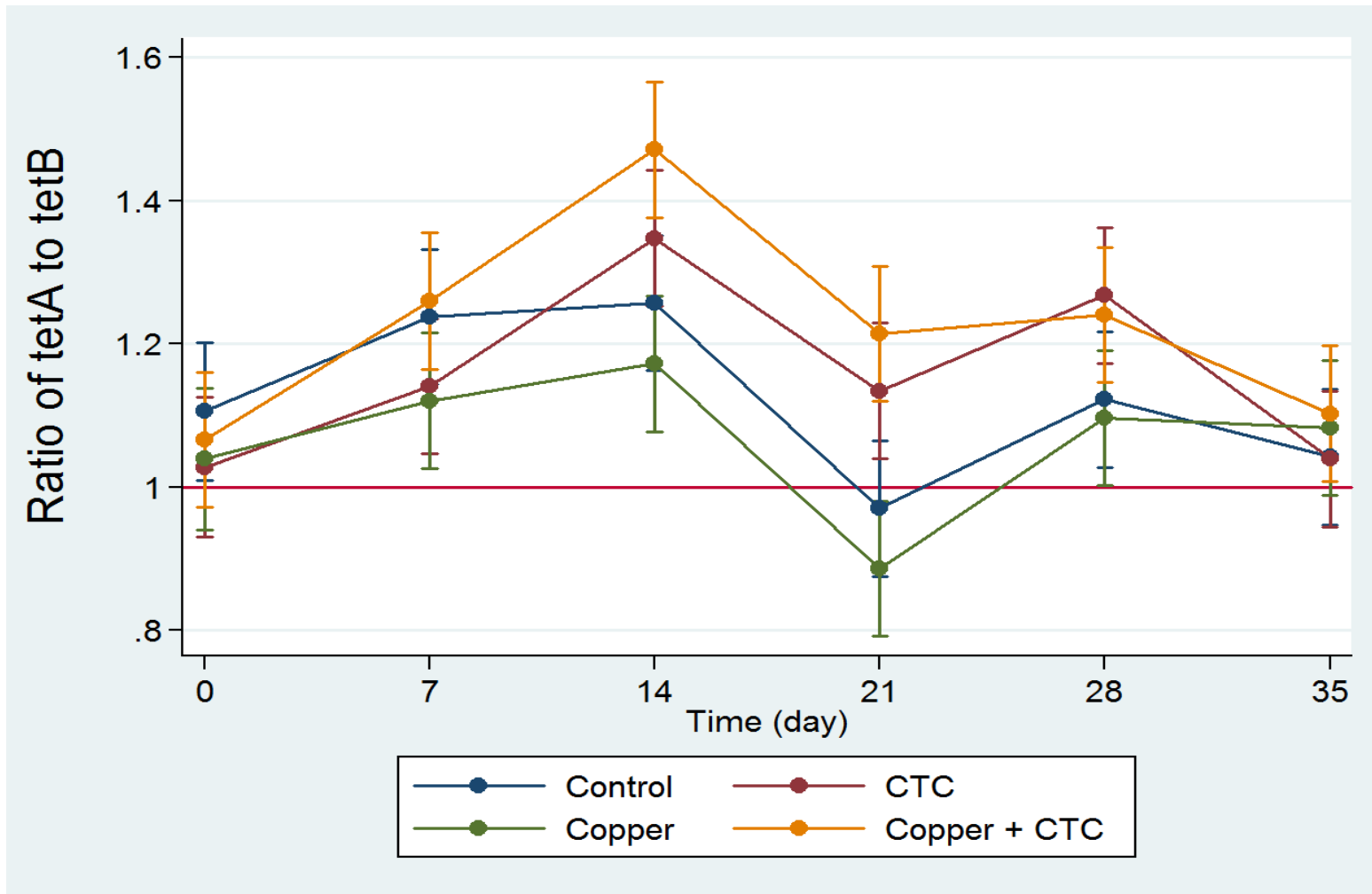
Figure 5.8. Ratios of *tet*A to *tet*B genes quantified from the fecal samples of weaned pigs supplemented with chlortetracycline, copper, both or neither over the treatment period.

The horizontal line serves as reference at ratio of 1.

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Table 5.1. Primers used in multiplex PCR assays for the detection of *tet* genes from fecal samples (n = 569) of weaned pigs supplemented with chlortetracycline, copper both or neither

Multiplex group	Resistance gene	Resistance mechanism	Primer sequence (5'-3')	Amplicon size (bp)	Genebank accession No.
I	*tet*B	Efflux pump	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659	X61367
	*tet*C	Efflux pump	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418	J01830
	*tet*D	Efflux pump	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787	J01749
II	*tet*A	Efflux pump	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210	L06798
	*tet*E	Efflux pump	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278	L06940
	*tet*G	Efflux pump	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	844	S52437
	*tet*K	Efflux pump	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169	S67449
III	*tet*L	Efflux pump	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267	U17153
	*tet*M	Ribosomal protection	CTG TTG AAC CGA GTA AAC CT GCA CTA ATC ACT TCC ATT TG	406	X90939
	*tet*O	Ribosomal protection	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515	Y07780
	*tet*S	Ribosomal protection	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667	X92946
IV	*tetA*(P)	Efflux pump	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	676	L20800
	*tet*Q	Ribosomal protection	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	904	X58717
	*tet*X	Enzymatic inactivation	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	468	M37699

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Table 5.2. Primers used for the quantification of resistance genes from fecal samples of weaned pigs supplemented with chlortetracycline, copper, both or neither

	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
*tet*A	*tet*A-F	GCT ACA TCC TGC TTG CCT TC		(194)
	*tet*A-R	CAT AGA TCG CCG TGA AGA GG	210	
*tet*B	*tet*B-F1	CAG CAA GTG CGC TTT GGA TGC TG		
	*tet*B-R1	TGA GGT GGT ATC GGC AAT GA	101	(153)
	585F	CAG ACG CGT CCT GCA ACC ATT AAA		
*bla*CMY-2[a]	1038R	TAC GTA GCT GCC AAA TCC ACC AGT	454	(154)
*pco*D[b]	*pco*D-F	ATC AGCA GGC AGG ACA ATA C		This study
	*pco*D-R	CTG ATG TGG GTA TTA GCT GGA TT	103	

[a]Positive *E. coli* strain for *bla*CMY-2 was obtained from University of Illinois (Odeh et al., 2002 (203)).

[b]Positive *E. coli* with pRJ1004 for *pco*D was obtained from Dr. Henrik Hasman (the National Food Institute, Technical University of Denmark).

Table 5.3. Prevalence (95% CI) of the various *tet* genes detected by multiplex PCR from total community DNA obtained from fecal samples (n = 569) of weaned pigs fed diets supplemented with chlortetracycline (CTC), copper, both or neither

tet genes	Treatment group	Treatment period[a]			
		Before (n = 24)[b]	During (n = 72)[b]	After (n = 48)[b]	P-value[c]
*tet*A	Control	91.3 (72.0–98.9)	68.1 (56.0–78.6)	68.8 (53.7–81.3)	0.071
	CTC	95.5 (77.2– 99.9)	73.6 (61.9–83.3)	77.1 (62.7–88.0)	0.082
	Copper	95.2 (76.2–99.9)	73.6 (61.9–83.3)	62.5 (47.4–76.0)	0.011
	Copper + CTC	100 (85.8–100)	83.1 (72.3–91.0)	81.3 (67.4–91.1)	0.053
	P-value [d]	0.591	0.206	0.164	
*tet*B	Control	87.0 (66.4–97.2)	63.9 (51.7–74.9)	35.4 (22.2–50.5)	< 0.001
	CTC	100 (84.6–100)	51.4 (39.3–63.3)	41.7 (27.6–56.8)	< 0.001
	Copper	90.5 (69.6–98.8)	66.7 (54.6–77.3)	35.4 (22.2–50.5)	< 0.001
	Copper + CTC	95.8 (78.9–99.9)	46.5 (34.5–58.7)	45.8 (31.4–60.8)	< 0.001
	P-value[d]	0.326	0.040	0.667	
*tet*C	Control	95.7 (78.1–99.9)	100 (95.0–100)	100 (92.6–100)	0.161
	CTC	100 (84.6–100)	95.8 (88.3–99.1)	100 (92.6–100)	0.401
	Copper	85.7 (63.7–97.0)	100 (95.0–100)	100 (92.6–100)	0.003
	Copper + CTC	95.8 (78.9–99.9)	95.8 (88.1 –99.1)	100 (92.6–100)	0.381
	P- value[d]	0.211	0.073	–	
*tet*L	Control	95.7 (78.1–99.9)	100 (95.0–100)	100 (92.6–100)	0.161
	CTC	100 (84.6–100)	98.6 (92.5–100)	100 (92.6–100)	1.00
	Copper	100 (83.9–100)	100 (95.0–100)	100 (92.6–100)	–
	Copper + CTC	100 (85.8–100)	100 (94.9–100)	100 (92.6–100)	–
	P-value[d]	0.733	1.00	–	
*tet*M	Control	100 (85.2–100)	100 (95.0–100)	97.9 (88.9–99.9)	0.497
	CTC	100 (84.6–100)	100 (95.0–100)	97.9 (88.9–99.9)	0.493
	Copper	100 (83.9–100)	97.2 (90.3–99.7)	100 (92.6–100)	0.650
	Copper + CTC	100 (85.8–100)	95.8 (88.1–99.1)	97.9 (88.9–99.9)	0.661
	P-value	–	0.087	1.00	
*tet*A(P)	Control	39.1 (19.7–61.5)	59.7 (47.5–71.1)	95.8 (85.7–99.5)	< 0.001
	CTC	31.8 (13.9–54.9)	66.7 (54.6–77.3)	79.2 (65.0–89.5)	0.001
	Copper	14.3 (3.0–36.3)	73.6 (61.9–83.3)	89.6 (77.3–96.5)	< 0.001
	Copper + CTC	37.5 (18.8–59.4)	85.9 (75.6–93.0)	81.3 (67.4–91.1)	< 0.001
	P-value[d]	0.252	0.003	0.050	

[a]P-values are based on Fisher's exact test or LR as appropriate. 95% confidence interval (CI) is an exact confidence interval based on binomial distribution.

[b]Number of fecal samples tested per treatment group. Ranged from 22–24 (pretreatment period), 71–72 (treatment period), and was constant at 48 post-treatment period.

[c]LR χ^2 p-value with 2 d.f. comparing treatment periods within treatment group.

[d]LR χ^2 p-value with 3 d.f. comparing treatment groups within treatment period.

Table 5.4. Frequency distribution of *tet* genes detected from fecal samples of pigs supplemented with chlortetracycline (CTC), copper, both or neither

Genotypic profiles[a]	Control[b] (n = 143)	CTC (n = 142)	Copper (n = 141)	Copper + CTC (n = 143)	Total (n = 569)
a_b_c_e_l_m_o_p_q_x	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_b_c_e_l_m_o_q_x	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_b_c_g_l_m_o_q_x	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
a_b_c_l_m_o_p_q_x	43 (30.1)	38 (26.8)	40 (28.4)	41 (28.7)	162 (28.5)
a_b_c_l_m_o_q_x	24 (16.8)	27 (19)	26 (18.4)	27 (18.9)	104 (18.3)
a_b_c_l_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
a_b_c_l_o_q_x_	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.2)
a_b_c_m_o_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
a_b_l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	2 (1.4)	3 (0.5)
a_b_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
a_c_g_l_m_o_p_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
a_c_l_m_o_p_q_x_	24 (16.8)	32 (22.5)	26 (18.4)	44 (30.8)	126 (22.1)
a_c_l_m_o_q_x_	9 (6.3)	10 (7)	7 (5)	4 (2.8)	30 (5.3)
a_c_l_o_p_q_x_	0 (0)	0 (0)	1 (0.7)	2 (1.4)	3 (0.5)
a_l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	1 (0.7)	2 (0.4)
a_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
b_c_l_m_o_p_q_x_	7 (4.9)	7 (4.9)	12 (8.5)	6 (4.2)	32 (5.6)
b_c_l_m_o_q_x_	7 (4.9)	4 (2.8)	3 (2.1)	1 (0.7)	15 (2.6)
b_l_m_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
c_l_m_o_p_q_x_	21 (14.7)	12 (8.5)	20 (14.2)	12 (8.4)	65 (11.4)
c_l_m_o_q_x_	3 (2.1)	7 (4.9)	1 (0.7)	1 (0.7)	12 (2.1)
c_l_o_p_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)
c_l_o_q_x_	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.2)
l_m_o_p_q_x_	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.2)
l_o_p_q_x_	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.2)
m_o_q_x_	1 (0.7)	0 (0)	0 (0)	0 (0)	1 (0.2)

[a]Lowercase letters represent the different *tet* genes (i.e., *tet*A, B, C, E, G, L, M, O, P, Q, X).

[b]The distribution of the genes is given as the frequency (percentage calculated from the total number of fecal samples tested per treatment group).

Chapter 6 - Summary and conclusions

Antimicrobial resistance (AMR) creates a challenge for the treatment of bacterial infections in both human and veterinary medicine; further, it is a global problem in both developed and developing countries alike. The epidemiology of AMR is complex and clearly calls for collaborative approaches among medical, veterinary and public health agencies in order to effectively address the issue. Thus, AMR truly represents a "One Health" problem. The magnitude of the AMR problem is directly related to the use of antibiotics for any purpose (whether therapy, prophylaxis, metaphylaxis or growth promotion). The use of antibiotics forms an integral part of livestock production: increased productivity, improved animal health, enhanced food safety and protection of the public health and the environment.

Paradoxically, such uses of antibiotics in agriculture can create a breeding ground for antibiotic resistant bacteria that can be disseminated in the environment and which can be further transferred to humans through the food chain or via direct contact with carrier animals. The use of in-feed antibiotics for growth promotion in animals is of particularly great concern. The use of antibiotics for growth promotion has been completely banned from EU countries and is increasingly being scrutinized in the United States (75). Thus, alternatives to antibiotics such as the use of heavy metals (copper and zinc) are actively being researched at this time. Previous reports have shown that the transferable copper resistance determinant (*tcr*B) in *Enterococcus* is linked with macrolide and glycopeptide resistance (41). We therefore hypothesized that experimental supplementation of copper either alone or in combination with CTC may also be associated with other antimicrobial resistances of *E. coli* in nursery pigs.

This PhD dissertation was aimed at experimentally investigating the impacts of supplementing feed-grade chlortetracycline (CTC) and elevated levels of copper in weaned pigs

on the AMR of the gut microbial flora. To quantify the effects, two approaches – culture dependent and culture independent methods – were used. For the culture based approach, *E. coli* isolates were derived from fecal samples and characterized for phenotypic resistance to 15 antibiotics and copper; and for genotypic resistance to 4 tetracycline (*tet*A, B, C and E), one plasmid borne copper resistance (*pco*D) and one ceftiofur (bla_{CMY-2}) resistance gene. The culture independent approach was based on the qualitative detection of 14 *tet* genes and quantification of the gene copies of each of *tet*A, *tet*B, *pco*D and bla_{CMY-2} arising from the fecal metagenome.

The phenotypic susceptibility results indicated that *E. coli* isolates were highly resistant to most of the antibiotics studied before the start of the experiment, and throughout the trial in the control pigs which were not exposed to antibiotics during the experiment. Resistance to antibiotics that have been used for a great many years (e.g., ampicillin, amoxicillin/clavulanic acid, sulfisoxazole, streptomycin and tetracycline) ranged from 58% (streptomycin) to 98% (tetracycline) before the start of the experiment. More pressing, was the observation that resistance to the relatively new antibiotics of the cephalosporin classes (cefoxitin, ceftriaxone and ceftiofur) was also similarly high (above 67% resistance) even in the absence of direct selective pressure from this class of antibiotics during the experiment. This high resistance, both to the older and relatively newer antibiotics, could be a result of co-and cross-resistance leading to multidrug resistance (MDR).

The *E. coli* isolates were of especially high counts of MDR: most of the isolates being resistant to 7 (out of 8) different antimicrobial classes. Tetracycline and ceftiofur resistances were significantly associated with each other, both phenotypically and genotypically indicating genetic linkage for co-selection. The high AMR observed in this study could be reflective of the unknown antibiotic use history of the breeder farm from which the experimental pigs were

acquired, or more likely could be associated with widespread dissemination of resistance determinants following the use of antibiotics for over 60 years, and especially evident in very young livestock. Though reducing unnecessary use of antibiotics, especially in the feed, would likely somewhat decrease the magnitude of AMR in pig production thus increase public confidence, it will not result in the elimination of the AMR problem. The use of antibiotic growth promotion (AGPs) in pig farms should be justified by evidence-based decisions. Recent studies have shown that in-feed antibiotics do not always significantly improve the growth of pigs under current pig production systems (73, 74). Existing evidence shows that the effect of in-feed antibiotics is the highest in the weaned pigs and thereafter decreases as pigs get older. Therefore, there is room to reduce antibiotic use by limiting the use of in-feed antibiotics to those in the early production phase, and only if their benefit is scientifically justified (9, 40, 71-73).

CTC supplementation was associated with increased phenotypic and genotypic resistance of *E. coli* to tetracycline, even beyond the already very high background levels of resistance. This increase in tetracycline resistance was usually mediated through *tet*B gene since CTC supplementation was also associated with an increased detection of *tet*B and with little or no effect on *tet*A. *tet*B also confers higher levels of tetracycline resistance than *tet*A (as measured via MIC distribution) and gene substitution at the population (metagenomic) level was observed as *tet*B detection increased over time while *tet*A decreased. Copper supplementation also increased *tet*B detection while paradoxically the combined supplementation of copper and CTC appeared to decrease its probability of detection. CTC supplementation was associated with increased MDR counts. At the genetic level, this association was largely due to *tet*A. *tet*A and bla_{CMY-2} genes were positively associated with each other reflecting their co-selection. These genes were significantly associated with higher counts of MDR. Of particular interest,

*tet*B and *pco*D were positively associated with each other and negatively associated with *tet*A and *bla*$_{CMY-2}$; furthermore, they were associated with lower MDR counts. It is possible that the use of low levels of CTC in the pig diet as AGP may well select for higher MDR by favoring co-selection of *tet*A with *bla*$_{CMY-2}$ gene.

Copper supplementation was associated with reduced AMR to most of the antibiotics, including the cephalosporins, over the treatment periods. Furthermore, *E. coli* from the CTC supplemented group exhibited modestly increased susceptibility to copper. Surprisingly, neither the distribution of copper MIC, nor the probability of detection of the *pco*D gene, was meaningfully affected by the level of copper supplementation in the experimental diet. This suggests that the *E. coli* population did not rely on acquired copper resistance that is mediated by *pco* gene in order to survive and proliferate in the presence of elevated copper supplementation. This could be due to the fact that either the level of copper used (125 ppm) in the experiment was not sufficient to favor any such acquired resistance, or it could reflect the generalized adaptability and tolerance of *E. coli* and other Enterobacteriaceae to high levels of copper. The findings could be also as result of the presence of other unknown mechanisms, besides the *pco* determinant, encoding for copper resistance. The presence of multiple chromosomally mediated intrinsic mechanisms of intracellular copper regulation in *E. coli* makes it particularly challenging for conducting epidemiological and other field-based studies. *In vitro* findings often do not translate well into *in vivo* findings, with even further difficulty in interpretation when taken further afield. It was reported elsewhere (156) that the Gram negative enteric bacteria (e.g., *Salmonella* and *E. coli*) were more tolerant to copper than their Gram positive counterparts (e.g., enterococci and staphylococci).

The fecal metagenome held a diverse set of tetracycline resistance (*tet*) genes, with 11 (out of 14) *tet* genes being detected from at least one fecal sample and with a median number of 8 *tet* genes detected per fecal sample. This diversity in *tet* genes detected from the fecal samples appeared to decrease over the subsequent treatment periods. Most of the *tet* genes were found in high prevalence. The prevalence of the Gram negative associated *tet* genes *tet*A and *tet*B (as opposed to the isolate based approach) significantly decreased over the treatment period, whereas that of the predominantly Gram positive associated *tet* genes *tet*C and *tet*P significantly increased. This finding could be explained by the gradual substitution of tetracycline resistant Gram negative bacteria by tetracycline resistant Gram positive bacteria in the gut microbial ecology of the pigs as they aged. CTC supplementation was associated with an increased detection of *tet*A, but with no apparent effect on *tet*B. This was in contrast to what was observed in the *E. coli* isolate analysis where CTC supplementation resulted in increased *tet*B detection in *E. coli*, with no apparent effect on *tet*A detection. As expected, the prevalences of *tet*A (77%) and *tet*B (57%) detected from the fecal metagenome were significantly higher compared to their prevalence from *E. coli* (66% and 48% respectively).

The mean $\log_{10}$ copies of *tet*A, *tet*B and *bla*$_{CMY-2}$ genes decreased over time while that of *pco*D increased during the first week of the experiment and later gradually decreased. Most likely this reflects the aging effect of the pigs and represents a gradual change in the microbial composition of the gut, with predominantly Gram positive bacteria expanding their dominance, since the four genes studied quantitatively largely confer resistance to Gram negative bacteria. In support of this hypothesis, we observed a decrease in the prevalence of Gram negative *tet* genes (*tet*A and *tet*B) while Gram positive *tet* genes were increased or else remained stable at a higher prevalence among the fecal samples. Similar to the isolate based approach, copper

supplementation tended to decrease the abundance of *tet*A while increasing that of *tet*B gene copies. CTC supplementation was associated with increased gene copies of *tet*A and bla_{CMY-2} as opposed to the isolate based finding. Similar to isolate based findings, gene copies of *pco*D were significantly higher in the control group when compared to the treatment groups, which remains inexplicable given its purported function.

Table 6.1 summarizes the resistance genes and their associations based on both isolate and metagenome based approaches across all *E. coli* isolates and fecal samples studied. *tet*A and *tet*B, either alone or in combination, were detected in 98% of phenotypically tetracycline resistant isolates. Similarly 97% of the *E. coli* isolates that were phenotypically ceftiofur resistant were also positive to the bla_{CMY-2} gene. In conclusion, antimicrobial resistance (both in isolate based and metagenomic approaches) reflecting the gut microbial ecology of the weaned pigs studied was very high at the baseline which then gradually decreased over time. Copper supplementation seemed on the surface to be a promising alternative to antibiotics since it was associated with decreased AMR, including to the critically important cephalosporins. However, the roles of copper supplementation and the *pco* gene, in particular, on the gut microbial ecology of pigs should be further studied under both laboratory and field conditions.

Table 6.1. Summary and comparison of the resistance genes based on isolate and metagenome approaches across all the *E. coli* isolates and fecal samples tested

Antimicrobial	Number (%) resistant	Gene	*E. coli* (n = 1,152)		Metagenome (n = 569)	
			Prevalence (%)[a] n = 1,152	% Phenotypically resistant[a]	Prevalence (%)[a] n = 569	Mean $\log_{10}$ gene copies/g[b]
Tetracycline	1,119 (97%)	*tet*A	66 (64-69)	67 (65-70)	77 (74-81)	5.2 (5.1-5.3)
		*tet*B	48 (45-51)	49 (46-52)	57 (53-61)	4.7 (4.6-4.8)
Ceftiofur	746 (65%)	*bla*CMY-2	72 (69-75)	97 (95-98)	N/A[d]	4.5 (4.4-4.6)
Copper[c]	1,076 (93%)	*pco*D	16 (14-18)	16 (14-18)	N/A[d]	5.0 (4.9-5.0)

[a] Values were expressed as percentage (95% confidence interval, CI) prevalence or percentage resistant

[b] Values were expressed as mean $\log_{10}$ gene (95% CI) copies/g of feces

[c] 18 mM concentration of copper was used as a non-standardized breakpoint

[d] N/A= Not tested

Chapter 7- References

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