

SELECTION AND CO-SELECTION OF ANTIMICROBIAL
RESISTANCE IN GUT ENTEROCOCCI OF SWINE AND
CATTLE FED DIETS SUPPLEMENTED WITH COPPER,
TYLOSIN, AND CHLORTETRACYCLINE

By

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AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Abstract

Copper, as copper sulfate, is often used as an alternative to in-feed antibiotics for growth promotion in both swine and cattle diets. Gut bacteria exposed to copper can acquire resistance, which among enterococci is conferred by a plasmid-borne transferable copper resistance gene (*tcrB*). The plasmid also carries tetracycline [*tet*(M)] and macrolide [*erm*(B)] resistance genes. Because of the genetic link between acquired copper (*tcrB*) and antibiotic resistance in *Enterococcus* spp., we hypothesized that copper supplementation may exert selection pressure for enterococci to become resistant to macrolides and tetracyclines, and possibly to other antibiotics. We conducted studies in cattle and swine to investigate the relationship between copper supplementation and the fecal prevalence of *tcrB*-positive enterococci, as well as its potential co-selection for macrolide and tetracycline resistance. The prevalence was higher in animals fed diets supplemented with elevated level of copper compared to normal level ($P < 0.05$). The *tcrB*-positive isolates belonged to either *E. faecium* or *E. faecalis*; the majority was *E. faecium*. All *tcrB*-positive isolates also contained both *erm*(B) and *tet*(M) genes; however, none of them harbored the *vanA* gene. Median minimum inhibitory concentrations (MIC) of copper for *tcrB*-positive and *tcrB*-negative enterococci were 22 mM and 4 mM, respectively ($P < 0.0001$). The overall prevalence of *erm*(B) and *tet*(M) genes among enterococcal isolates of cattle were 46.8 % and 57.5%, respectively; in contrast, 100% of the swine isolates were positive for both *erm*(B) and *tet*(M) genes. The transferability of the *tcrB* gene was demonstrated by filter mating assay. Multi-locus variable number tandem repeat analysis revealed a genetically diverse population of enterococci. The finding of a strong association between the copper resistance gene and other antibiotic (tetracycline and tylosin) resistance determinants is

significant because enterococci are potential pathogens and have the propensity to transfer resistance genes to other bacteria in the gut. The occurrence of vancomycin resistant enterococci in swine in the US is very rare. Strains of *E. faecium* positive for *vanA*, that confers resistance to vancomycin, were isolated and characterized from swine feces. The swine strains belonged to clonal complex 17, a well-adapted hospital clone throughout the world.

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Acknowledgements

First, I would like to take this opportunity to thank my advisor, Dr. H. Morgan Scott, for his vision and determination, which brought this project into reality. He challenged me throughout the duration of my studies, and as a result, I have a much broader understanding of the concepts of statistics, ecology, and epidemiology and the form and structure that scientific literature should take. I would like to thank him for his erudite guidance, constant support, encouragement, constructive criticism, and the intellectual freedom that he allowed me in day-to-day work. I always look up to him for his industry and perseverance. I have learnt a lot from him both professionally and personally. I am greatly indebted to him forever.

I also take this opportunity to thank my beloved department head, Dr. M. M. Chengappa. He was instrumental in guiding me through the tough phase of my career, both personally and professionally, and without his moral support and valuable guidance this work would not have seen the light of the day. I am always indebted to him for providing intellectually stimulating and personally gratifying scientific environment. He always supported my efforts, strengthened my resolve and congratulated me on my accomplishments.

My sincere appreciation and thanks to Dr. T. G. Nagaraja. Words cannot express my gratitude for the professional and personal guidance I have received from him. He was always available to discuss any questions I had and to resolve any problems that came my way. He also taught me how to successfully navigate the world of scientific journals and peer review-based publishing. I am truly grateful and fortunate to have had such an accomplished academic professor as my mentor. His infinite patience, encouragement, support, and optimism are appreciated beyond words. Keep your interest broad, remain diligent in reading the literature,

and be tenacious at the laboratory bench, are the guiding principles I imbibed from Dr. T. G. Nagaraja.

I would also like to express my gratitude to my committee members, Dr. Mike Tokach, and Dr. Lynn Hancock, who have always been there to advise me and answer my queries. Their support and commitment to my graduate work is appreciated. As in all scientific endeavors, nothing great can ever be accomplished without the support and ideas of wonderful collaborators; therefore, I would like to thank my collaborators, Dr. Jim Nelssen, and Dr. Steve Dritz, for their support and involvement in my research. The success of these projects was made possible by working closely and collaborating with such a great group of colleagues.

These projects could not have been completed without the help of several people. My heartfelt thanks to Tiffany, Melody, Debra, Kevin, Eunice, Josh, Jasmine, Brittany, Nick, Celine, Lia, Flavia, Cadra, Christian, students of the KSU Swine teaching and research unit and KSU Beef cattle research center, for their unending assistance and technical support in the field as well as in the lab. I wish to acknowledge Javier Vinasco, Neil Wallace, and Mal Hoover for their constant support, sharing ideas, technical assistance, and for their valuable contribution towards my graduate research. There are ups and downs, turns and twists, and plenty of ruts and bumps along the way. Therefore, I would like to express special thanks to the many people who helped me to navigate all the obstacles, ensuring that I would cross the finish line. I am always indebted to them forever.

Dedication

The road that I have taken which has brought me to this stage has involved some straightforward decisions and some quirky coincidences. Through it all, I have had the support of my family, friends, and colleagues, and I would like to dedicate my work to some of those people here.

- 1) Beloved family members; M. B. Jayashree and Gurusiddashetty, Dr. B. S. Krishna Prasad and Kusuma Prasad, and my wife, Amoolya Prasad for their love, understanding, support, and sacrifice.
- 2) Dr. B. C. Ramakrishna
- 3) Shekar Jannah and Barbara Jannah
- 4) G. Shivamallu and Nagalambike
- 5) Dr. H. M. Nanjappa, Dr. Ramesh Rao, and Muralidhara

The faith and support that my family and friends have provided has been an integral to reaching my goals, I could not have accomplished all of this without them.

CHAPTER 1 - Antimicrobial Agents in Food Animal Agriculture: Use, Resistance, and Alternatives in Swine and Cattle

Introduction

Antimicrobial agents have been widely used both in human and veterinary medicine for more than 60 years in the United States as well as in other countries. Since that time, food animal production has increasingly included larger farms and greater animal densities, requiring a greater need for disease management (Mathew et al., 2007). Food animal production often involves intensive optimization in every step of the production process to maximize the efficiency of production (Aarestrup and Jensen, 2007). The early indications of antimicrobials beneficial effects are on production efficiency in poultry and swine were reported by Moore et al. (1946) and Jukes et al. (1950). Improved health management in modern livestock production has occurred, in part, due to the use of antimicrobial products (NRC, 1999). The use of antimicrobial compounds in food animal production provides demonstrated benefits, including improved animal health, higher production, and reduction in food borne pathogens in some cases (Mathew et al., 2007). Agricultural use of antibiotics accounts for at least half of antibiotics produced in the United States (CSPI, 2013). Antibiotics or antimicrobials are routinely used in livestock production to treat and prevent diseases. In addition, subtherapeutic concentrations of antimicrobials are commonly used as growth promoters in feed and drinking water (Chee-Sanford et al., 2009). In food animal production, the antimicrobial agents are used in one of the four different ways (Aarestrup, 2005).

1. Therapy: Treatment of infectious and clinically sick animals, preferably with a bacteriologic diagnosis.

2. Metaphylactics: Treatment of clinically healthy animals belonging to the same flock or pen with animals with clinical symptoms.
3. Prophylactics: Treatment of healthy animals to prevent disease in a period where they are stressed.
4. Growth promotion: Continuous inclusion of antimicrobial agents in the feed to improve growth.

The use of antimicrobial agents as growth promoters is often justified in a number of different ways, but primarily on economic grounds. Growth promoters are generally inexpensive, safe, and easy to use and they tend to improve the growth performances in a constant manner regardless of the husbandry practices (MacKinnon, 1987).

Antimicrobials and Antimicrobial Resistance

The term ‘antimicrobials’ refers broadly to compounds with activity against microorganisms, i.e., bacteria, viruses, fungi, and protozoa. More often, the term is commonly used in reference to drugs with activity against bacteria (Guidance of Industry # 209; FDA, 2012). Antimicrobials include both naturally derived and many synthetic agents that are active against a broad range of microbes (from viruses to parasites). Antibiotics, which are a subset of antimicrobials, are derived from a microbial source (e.g., fungi or bacteria) and are active against various classes of bacteria. Antibiotics include aminoglycosides, beta-lactams (e.g., penicillins and cephalosporins), macrolides, and tetracyclines. Antimicrobials also include other agents such as heavy metals (e.g., copper and zinc), common disinfectants such as quaternary ammonium compounds (QACs), and even lactic acid to name a few examples. Antimicrobial agents are widely employed in food producing animals for growth promotion purposes. The antimicrobial agents meant for production purposes are typically administered via feed or water on a herd or

flock wide basis and are approved for uses such as increasing rate of weight gain or to improve feed efficiency. Antimicrobials meant for ‘production’ purposes are also referred to as ‘nontherapeutic’ or ‘subtherapeutic’ uses. The drugs intended for production purposes are not meant to manage a specific disease that may be ongoing or at risk of occurring (Guidance for Industry # 209; FDA, 2012). Antimicrobials at therapeutic levels are used in food animal agriculture to treat, prevent, and control diseases. Therapeutic use is at a higher dose than “subtherapeutic” purpose and is administered in water or feed or by injection or in feed. The use of antimicrobials in feed is a regulated activity under the Code of Federal Regulations, Title 21, Part 558 (CFR, FDA) accessed at <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=558&showFR=1>. Both prophylactic and growth promotion use of antimicrobials have come under increasing scrutiny in recent years because of concerns over antimicrobial resistance (AMR) among pathogenic bacteria with potential public health consequences. Animal agriculture has been blamed both for the emergence and for the widespread dissemination of antimicrobial-resistant bacteria. The use of antibiotics at sub therapeutic concentrations has been prohibited in European Union countries since 2007, because of concerns about antibiotic resistance (Aarestrup et al., 2008).

Antimicrobial resistance reflects the ability of microbes to resist the effects of a drug, and the manifestation of resistance occurs when there is a change in bacteria, loss in effectiveness, or any other agents to treat bacterial infections (Guidance for Industry # 209; FDA, 2012). In recent years, the spread of antibiotic resistance determinants among pathogenic, commensal, and environmental bacteria has reached a global dimension. The most overt example of evolution driven by selection is the selection for antibiotic resistance in pathogens (Baquero et al., 2011). Commensal bacteria such as, *E. coli* and *Enterococcus* spp. can serve as vectors for the

transmission of resistance genes between environmental and pathogenic microorganisms (Martinez, 2009). The accumulation of antimicrobial resistance genes in pathogens has generated a world-wide crisis in the management of infectious disease (Davies, 2007). Thus, leading to global wide dissemination of antibiotic resistance. Antibiotic resistance continues to become worse, despite the ever-increasing resources devoted to combat the problem (Stokes and Gillings, 2011). Earlier researchers have hypothesized that these antibiotic resistance determinants are already present in the environment in genetic platforms compatible with both commensal and pathogenic bacterial pathogens and thus ready to be transferred by horizontal gene transfer under selection pressure of antibiotics and or antimicrobials. Both ecology and evolution play a vital role in linking antibiotic resistance and bacterial virulence (Martinez and Baquero, 2002; Baquero et al., 2011). The emergence of resistance has revealed multiple and complex mechanisms by which resistance genes spread across the bacterial kingdom, with apparent disregard for species barriers. But the bacterial evolutionary response has not been limited to the acquisition of resistance genes. Bacteria have also developed means for stabilizing the resistance phenotype, thus thwarting initial hopes of reversing resistance by simply reducing antibiotic use (Baquero et al., 2011).

Experiences from the ban of antibiotic growth promoters in the EU, and from antibiotic-free/organic production systems in the US, suggest that marked decreases in prevalence of resistance can be expected for some bacteria-antimicrobial combinations (@ H. M. Scott), while others have changed very little (Aarestrup et al., 2001; Bunner et al., 2007). When reductions are seen, they usually take several years to occur; especially with respect to enteric bacteria harboring multiple antimicrobial-resistance determinants. This illustrates the complexity of the antimicrobial resistance in bacteria. Reductions in the use of antimicrobial growth promoters in the EU has increased the search for alternative feed additives, both to improve the feed efficiency

and growth rate in food producing animals and to control subclinical and clinical disease. Finding replacements for antibiotic growth promoters and making key management changes will play a key role in maintaining animal productivity in their absence. It is unlikely that any single replacement will be found that will prove to be as a panacea for all purpose and with no downside. The need for multiple alternative feed additives increases with the phasing out or banning of antibiotic growth promoter use in food producing animals. The main focus of this dissertation is to evaluate the effect of feeding copper and other feed grade antimicrobials on the resistance of *Enterococcus* spp., and its co-selection with other antibiotics within cattle and swine production systems.

Antibiotic Growth Promoters

Antibiotic growth promoter (AGP) is defined as a group of antibiotics which are supplemented via feed at subtherapeutic levels to improve weight gain and feed efficiency in food production animals. Given the antibacterial nature of AGPs and important role of intestinal microbiota in host nutrient assimilation and immune defense, it is widely accepted that the growth promoting effect of AGPs is likely mediated through enhanced energy harvest and the control of gastro intestinal infections due to altered microbiota in the intestine (Dibner and Richards, 2005). However, the precise mechanisms of AGPs are still largely unknown. It has been proposed that AGPs may improve animal growth performance by reducing the maintenance cost of gastrointestinal tract system via reduction of total gut bacteria (Collier et al., 2003; Gaskins et al., 2002) or by reshaping the microbial diversity and relative abundance in the intestine to achieve optimal microbiota for growth (Dibner and Richards, 2005). However, use of AGPs is associated with the emergence of antibiotic resistant bacteria that may be transmitted

to humans, posing a significant threat to public health (Marshall and Levy, 2011). Therefore, development of alternatives to AGPs is an important mitigation strategy for controlling antimicrobial resistance in foodborne pathogens.

Antibiotic Alternatives for Growth Promotion in Animals

The need for alternatives to antimicrobials increased with the phasing out or banning of antimicrobial growth promoters use in food producing animals. To be effective, alternative feed additives should generate similar benefits as the antibiotics which are currently used as growth promoters in swine/cattle production. Some of the commonly used alternatives are as follows:

1. **Probiotics and competitive exclusion:** Probiotics are live cultures of microbes, often lactic acid bacteria but also other bacterial species, which are fed to animals to improve health and growth by altering intestinal microbial balance. Some of the bacterial cultures are used specifically for competitive exclusion, which are bacterial cultures that are allowed to establish in intestinal flora to prevent colonization of pathogenic bacteria (Abe et al., 1995). Probiotic microorganisms, added to the feed, may protect piglets and calves from intestinal pathogens by several possible mechanisms:
 - i. Adherence to intestinal mucosa thereby preventing attachment of pathogens,
 - ii. Production of antimicrobial compounds such as bacteriocins and organic acids,
 - iii. Competition with pathogens for nutrients,
 - iv. Stimulation of intestinal immune responses.
2. **Enzymes:** The addition of enzymes to feed helps to increase its digestibility. Studies reported that average daily growth rate and feed conversion rate increased in piglets significantly (Baidoo et al., 1998) with enzyme supplementation.

3. **Immune modulators:** Immunologically active compounds affect the immune system and enhance host resistance to disease agents. These substances include extrinsic antibodies, cytokines, spray dried plasma, and other compounds. The growth promoting effects of antibodies or other immune active compounds has been reported to be similar to that of feeding subtherapeutic antibiotics (Coffey and Cromwell, 1995; Chae et al., 1999).
4. **Organic acid:** Organic acids are added to the feed to maintain low pH, so that the digestion of proteins and the population of beneficial bacteria are maximized and harmful bacteria are inhibited. Diets fed to piglets often have a high buffering capacity, which can further reduce stomach acidity (Gedek et al., 1992). A study has reported that organic acids also improve the digestibility and absorption of proteins, minerals, and other nutrients in the diet (Overland et al., 2000).
5. **Essential oils:** Essential oils (EO) are aromatic, volatile liquids obtained mainly from plant sources (flowers, buds, seeds, leave, twig, barks, fruits, and roots) that consist of terpenes and phenylpropane derivatives as their principal components (Janczyk et al., 2009). Paracelsus von Hohenheim, Swiss reformer of medicine from 16th century was the first one to name the effective component of drugs *Quinta essential* or ‘essential oil’ (Cho et al., 2006). EO are known to protect plant tissues against bacteria and parasites. The composition of EO may vary depending on the species of plants, geographical origin, and its vegetative stages (Aligiannis et al., 2001; Hudaib et al., 2002). Recently, EO has been promoted as natural feed additives in many parts of the world since the use of antibiotic additives has become increasingly controversial (Yang et al., 2010). EO are considered to be one of the more promising alternatives to in-feed antibiotic growth promoters in food animal production, and especially so in cattle and swine. EO are

known to have myriad of beneficial effects in food animal agriculture such as an increase in feed intake, secretion of digestive enzymes, immune stimulation, antibacterial, antioxidant, antiviral and coccidiostatic properties (Wenk, 2003).

6. **Beta-2- Adrenergic Agonists:** The adrenergic receptors are members of the superfamily of cell surface receptors that carryout signaling via coupling to guanine nucleotide binding proteins (G-proteins). There are two main types of adrenergic receptors; α and β . The receptors have two different subtypes, $\alpha 1$ and $\alpha 2$, whereas the β receptors have three different subtypes, $\beta 1$, $\beta 2$, and $\beta 3$. The $\beta 2$ receptor is mainly involved in the anabolism in skeletal muscle and lipolysis in adipose tissue (Lynch et al., 1994; Small et al., 2003). The efficiency and scale of conventional food animal production has been greatly enhanced through the usage of growth promoting agents such as feed grade antibiotics (Stock et al., 1995). One alternative to antibiotics as growth promoters is the class of beta-2-adrenergic agonists (BAA). BAA, also known as phenethanolamines, are compounds similar in structure to naturally occurring catecholamines such as dopamine, norepinephrine, and epinephrine (NRC, 1994; Bell et al., 1998; Scramlin et al., 2010). Ractopamine hydrochloride (RAC) is a Category I BAA which is commonly employed in food animals to improve growth performance and carcass characteristics (Pringle et al., 1993; Crome et al., 1996) by augmenting protein synthesis. RAC is known to improve growth performance, dressing percent, and carcass leanness (Gu et al., 1991; Crome et al., 1996). The use of RAC as a means of increasing growth and lean tissue gain is very well documented in swine (Olayiwala et al., 1990; Watkins et al., 1990; See et al., 2004). RAC was later approved for use in cattle in the United States in 2003, and zilpaterol hydrochloride (ZH), another BAA compound, was approved in cattle only in the year 2006 for increased weight gain, improved feed efficiency, and increased carcass leanness

(Avendano-Reyes, 2006) by the US Food and Drug Administration. Zilpaterol hydrochloride is a Category II BAA, which functions through increased protein synthesis and decreasing protein degradation (Mersmann, 1998; Moody et al., 2000).

7. **Other feed supplements:**

- a) **Minerals:** Copper and zinc added to piglet diets have significantly improve average daily weight gain, feed intake, and feed efficiency.
- b) **Vitamins:** Vitamin E supplements are used to prevent weaning diarrhea in pigs (Lamberts, 1997).
- c) **Conjugated linoleic acid:** Conjugated linoleic acid refers to a mixture of isomers of linoleic acid with conjugated double bonds in the position 8-13. Piglets fed diets with 1-3% of conjugated linoleic acid known to increase weight gain (Muller et al., 2000).
- d) **Phospholipids:** Lysoforte, a phospholipid product, significantly improved growth and feed conversion in piglets (Schwarzer & Adams, 1996).
- e) **Amino acids:** Supplementation of limiting amino acids to diets known to improve animal performance (Thomke & Elwinger, 1998).
- f) **Carnitine:** Carnitine is synthesized from lysine and methionine in the body. Carnitine supplementation known to improve daily growth and piglet survival rate (Lobo, 1999).
- g) **Carbohydrates:** Mainly polysaccharides and fibers from the plants or feed known to increase weight gain and feed efficiency. The steam flaking process makes these feed more digestible and there is less non-starch polysaccharide passing through the large intestine (Pluske et al., 1996; Medel et al., 1999)

- h) **Herbs:** Herbs are known to contain compounds with antibacterial effects, and also known to increase the palatability of diets there by increase feed intake. In piglets, oregano and a mixture of Aromex known to increase growth and reduce diarrhea (Mellor, 2000).

Heavy metals

A number of heavy metals (copper and zinc) are used in trace quantities in food animal production to maintain the normal physiology and the health of animals. Resistance to antimicrobials, including heavy metals, is important for bacterial survival in competitive environments such as the gut lumen. Often, heavy metal and antibiotic resistance genes are co-located on the same mobile genetic elements such as plasmids, transposons, and integrons (Aminov and Mackie, 2007). Therefore, there is a distinct possibility that both natural (environmental) and artificial (feed supplementation) selection pressures imposed by heavy metals may indirectly select for resistance to antibiotics.

Metals are integral constituents of the earth's crust. All living organisms have evolved to use some metals, even though they are toxic at relatively low doses. The primary source of heavy metals in all ecosystems is the underlying bedrock of the planet. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals, and the remaining 53 are grouped as heavy metals (Nies, 1999). Heavy metals are metals with a density above 5 g/cm^3 , with most of the heavy metals containing an incompletely filled diffuse (d) orbital, so they can easily give and take electrons. Thus heavy metal cations play an important role as "trace elements" in biological reactions (Nies, 1999). A large number of metals are used in trace amounts in food animal production to maintain the normal physiology and healthy status of animals. Two of the trace elements, copper and zinc, are often supplemented in animal feeds in concentrations higher than

their physiological requirement (Hasman et al., 2006). Copper and zinc are essential trace elements; in addition to their role in the normal growth of the animal they also have additional benefits when supplemented in high doses (Hasman et al., 2006). In the United States, in addition to copper and zinc, livestock producers also use the nonessential metal arsenic as a feed supplement in chickens, turkeys, and pigs (Hasman et al., 2006).

Resistance to Heavy Metals

It is necessary to make a distinction between two types of heavy metals; those that are toxic *per se* and those that are essential for growth and maintenance, but are toxic in excess (Choudary and Srivatsava, 2001). Though many of the metals are essential for life, all the metals are toxic at some level. Metals with high atomic masses tend to bind strongly to sulfide groups. Exceptions to that being, the divalent cations of cobalt, copper, nickel, and zinc are medium sulfur liking metals and these metals function at low concentrations, but are toxic at high concentrations. It is necessary to achieve homeostasis of these metals to avoid either metal deprivation or metal toxicity.

Bacteria do acquire resistance to some of the heavy metals which are physiologically required. Generally, five mechanisms are proposed for heavy metal resistances in bacteria:

- 1) Exclusion of the metal by a permeability barrier (Nies, 1992),
- 2) Exclusion by active transport of the metal from the cell (Nies, 1992; Mago et al., 1994),
- 3) Intracellular physical sequestration of metal by binding proteins or ligands to prevent them from damaging metal-sensitive cellular targets (Gilotra and Srivatsava, 1997),
- 4) Extracellular sequestrations (Diels et al., 1995; Nies, 2000), and
- 5) Transformation and detoxification (Rouch et al., 1995; Ji and Silver, 1995).

Copper

Copper is an essential micronutrient, is required for the synthesis of hemoglobin and also for the synthesis and activation of several oxidative enzymes necessary for metabolism (NRC, 1998). Copper is also necessary for the normal immune function of swine and cattle. Copper is an integral part of several enzymes, cofactors, and metalloproteins in biological systems. A copper deficiency leads to poor iron mobilization, abnormal hemopoiesis, and poor keratinization and synthesis of collagen, elastin, and myelin (NRC, 1998). Copper deficiency signs include microcytic, hypochromic anemia, bowing legs, spontaneous fractures, cardiac and vascular disorders, and depigmentation (Hill et al., 1983). Copper may be toxic if supplemented in excess or when dietary levels exceeding 250 ppm are fed for extended periods of time (NRC, 1998). Copper toxicity signs include depressed hemoglobin levels and jaundice, mainly because of the excess copper accumulation in the liver and other vital organs.

Supplemental copper level of 5-6 ppm in the diet is sufficient to meet basal requirements for neonatal pigs (Okonkwo et al., 1979; Hill et al., 1983). Earlier studies have reported that 60 ppm of copper fed to sows improved piglet weight at birth and at weaning (Lillie and Frobish, 1978). Copper can be used in the diet of piglets as copper sulfate, carbonate, and chloride salts (Cromwell et al., 1998). Copper, when fed at the rate of 100-250 mg/kg as copper sulfate is known to stimulate growth in pigs (Barber et al., 1955; Braude, 1967). In cattle, copper is included at concentrations of 10 (basal level) to 100 (growth promotion level) mg/kg of diet to reduce mortality and morbidity in calves and for growth promotion in feedlot cattle (NRC, 2000). The mechanism through which copper promotes growth remains unknown.

Use of Copper as a Growth Promoter

In livestock, copper requirements are often met by inclusion of copper, as copper sulfate, as part of a multi-mineral supplement in the diet. Inclusion of copper in diets at elevated concentrations, particularly in cattle and swine, has documented growth promotion effects (Hasman et al., 2006). Copper, as copper sulfate, in diets of weaned piglets (Braude, 1967) and cattle diets (NRC, 2000) has been known to exert growth promoting effects. Copper at high concentrations has antimicrobial activity primarily due the production of free hydroxyl radicals by copper ions that block functional groups of proteins and enzymes inhibit or alter synthesis of nucleic acids, and modify bacterial cell wall synthesis (Gant et al., 2007). Earlier studies have focused on either the growth stimulating effects of copper on daily gain or feed efficiency or on the microbial ecosystem in the gut mainly via the effects on the fecal micro flora (Fuller et al., 1960; Kellogg et al., 1964; Jensen, 1998). However, no study has shown a definitive link between the effects of copper on the micro flora and growth promotion. Despite this, it is generally accepted that the action of copper is attributable to its antimicrobial activity. Højberg et al. (2005) has shown that elevated levels of copper inhibit coliforms. Li et al. (2006) and Shurson et al. (1990) found a positive effect of high dietary copper on daily growth rate and feed conversion rate. Copper supplementation is known to reduce the crypt depth in the duodenum of 10- day- old piglets after weaning and tends to increase the villous height of the jejunum (Zhao et al., 2007). This latter study also showed an increase in growth rate and feed intake in dose response increased amount of copper in the diet. The growth stimulating effect of feeding pharmacological concentrations of copper to swine is well documented (NRC, 2005). Generally, the response of weanling pigs to growth promoter is more pronounced in conventional farm nurseries than in experimental nurseries (Coffey and Cromwell, 1995; Zhao et al., 2007). This

may be suggestive of differences in hygiene and sanitation and subclinical pathogen exposures between farm types (Le Floch et al., 2006).

Copper Homeostasis

Copper is an essential micronutrient for both prokaryotes and eukaryotes. The biological properties of copper are attributed to its transition state. Generally, copper exists in two oxidation states, Cu^+ (Copper I) and Cu^{++} (Copper II). Free copper ions (copper II) are stable in neutral, aqueous solutions exposed to atmosphere, while copper (I) ions can only be maintained in solutions at a very acidic pH or else in a complexed form. Because of its two oxidation states, copper participates in biological redox reactions, thereby taking up and releasing electrons. However, copper homeostasis has to be tightly regulated, because it also is toxic to the cells (Solioz and Stoyanov, 2003). The mechanism of copper toxicity is still not fully understood. Copper toxicity occurs via a Fenton-type reaction leading to the production of hydroxyl radicals (OH^\cdot).



These hydroxyl radicals (OH^\cdot) are highly reactive, which in turn damage bacterial cell phospholipids, enzymes, may attack DNA bases preferably guanine residues, and break phosphodiester bonds (Yoshida et al., 1993). For the safe management of intracellular copper concentrations, both prokaryotes and eukaryotes have developed variety of homeostatic mechanisms.

Copper homeostasis in Gram-positive bacteria (*Enterococcus hirae*)

Copper homeostasis in *Enterococcus hirae* is one of the best understood prokaryotic homeostasis systems (Solioz et al., 1994; Wunderli-Ye and Solioz, 1999). Copper homeostasis

in *E. hirae* is maintained and regulated by a four gene operon, the *copYZAB* operon (Fig. 1.1). The extracellular copper reductase converts Cu (II) to Cu (I), thereby facilitating the supply of Cu I to the CopA protein. The CopA (727 amino acids) protein takes up copper when copper is limited and is encoded by the *copA* gene. The CopB protein (745 amino acids), coded by the *copB* gene, extrudes copper when it reaches a toxic levels. Both CopA and CopB act as copper transporting ATPases. Inside the cell, copper is transported via CopA ATPses to CopY (145 amino acids), which acts as a repressor and regulates the expression. CopZ protein (69 amino acids), acts as copper chaperone, which helps in intracellular delivery of copper from CopA to CopY (Odermatt et al., 1993; Odermatt and Solioz, 1995; Magnani and Solioz, 2005; Solioz and Stoyanov, 2003).

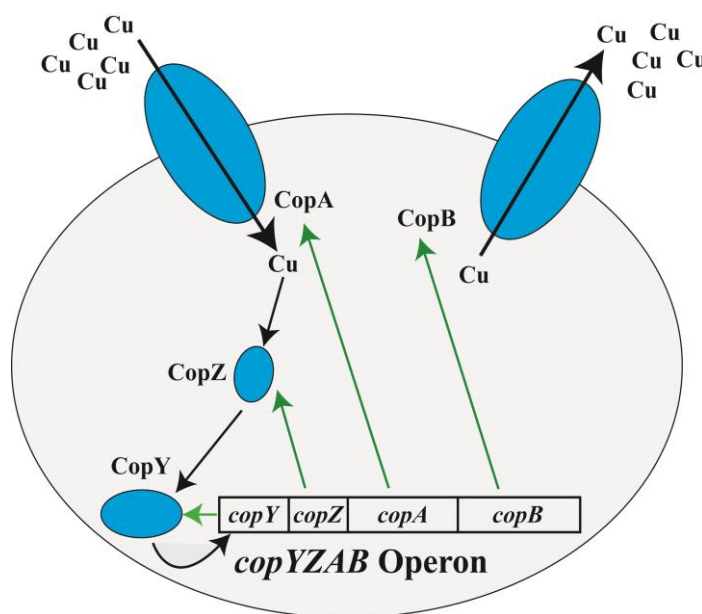


Figure 1.1: The cop operon and copper homeostasis in *Enterococcus hirae*
(Adapted from Solioz and Stoyanov, 2003)

The heavy metal ATPases contains aspartic acid of the universally conserved sequence DKTGT in the ‘phosphorylation domain’, which forms a phosphorylated intermediate, hence the name P-type ATPases (Pedersen and Carafoli, 1987). The copper transporting ATPases, CopA

and CopB, belong to this class of P-type ATPases (Lutsenko and Kaplan, 1995). Because of their characteristic phenomenon of possessing intramembranous CPC or CPH motifs, of heavy metal ATPases, these are also called as CPx-type ATPases (Solioz and Vulpe, 1996). Copper ATPases are surprisingly conserved from bacteria to humans. The distinguishing CPC/CPH or SPC motifs of the CPx-type ATPases are located in the middle of membrane helix at the most conserved structure of these ATPases. All these ATPases have conserved cysteine and proline in the motif (Solioz and Stoyanov, 2003).

Copper resistance in Gram-positive bacteria

Copper resistance in the gram-positive bacterium, *Enterococcus hirae*, occurs through the efflux mechanisms mediated by P-type ATPases. P-type ATPases are the ion pumps that carry out many fundamental processes in biology and medicine, ranging from the generation of membrane potential to muscle contraction and the removal of toxic ions from cells (Kuhlbrandt, 2004). P-type ATPases are ubiquitous and present virtually in all living organisms. Members of P-type ATPases carry putative heavy metal binding sites in the polar amino-terminal region, and have conserved intramembrane CPC, CPH or CPS motifs (CPx motif), hence the name CPx-type ATPases, they also contains a conserved histidine-proline dipeptide (HP locus), which has 33 to 43 amino acids carboxy-terminal to the CPx motif, and possess unique numeric topology of membrane spanning proteins.

Copper resistance in the gram-positive bacterium, *Enterococcus hirae*, has led to the discovery of two putative copper transporting ATPases (*copA* and *copB*). However, in the case of gram-negative bacteria such as *E. coli* and *P. syringae*, there is no evidence for ATP driven copper transport (Solioz et al., 1994). Hasman and Aarestrup (2002) have identified a gene responsible for a strong copper resistance phenotype; a large plasmid (175 kb) was isolated from

a copper resistant *E. faecium* pig isolate (A17sv1). The plasmid also contains genes for glycopeptide and macrolide resistance; further the transferability of the plasmid was confirmed by conjugation assay. The sequence homology and constructed DNA library revealed that the part of the open reading frame had a strong homology to a previously sequenced gene from the *E. coli* coding protein (HRA-1) of the CPx type ATPase family (Trenor, et al., 1994). These studies gave a solid foundation to sequence the open reading frame (*orf710*), which codes for a putative protein of 710 amino acids showing 92% identity to the HRA-1 (Hidden in Reading Frame Antisense-1) protein and 46% identity to the CopB protein of *E. hirae*.

The close homology of the *tcrYAZB* operon to the *copYZAB* operon makes the structural relationship evident, and gives an indication of the function of the plasmid-located *tcrYAZB* genes within the cell; however, the origin of the operon remains elusive. The organization of genes in *tcrYAZB* is different from that of *copYZAB* operon. The *tcrZ* gene in *tcrYAZB* is located between *tcrA* and *tcrB* genes, whereas *copZ* is located between the repressor *copY* and *copA* in *E. hirae*. Evolutionary evidence suggests that location of *copZ* gene after the *copA* gene can be seen among the copper-homeostasis genes from *Streptococcus mutans* and *S. gordonii*.

***tcrYAZB* Operon**

The plasmid localized *tcrB* (transferable copper resistance gene B) gene from *E. faecium* and *E. faecalis* was identified to be a part of an operon called the *tcrYZAB* operon. The genetic organization of the *tcrYZAB* operon (Fig. 1.2) is similar to the *copYZAB* copper-homeostasis gene cluster from *E. hirae* (Hasman, 2005), who first characterized the *tcr* gene operon. Sequence studies of the flanking regions of the putative promoter and the repressor binding sites revealed strong homology to the well characterized copper-homeostasis *copYZAB* operon from

E. hirae. By analogy to the *E. hirae* counterparts, the genes are thus named as *tcrY*, *tcrA*, *tcrZ*, and *tcrB*.

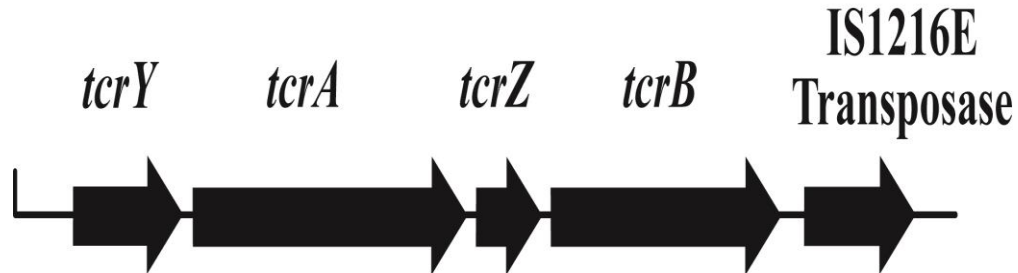


Figure 1.2: Organization of *tcrYAZB* operon including the flanking regions in *Enterococcus faecium*

The first gene of the operon, *tcrY*, has 453 bp encoding for a 151 amino acid putative protein called TcrY. TcrY is homologous to the CopY repressor from *E. hirae*, and consists of CXCX₄CXC in the C-terminal part of the protein. This domain has been known to contain zinc and copper binding domains common to all CopY-like repressors (Lu and Solioz, 2002), which indicates that *tcrY* is responsible for expression control of the operon. The *tcrA* gene encodes for a putative copper-influx CPx-type ATPase called TcrA, made up of 2,433 bp and 811 amino acids. The *tcrZ* gene is 204 bp in length, which encodes a putative chaperone protein called TcrZ with 68 amino acids, with homology to other copper chaperones, including CopZ from *E. hirae*. The *tcrB* gene, the last gene of the *tcrYAZB* operon, encodes for a copper efflux pump, TcrB protein, homologous to CopB from *E. hirae*. The close homology to the *copYZAB* operon from *E. hirae* gives evidence of structural relationship, and also an indication of the function of the plasmid-borne *tcrYAZB* genes within the cell; however, origin of the operon remains elusive, as the order of the genes is not the same as the *cop* genes of *E. hirae*.

Table 1.1: Nucleotide and protein identities (%) between the genetic elements of the *tcrYAZB* and the *copYZAB* operons of *E. hirae*

Genes	DNA	Protein
P_{cop} vs P_{tcr}	70.0	NA
<i>copY</i> vs <i>tcrY</i>	56.2	44.4
<i>copA</i> vs <i>tcrA</i>	51.9	49.3
<i>copZ</i> vs <i>tcrZ</i>	42.7	27.5
<i>copB</i> vs <i>tcrB</i>	56.9	46.3

The nucleotide and protein identities between the *tcrYAZB* operon and the *copYZAB* operon of *E. hirae* are shown above (Table 1.1). The truncated ISS-1 type transposase was located upstream of the *tcr* promoter, and an IS *1216E* element was located downstream of the four *tcr* genes, indicating the termination of the operon. This was further supported by the fact that intergenic region between *tcrB* and the IS *1216E* contained a strong dyad symmetry region which can form a loop structure and function as a rho factor-independent transcriptional terminator (Hasman, 2005).

Acquired copper resistance, mediated by *tcrB* gene, in *E. faecium* was strongly correlated to macrolide and glycopeptide resistance in isolates from pigs, and the *tcrB* gene was shown to be located on the same conjugative plasmid of 175 kb. Copper resistance in *E. faecium* isolates of both animal and human reservoirs was genetically linked to macrolide and glycopeptide resistance in *E. faecium* isolates from pigs (Hasman and Aarestrup, 2002).

Antimicrobial Use and Resistance Associated with Swine Production

The use of in-feed antimicrobials for growth promotion and disease prevention is an integral part of swine production and management. In the US, antimicrobial feed additives are commonly used in 90% of nursery, 75% of grower, and more than 50% of finisher pigs (Apley et al., 2012). According to NAHMS (USDA-NAHMS, 2005), 92% of the swine farms surveyed use antibiotics, most delivered through feed. More than 85% of the farms used in-feed antibiotics in the grower or finisher phase. However, recent changes in the swine industry and a growing concern over the non-therapeutic use of antimicrobials, along with changing trends in therapeutic and non-therapeutic regimens, may have caused a change in the overall use of antibiotics, particularly in grower or finisher units (Cromwell, 2001; Dritz et al., 2002). A link between antibiotic use in swine and increased prevalence of resistant bacteria has been demonstrated through several studies (Mathew et al., 2001; Mathew et al., 2005). These studies have shown significant increase of resistance in the gut flora following the use of antibiotics, and also have shown that the rapid reversion of susceptibility in commensal microflora following the drug withdrawal may also occur in some cases, but it depends on the type of antibiotics.

Any usage of antimicrobials, even in subtherapeutic concentrations, will select for antimicrobial resistance. Studies have shown that antimicrobial resistant bacteria and antimicrobial resistant genes are exchanged between both human and animal reservoirs (Mathew et al., 2007). The termination of the use of antimicrobial growth promoters increases the need for other feed additives or alternatives to control disease outbreaks, and to improve the growth rates in food producing animals. Often, heavy metals such as copper and zinc are utilized for these purposes, as they possess both antimicrobial as well as growth promotion properties. These heavy metals are most often employed during the first week of weaning, in piglets to

control diarrhea or scouring, and to improve the performance of piglets. Piglets are subjected to many stressors at weaning. The diet changes along with the shift from sow milk to a diet composed of cereal grains supplemented with protein sources, such as soybean meal, skim-milk powder, and or fish meal. These diets are normally supplemented with minerals and vitamins according to current recommendations (NRC, 1998). Antibiotics and other alternatives like copper are extensively used in swine production to improve growth and nutrient utilization. It is generally hypothesized that the altered gut micro flora in response to in-feed usage leads to improvement in growth and efficiency of feed utilization. Antimicrobials also could act by inhibiting the growth of opportunistic gut pathogens in animals (Kim et al., 2012). Copper promotes growth of piglets independent of growth promotion by antibiotics (Cromwell, 1998). Chlortetracycline and tylosin are the most commonly used in-feed antibiotics for growth promotion at subtherapeutic dose in the swine industry (Dritz et al., 2002). Chlortetracycline, with broad spectrum activity, is used to treat or control respiratory and enteric bacterial infections. Tylosin, a macrolide antibiotic is used to treat or prevent swine dysentery, and other bacterial infections, including arthritis, ileitis, and erysipelas in swine (Dritz et al., 2002). The supplementation of these antimicrobials (chlortetracycline or tylosin) along with copper has been shown to increase feed intake, growth rate, and feed efficiency indicating that the effects of copper and antibiotics are additive (Stahly et al., 1980). The table on information on the FDA approved antimicrobials which are commonly used in swine production is shown in table 1.2.

Table 1.2: FDA approved antimicrobial feed additives of swine intended for improved growth rate and feed efficiency

Antimicrobial	Inclusion level	Withdrawal time (Days)
Arsanilic acid	45-90 g/ton of feed	5
Bacitracin		
i) Methylene disalicylate	Nursery pigs: 10-30 g/ton	0; not for use in swine weighing more than 250 lb
	Grow to finish pigs: 250 g/ton	0
ii) Chlortetracycline (CTC)	Bacitracin @ 10-30 g/ton CTC @ 400 g/ton	0
iii) Zinc	10-50 g/ton	0
Bambermycins	2-4 g/ton	0
Carbadox	10-25 g/ton	42
Chlortetracycline	10-50 g/ton	Voluntary withdrawal to meet market needs
Chlortetracycline	+ 100 g/ton	7
Sulfamethazine +	100 g/ton	
Procaine Penicillin	50 g/ton	
Lincomycin	20 g/ton	0
Neomycin/Oxytetracycline	10-50 g/ton	5
Oxytetracycline	10-50 g/ton	0
Roxarsone	22.7-34.1 g/ton Growing and finisher pigs	5
Tylosin	Nursery pigs @ 20-100 g/ton	0
	Growers @ 20-40 g/ton	0
	Finishers @ 10-20 g/ton	0
Tylosin / Sulfamethazine	100 g/ton	15
Virginiamycin	5-10 g/ton To maintain feed efficiency in the presence of atrophic rhinitis	0

(Adapted from Jacela et al., 2009; Feed Additive Compendium, 2014)

Antimicrobial Use and Resistance Associated with Beef Cattle Production

Feedlots provide a focal point for development and dissemination of antimicrobial resistance, due to high density housing of animals and usage of various antimicrobials. Generally, more than 80% of the feedlots in the US use antimicrobials for growth promotion or prophylaxis (McEwen and Fedorka-Cray, 2002). Feed grade antimicrobials are supplemented in feedlot cattle diets to promote growth, improve feed efficiency and to reduce the incidence and severity of respiratory infections, foot rot, and liver abscesses (Nagaraja, 1995; Butaye et al., 2003).

Table 1.3 gives information on the FDA approved antimicrobials intended for improved growth rate and feed efficiency in feedlot cattle. In cattle, tylosin is included in the feed to reduce the incidence of liver abscesses (Nagaraja and Chengappa, 1998). The below table (1.4) gives information on the FDA approved antimicrobials used to prevent liver abscesses in feedlot cattle. Tetracyclines (chlortetracycline and oxytetracycline) are also widely used in feedlot cattle for improved growth rate and feed efficiency, to prevent liver abscesses, to treat bacterial enteritis, and for the control of respiratory disease associated with transportation stress (Nagaraja, 1995).

Copper, generally as copper sulfate, is supplemented in the diet at a concentration of 10-100 mg/kg (NRC, 2000). The effects of these antimicrobials on the susceptibility of both commensal and food-borne bacterial species are heavily debated and have potentially important ramifications for food safety. An aspect of food safety is the emergence in and dissemination of antimicrobial resistance from food-borne bacteria. There is a potential public health hazard from these antimicrobial resistance pathogens and commensal organisms to disseminate to humans via direct contact with animals (Price et al., 2007; Call et al., 2008) or through food chain (Silbergeld et al., 2008). Because in-feed antimicrobials provide a continuous selective pressure, it is not

unreasonable to believe they have an effect on the susceptibility of bacteria with which they associate; studies examining the effect of removing in-feed antimicrobials on bacterial susceptibility from feedlot cattle are not abundant. The potential for selection and expansion of AMR bacteria is as germane today as it was six decades ago. Co-selection of AMR traits is an obvious case whereby AMR traits are genetically linked so that selection of one antibiotic resistance trait maintains the unrelated AMR trait (Borgen et al., 2002). Similar genetic linkages have been described for heavy metal resistance (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2011 & 2013).

The National Antimicrobial Resistance Monitoring System (NARMS), which was established in 1996 in collaboration with Food and Drug Administration (FDA), which provides an early warning system for detecting any change in bacterial resistance dynamics or trends (Accessed at www.fda.gov/cvm/narms_pg.html). The FDA's Guidance For Industry (GFI # 152) adds an additional safety measure to prevent antimicrobial resistance that may result from the use of antimicrobial drugs in production animals. On the other hand, the beef industry is striving very hard to curtail antimicrobial resistance. As a consequence, beef industry has funded comprehensive research projects to enhance the understanding of the basic science of resistance development, as well as collect information on the effects of beef production practices on resistance development in foodborne pathogens (www.beefresearch.org). Longitudinal studies that compare effects and levels of antimicrobial use on the prevalence and co-selection of resistance determinants would be particularly useful for understanding the risks and consequences of different policy choices for limiting antimicrobial resistance (Klement et al., 2005). Observational studies can also demonstrate associations but are not able to show direct causal links between antimicrobials and the development of antibiotic resistance at the individual animal level (Berge et al., 2005). Reducing AMR in cattle populations may or may not have

important public health ramifications, but given the uncertainty principle underlying its selection, efforts are made to reduce AMR populations in production environments (Call et al., 2008). Major contributing factors such as transient selection, genetic linkage to specific resistance traits, and the involvement of multifactorial variables contribute to the persistence of AMR in cattle production systems. Careful management practices can reduce the need for antimicrobials and also satisfy the prudent use principle.

Table 1.3: FDA approved medicated feed additives of cattle intended for improved growth rate and feed efficiency

Antimicrobial	Inclusion level	Withdrawal (Days)	time
Bacitracin (zinc)	35-70 mg/head/day	0	
Bambermycin	1-4 g/ton (10-20 mg/head/day)	0	
Chlortetracycline	Calves under 250 lbs: 0.1 mg/lb body weight/day Calves 250-400 lbs: 25-70 mg/head/day Growing cattle over 400 lbs: 70 mg/head/day	0	
Laidlomycin propionate	Feedlot cattle meant for slaughter: 5 g/ton (30-75 mg/head/day) Pasture cattle meant for slaughter: 30-150 mg/head/day	0	
Lasolacid	Feedlot cattle meant for slaughter: 25-30 g/ton (250-360 mg/head/day) Pasture cattle meant for slaughter: 60-300 mg/head/day	0	
Monensin	Feedlot cattle: 5-40 g/ton (50-480 mg/head/day) Pasture cattle: 25-400 g/ton For first five days, no more than 100 mg/head/day	0	
Oxytetracycline	Calves less than 250 lbs: 0.05-0.1 mg/lb body weight/day Calves between 250-400 lbs: 25 mg/head/day Growing cattle over 400 lbs: 75 mg/head/day	0	
Virginiamycin	16-22.5 g/ton to provide 100-340 mg/head/day	0	

(Adapted from Feed Additive Compendium, 2014)

Table 1.4: FDA approved feed additives used to prevent liver abscesses in feedlot cattle

Antimicrobial	Inclusion level	Withdrawal (Days)	time
Bacitracin methylene disalicylate	70 mg/head/day continuously or 250 mg/head/day for 5 days	0	
Chlortetracycline	70 mg/head/day	0	
Oxytetracycline	75 mg/head/day	0	
Tylosin	60-90 mg/head/day (8-10 g/ton)	0	
Virginiamycin	85-240 mg/head/day (13.5-16 g/ton)	0	

(Adapted from Feed Additive Compendium, 2014)

CHAPTER 2 - Epidemiology of Antimicrobial Resistant Enterococci, Vancomycin Resistant Enterococci, and their Virulence Characteristics

Enterococcus spp.

Enterococcus is an extremely versatile bacterial genus. Enterococci, previously known as fecal streptococci, represent part of the normal commensal flora of both human and animal gut microbiota, and some of the strains of this genus are employed in food manufacturing industry or as probiotics (Facklam, et al., 2002; Simjee, et al., 2006). Enterococci are gram-positive cocci that occur singly, in pairs, or as short chains. They are facultative anaerobes with an optimum growth temperature of 35° C and a growth range from 10 to 45° C. All species belonging to this genus grow in broth containing 6.5% NaCl, and they hydrolyze esculin in the presence of 40% bile salts (Facklam and Elliott, 1995). Enterococci are intrinsically resistant to a broad range of antimicrobial agents, thus leading to limited choice of agents against these organisms. Enterococci have also evolved and acquired resistance to many antimicrobial agents by acquisition of plasmids or transposons from Gram-negative as well as Gram-positive bacteria (Kak and Chow, 2002). Thus, enterococci have emerged as important nosocomial pathogens (Malani et al., 2002).

This ability of enterococci to acquire antimicrobial resistance has made it an obvious choice as indicator organism for antimicrobial resistance in gram-positive bacteria, and the prevalence of resistance has been monitored in surveillance programs

(www.cdc.gov/ncidod/hip/Surveill/nnis.htm). The enterococci are present in high numbers in

foods of animal origin (Klein, 2003) and have been also associated with food-borne outbreaks due to their capacity to carry and disseminate antibiotic resistance genes (Simjee et al., 2006). Studies (Hasman & Aarestrup, 2002; Hasman et al., 2006) have reported that enterococci have been known to harbor resistance genes to copper, which genetically co-exist with macrolide [*erm*(B)] and glycopeptide (*vanA*) resistance. This was first reported from Denmark in enterococcal isolates of piglets, poultry, and calves (Hasman & Aarestrup, 2002).

Enterococcal infections

Enterococci occur in a remarkable array of environments. They are found in soil, water, food, and make up a significant portion of the normal gut flora of humans and animals. Like other gut bacteria, enterococci can also cause infectious diseases. Twelve different enterococcal species can cause infections, including *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, and *E. salitarius* (Malani et al., 2002). Most of the infectious diseases are caused by *Enterococcus faecalis*, which account for 80-90% of the clinical strains. Enterococci are very robust and resistant to various routinely used antimicrobials, both by intrinsic and acquired mechanisms (Table 2.1). Enterococcal infections are more commonly seen in hospitalized individuals with underlying conditions comprised of a wide spectrum of severity of illness and immune modulation. Table 2.2 gives the Clinical and Laboratory Standard Institute's breakpoints used for susceptibility testing of *Enterococcus* spp. Enterococci are ranked second or third among the bacteria isolated from hospitalized patients (Kayser, 2003; Schaberg et al., 1991).

The most frequent infection caused by enterococci is as follows:

- a) Urinary tract infections: Lower urinary tract infections, such as cystitis, prostatitis, and epididymitis are common in older people (Graninger and Ragette, 1992).

However, upper urinary tract infections are more common in young people, which can lead to severe bacteremia in later stages.

b) Bacteremia: Enterococci account for approximately 6-7% of all the bacteria isolated from the blood stream (NNISS, 1999). Bacteria can get into the blood via urinary tract, intra-abdominal infections, burn wounds, diabetic foot infections, or intravascular catheters.

c) Intra-abdominal and pelvic infections: Enterococci are often found as part of mixed aerobic or anaerobic flora in intra-abdominal and pelvic infections. These infections are more common in patients with nephritic syndrome or cirrhosis, where enterococci can cause serious peritonitis (Graninger and Ragette, 1992).

d) Wound and tissue infections: Enterococci are always found associated with the mixed cultures together with gram-negative aerobic rods and anaerobes. Most commonly encountered in burn patients and diabetic foot infections.

e) Endocarditis: Enterococci account for approximately 5-15% of all cases bacterial endocarditis (Murray, 1990). Common sources of infections are via genitourinary, biliary portals, and also in drug addicts.

f) Rare enterococcal infections: Rare infections include meningitis in normal adults with some anatomic defects, prior neurosurgery or head trauma. Enterococci are also seen unusually in respiratory tract infections.

Virulence factors in *Enterococcus* spp.

The virulence factors produced by enterococcus are divided into three major categories:

Secreted Proteins

- **Cytolysin (Cyl):** Novel bacterial toxin expressed by some strains of *E. faecalis*, encoded within a large, pheromone-responsive plasmid (Clewell, 1981; Ike et al., 1990) and or on the chromosome (Ike and Clewell, 1992) within pathogenicity islands. Cytolysin displays both hemolytic and bactericidal activity (Gilmore et al., 1994).
- **Gelatinase (Gel):** Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase (Koch et al., 2004), with an ability to liquefy gelatin. Gelatinase (*gelE*) is a protease produced by *E. faecalis* that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides (Kreft et al., 1992). The main role of gelatinase in enterococcal pathogenesis is to provide nutrients to the bacteria by degrading host tissue, and also aid in biofilm formation (Mohamed and Huang, 2007).
- **Serine Protease (Spr):** Protease with proteolytic activity similar to gelatinase. Both these proteolytic enzymes are regulated by *fsr* quorum-sensing system and contribute to virulence in an animal peritonitis model (Qin et al., 2000; Sifri et al., 2002). Transcription of the *gelE-sprE* operon is positively regulated in a growth phase-dependent fashion by the *fsr* locus, the components of which have been termed *fsrA*, *fsrB*, and *fsrC* (Qin et al., 2001).

Adhesins

- **Aggregation substance (Asa):** Aggregation substance is a surface localized protein encoded by pheromone-responsive, self-transmissible plasmids that mediate binding of donor bacterial cells with plasmid free recipient cells, which aid in conjugal transfer of genes (Clewell, 1993). Asa is a pheromone-inducible surface protein and mediates aggregate formation during conjugation, thus aiding

in plasmid transfer as well as adhesion to an array of eukaryotic surfaces (Koch et al., 2004). Pulsed-field gel electrophoresis analysis of clinical isolates of *E. faecalis* showed that the gene encoding Asa was not present in *E. faecium* isolates (Hällgren et al., 2008).

- **Enterococcal surface proteins (Esp):** Extracellular surface protein (Esp) is a cell-wall-associated protein first described in *Enterococcus faecalis* (Shankar et al., 1999). Esp contributes to enterococcal biofilm formation, which could lead to resistance to environmental stresses, and adhesion to eukaryotic cells such as those of the urinary tract (Borgmann et al., 2004). Esp is also thought to promote adhesion, colonization and evasion of the immune system, and to play a role in antibiotic resistance (Foulquie Moreno et al., 2006).
- **Ace antigens:** Ace refers to Adhesin to collagen of *E. faecalis*. Collagen-binding protein, belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family. The *ace* gene is ubiquitous in *E. faecalis* and known to express conditionally after growth in serum or in the presence of collagen (Singh et al., 2010).
- **EfaA antigens:** EfaA refers to *E. faecalis* antigen A. EfaA is a major surface antigen of *E. faecalis* and was identified using sera from patients with known *E. faecalis* endocarditis (Lowe et al., 1995). EfaA has a sequence identity with group of streptococcal ABC transporter proteins, majority of which are identified as prominent surface adhesins and play a vital role in pathogenesis (Viscount et al., 1997; Kolenbrander et al., 1998). Several studies have shown that *efaA* gene is ubiquitously present in all *E. faecalis* isolates and also reported an equally

prevalent homolog in *E. faecium* isolates (Eaton & Gasson, 2001; Singh et al., 1998).

- **Pili:** Ebp, endocarditis and biofilm associated pili. Expression of pili at the bacterial cell surface facilitates adhesion, first step in biofilm formation. It was in 2006 (Nallappareddy et al., 2006), a three-gene *ebpABC* locus and an adjacent downstream sortase-encoding gene, *bps* (biofilm and pilus associated sortase) that are necessary for the assembly of pili on the surface of *E. faecalis* OG1RF strain were discovered. The Ebp revealed multifunctional role in host pathogen interaction. Ebp known to play a major role in virulence associated properties, such as adherence to various host tissue components, biofilm formation, and a role in animal infection models (Sillanpää et al., 2010).

Cell wall polymers

- **Capsule (Cps):** Capsule is a major contributor of virulence in most of the organisms. The capsule allows the microbes to escape and evade by the host immune system (Hancock and Gilmore, 2002; Thurlow et al., 2009).
- **Lipoteichoic acid (LTA):** Lipoteichoic acid (LTA) is a highly diverse class polymers containing sugar and phosphate backbone. LTAs are anchored in the cytoplasmic membrane by hydrophobic interactions. LTAs are generally released from the bacterial surface due to the action of cell wall active antibiotics and during sepsis due to inflammatory processes (Cleveland et al., 1996).
- **Cell wall antigen:** Enterococcal polysaccharide antigen (*epa*) appears to be widespread in *E. faecalis*. In *E. faecalis*, *epa* gene cluster is involved in the biosynthesis of an enterococcal polysaccharide antigen that is produced during infection in humans.

To conclude, enterococci have evolved different mechanisms to thrive in harsh environment. Their genome plasticity, propensity for horizontal gene transfer, and ability to turn on the genetic switches or gene/s make them important nosocomial pathogens

Table 2.1: Intrinsic and acquired antimicrobial susceptibility of enterococci

	Antimicrobials	Mechanism of resistance
Intrinsic resistance	β-lactams	
	Penicillin (low level)	Low-affinity penicillin binding proteins (PBP)
	Carbapenems(moderate level)	
	Cephalosporins (high level)	
	Aminoglycosides	
	Low level	Inefficient uptake
	High level	Production of chromosomal AAC(6')II enzyme
	Moderate level	
	Clindamycin	Mutation, substitutions, and or deletion of macrolide inducible DNA sequences
	Fluoroquinolones	Mutational changes to topoisomerase IV (Werner et al., 2010)
Acquired resistance	Glycopeptides	Production of D-Ala-D-Ser ending peptidoglycan precursors
	Lincosamides and Streptogramin A	Efflux
	Trimethoprim-sulfamethoxazole	Mutational changes in the dihydrofolate reductase (Wisell et al., 2008)
	Ampicillin	Overproduction or alterations of PBP
	Aminoglycosides	Aminoglycoside modifying enzymes [AAC(6')-APH(2'')]
	Chloramphenicol	Chloramphenicol

	acetyltransferase (CAT) encoding enzymes
Erythromycin	Ribosomal methylation
Fluoro quinolones	Mutational changes to topoisomerase IV (Werner et al., 2010)
Fusidic acid	Mutations of the EF-G encoding gene
Glycopeptides (vancomycin, teicoplanin)	Peptidoglycan precursor modification
Nitrofurantoin	
Oxazolidinones	Mutation in the loop of domain V of 23S rRNA gene
Rifampin	Mutation in <i>rpoB</i> gene (Enne et al., 2004)
Tetracycline	Modification of ribosomal protein
Quinolones	Alterations in DNA gyrase and Topoisomerase IV

This table is adapted from Cetinkaya et al., 2000; Enne et al., 2004; Wisell et al., 2008; Werner et al., 2010; Top et al., 2008.

Table 2.2: CLSI breakpoints used for susceptibility testing of *Enterococcus* spp.

CLSI Subclass	Antimicrobial Agent	WHO Classification of Antimicrobials	Antimicrobial concentration range (µg/ml)	Breakpoints (µg/ml)		
				Susceptible	Intermediate	Resistant
Aminoglycoside	Gentamicin	Critically Important	128 - 1024	≤ 500	N/A	≥ 500
	Kanamycin	Critically Important	128 - 1024	≤ 512	N/A	≥ 1024
	Streptomycin	Critically Important	512 - 2048	≤ 1000	N/A	≥ 1000
Glycopeptide	Vancomycin	Critically Important	0.25 - 32	≤ 4	8 – 6	≥ 32
Glycylcycline	Tigecycline	Critically Important	0.015 - 0.5	≤ 0.25	N/A	N/A ¹
Lincosamides	Lincomycin	Highly Important	1 - 8	≤ 2	4	≥ 8
Lipopeptide	Daptomycin	Critically Important	0.25 - 16	≤ 4	N/A	N/A ²
Marolide	Erythromycin	Critically Important	0.25 - 8	≤ 0.5	1 – 4	≥ 8
	Tylosin	Critically Important	0.25 - 32	≤ 8	16	≥ 32
Nitrofurantoin	Nitrofurantoin	Important	2 - 64	≤ 32	64	≥ 128
Oxazolidinones	Linezolid	Critically Important	0.5 - 8	≤ 2	4	≥ 8
Penicillin	Penicillin	Critically Important	0.25 - 16	≤ 8	N/A	≥ 16
Phenicol	Chloramphenicol	Highly Important	2 - 32	≤ 8	16	≥ 32
Quinolone	Ciprofloxacin	Critically Important	0.12 - 4	≤ 1	2	≥ 4
Streptogramin	Quinupristin/Dalfoprisitin	Highly Important	0.5 - 32	≤ 1	2	≥ 4
Tetracycline	Tetracycline	Highly Important	1 - 32	≤ 4	8	≥ 16

¹ For tigecycline, only a susceptible breakpoint (≤ 0.25 µg/ml) has been established. In this report, isolates with an MIC ≥ 0.5 µg/ml are categorized as resistant

² For daptomycin, only a susceptible breakpoint (≤ 4 µg/ml) has been established. In this report isolates with an MIC ≥ 8 µg/ml are categorized as resistant

N/A – Not applicable; NT – Not tested

Adapted from WHO Report, 2012; and CLSI M100 document (CLSI, 2012).

Vancomycin Resistant Enterococci

Enterococci have become an important nosocomial pathogen because of their intrinsic ability to acquire as well as transfer resistance genes to other gut commensal via plasmids, transposons etc. In the United States, enterococci are the second most common nosocomial pathogens isolated from catheter associated infections of the bloodstream, endocarditis, and urinary tract infections (Arias and Murray, 2008; Hidron et al., 2008). These organisms have survived in hospital environment because of their intrinsic resistance to most of the routinely used antibiotics, and also their ability to acquire resistance all currently available antibiotics either by mutation or horizontal gene transfer of genetic material via plasmids and or transposons (Clewell, 1990). Vancomycin, a glycopeptide antibiotic with gram positive antibacterial spectrum, was virtually the only drug of choice for the treatment of enterococcal infection for more than 3 decades without the emergence of resistance (Cetinkaya et al., 2000; Ingberman and Santoro, 1989). Resistance to glycopeptides is due to the production of low-affinity pentapeptide precursors ending either D-lactate (D-lac) or D-serine (D-ser) instead of high-affinity precursors ending in D-Ala-D-Ala (Courvalin, 2006; Walsh, 2000).

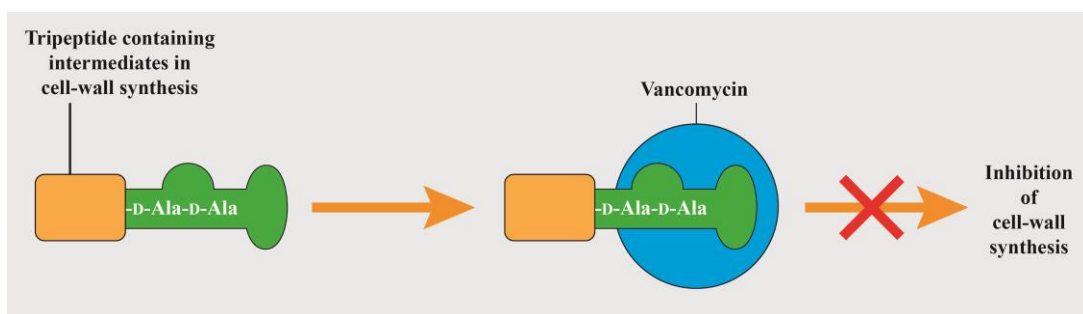


Figure 2.1: Mechanism of action of vancomycin, Vancomycin-Susceptible Enterococci (Adapted from Murray, 2000).

The origin of glycopeptide resistance among clinical enterococcal isolates remains uncertain. The first VRE isolates were reported in 1988 in the UK and in France (Uttley et al., 1988; Leclercq et al., 1988).

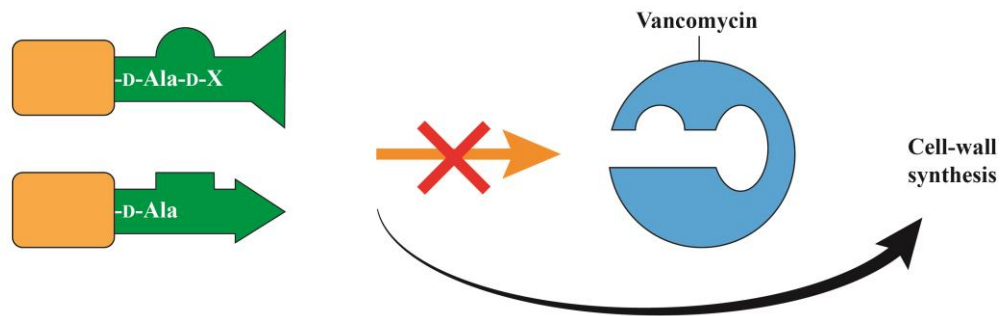


Figure 2.2: Mechanism of action of vancomycin, Vancomycin-Resistant Enterococci (Adapted from Murray, 2000).

The role of avoparcin, a glycopeptide antibiotic used as a growth promoter in food animals since 1970 in most of the European countries, was suspected as culprit for the selection of VREs, which could potentially be transmitted to humans via food chain (Bates et al., 1995). The ban on avoparcin resulted in a significant decline in VRE isolated from chickens from Denmark (Bager et al., 1999). However, VREs still persist even after the ban of avoparcin which is attributed to higher biological cost of vancomycin resistance (Johnsen et al., 2011). In the USA, the situation is entirely different because avoparcin was never approved for use in food animals. The VREs were first reported in the US in 1989 in New York City (Frieden et al., 1993). Molecular epidemiological studies on VREs have revealed the existence of a genetic lineage labelled as clonal complex 17 (CC17). This clonal complex is usually associated with hospital outbreaks of VREs worldwide and is characterized by distinct markers such as ampicillin and high-level fluoroquinolones (Willems et al., 2005). Furthermore, most of the CC17 contain mobile genetic

elements, phage genes, genes encoding for membrane proteins, regulatory genes, pathogenicity islands, and megaplasms (van Schaik et al., 2010). As of now, eight types of acquired resistance to glycopeptides have been reported on the basis of phenotypic and genotypic characteristics (Table 2.3).

Table 2.3: Characteristics of glycopeptide resistant enterococci

Characteristics	VanA	VanB	VanC	VanD	VanE	VanG	VanL	VanM	VanN
MIC (µg/ml)									
Vancomycin	64-1000	4-1024	2-32	64-128	8-32	16	8-16	>16	8-16
Teicoplanin	16-512	0.5-1.0	0.5-1.0	4-64	0.5	0.5	Susceptible	Resistant	Susceptible
Modified target	D-Alanine-D-Lactate	D-Alanine-D-Lactate	D-Alanine-D-Serine	D-Alanine-D-Lactate	D-Alanine-D-Serine	D-Alanine-D-Serine	D-Alanine-D-Serine	D-Alanine-D-Lactate	D-Alanine-D-Serine
Expression	Inducible	Inducible	Constitutive, Inducible	Constitutive	Inducible	Inducible	Inducible	Unknown	Constitutive
Location	Plasmid Chromosome	Plasmid Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Unknown	Plasmid	Chromosome
Resistance level	High	Variable	Low	Moderate	Low	Low	Low	High	Low
Genetic determinant	Acquired	Acquired	Intrinsic	Acquired	Acquired	Acquired	Intrinsic	Acquired	Acquired
Conjugation	Positive	Positive	Negative	Negative	Negative	Positive	Negative	Positive	Positive
Mobile element	Tn1546	Tn1547 Tn1549- Tn5382	Not applicable	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

Adapted from Cetinkaya et al., 2000; Top et al., 2008; Courvalin, 2006; Cattoir and Leclercq, 2013.

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Dissertation Outline

High concentrations of copper, fed as copper sulfate, are often used to increase growth rates in swine. Bacteria exposed to copper may acquire resistance, and in *Enterococcus faecium* and *E. faecalis*, a plasmid-borne transferable copper resistance (*tcrB*) gene that confers copper resistance has been reported. The plasmid also carries macrolide [*erm*(B)] and glycopeptide (*vanA*) antibiotics resistance genes, suggesting a potential linkage of copper resistance to antibiotic resistance. Because *tcrB* in enterococci has only been reported in Europe, we conducted a study to determine the occurrence of *tcrB* in fecal enterococcal isolates of piglets supplemented with elevated levels of copper and relate *tcrB* to phenotypic susceptibilities to copper, erythromycin, and vancomycin. Also, the transferability of *tcrB* from *tcrB*-positive to *tcrB*-negative enterococci strains was determined (Amachawadi et al., 2010). We have also shown that the *tcrB* gene is carried on a transferable plasmid and provide evidence for interspecies co-transfer of *tcrB* and *erm*(B) genes in enterococci (Amachawadi et al., 2011). In our study, all *tcrB*-positive isolates contained the *erm*(B) gene and were phenotypically resistant to erythromycin. Enterococcal isolates in our studies were also positive for *tet*(M) and phenotypically resistant to tetracyclines. The potential link between *tcrB* and antimicrobial resistance genes and the propensity of enterococci to transfer *tcrB* to other strains raises the possibility that copper supplementation may exert selection pressure for antimicrobial resistant enterococci. Because tetracycline resistance is provided in so many different ways, coded by many different genes located in a variety of locations within the bacterial genome and on plasmids, there is varied fitness costs associated with their presence. These fitness costs help

determine the baseline prevalence of strains of bacteria harboring these resistance factors when comparing these to strains competing for space and resources within ecological niches in the gut. Further, stress exerted by antimicrobial use also differentially selects across those strains harboring different resistance genes and mechanisms. This multitude of factors makes stable prediction of effects of co-selection a risky proposition at best. Platt et al. (2008) showed that chlortetracycline administration increased the probability of recovering *E. coli* and *Enterococcus* spp. exhibiting reduced susceptibility to tetracycline (Platt et al., 2008). The potential link between copper resistance (*tcrB*) and tetracycline and macrolide resistance genes raises the possibility that copper supplementation may exert selection pressure for antimicrobial resistant enterococci. Because tetracycline and erythromycin resistances are conferred by multiple mechanisms (especially, in gram-positive bacteria), coded by many different genes located within the bacterial genome or on plasmids, there is varied fitness costs associated with their presence (Rice, 1998). These fitness costs help determine the baseline prevalence of strains of bacteria harboring these resistance factors when competing for space and resources within ecological niches in the gut. Therefore, a longitudinal study was conducted to investigate the effects of in-feed copper, chlortetracycline, and tylosin alone or in combinations on selection and co-selection of resistance strains of fecal enterococci (**Chapter 3**).

Since the prevalence of the *tcrB* gene is not yet reported in cattle we conducted a study in feedlot cattle to investigate the effects of feeding copper on the prevalence of copper resistant enterococci isolated from cattle feces. The present study was conducted to determine the occurrence of *tcrB* gene in fecal enterococcal isolates of cattle supplemented with elevated (100 mg/kg) levels of copper in the diet and to determine phenotypic susceptibilities to copper, tetracycline, tylosin, and vancomycin (**Chapter 4**).

The high propensity of enterococci to acquire, express and transfer antimicrobial resistance determinants (horizontal gene transfer) enhances their ability to sustain antibiotic selection pressure, and therefore, have become important opportunistic and nosocomial pathogens in humans. Because antibiotic resistance genes are co-located with *tcrB* on the same plasmid, it is possible that feeding elevated level copper in the diet may co-select for enterococci resistant to antibiotics in the absence of antibiotic selection pressure. The presence of both antibiotic and heavy metal resistance genes may help in the plasmid maintenance in environments with no selection pressure of antibiotics. Because of the potential link between *tcrB* with *erm(B)*, we hypothesize that copper supplementation may exert selection pressure for enterococci to become resistant to macrolides (**Chapter 5**).

Vancomycin resistant enterococci are among the most common nosocomial pathogens in several European Union countries and in the United States. VRE are frequently isolated from farm animals, pets, and retail food products in Europe. In the US, VRE are rare in animals and as a source of community infections in humans. The reason is that avoparcin, a glycopeptide growth promoter closely related to vancomycin, was never approved for use in animals in the US. Only recently the occurrence of VRE in swine was reported (Donabedian et al., 2010). We hypothesize that there are differences in molecular characteristics of VRE to explain this geographical disparity and that understanding these differences is essential for maintaining low levels of VRE in community and agricultural settings in the US. In our repository, we had samples and or isolates originated from human and swine cohorts in a semi closed integrated agri food system, Texas in the year 2004 and 2005. We were able to recover VREs from frozen swine feces collected back in 2004 and 2005; technically this is the first report of VRE from swine in the US. These isolates were used to characterize VREs to explore possible differences

in resistance determinant carriage and elucidate the genetic and clonal differences to address the global epidemiological status of each level of genetic aggregation, and also help us to understand the evolutionary dynamics in the dissemination of VREs in humans and animals (**Chapter 6**).

CHAPTER 3 - Effects of in-feed copper, chlortetracycline, and tylosin on the prevalence of transferable copper resistance gene, *tcrB*, among fecal enterococci of weaned piglets

Introduction

The use of in-feed antimicrobials for growth promotion and disease prevention is an integral part of swine production and management. In the US, antimicrobial antibiotic feed additives are commonly used in 90% of nursery, 75% of grower, and more than 50% of finisher pigs (Apley et al., 2012). Because of concerns that bacteria, including pathogens, could develop resistance to antibiotics and lead to exposure among humans via the food chain (Marshall and Levy, 2011), alternatives to antibiotics, such as heavy metals, are increasingly employed in swine production systems. Copper is supplemented in swine diets, particularly in nursery pigs, at concentrations of 100-250 ppm, to reduce mortality and morbidity associated with bacterial enteric infections, and for growth promotion (NRC, 1998). The suggested mechanisms of growth promotional effects of copper include altered gut microbial flora to reduce fermentation loss of nutrients, and suppression of gut pathogens (Højberg et al., 2005). However, high concentrations of copper are toxic to bacteria and therefore copper ion homeostasis is important to ensure its use as a co-factor while limiting cellular damage (Shafeeq et al., 2011). Acquired copper resistance has been reported in both gram-positive (Solioz et al., 2010) and gram-negative bacteria (Brown et al., 1995). A plasmid-borne gene that confers copper resistance, designated as transferable copper resistance (or *tcrB*) has been identified among enterococcal species, particularly *E. faecium* and *E. faecalis* in Denmark and U.S. (Hasman and Aarestrup, 2002; Hasman et al., 2006;

Amachawadi et al., 2011). The plasmid size varied in Danish (175 kb; Hasman et al., 2006) and US enterococcal isolates (194 kb; Amachawadi et al., 2011). Interestingly, the plasmids in both countries also carried genes for antibiotic resistance. The plasmids in Danish isolates carried *erm(B)* and *vanA* (Hasman and Aarestrup, 2002; Hasman et al., 2006) genes that encode resistance to macrolides and glycopeptides, respectively; in the US, *tcrB*-borne plasmids carried *erm(B)*, and a tetracycline resistance gene, *tet(M)* (Amachawadi et al., 2011), suggesting a potential linkage of copper resistance to antibiotic resistance.

In the U.S., tetracyclines and macrolides are the most widely used antibiotics in swine production (Holman & Chenier, 2013; Deckert et al., 2010). Chlortetracycline is the most commonly used antibiotic in both nursery and grower/finisher pigs, followed by oxytetracycline, tilmicosin, and tylosin (Apley et al., 2012; Dritz et al., 2002). The potential link between copper resistance (*tcrB*) and tetracycline and macrolide resistance genes raises the possibility that copper supplementation may exert selection pressures favoring antimicrobial resistant enterococci. Because tetracycline and erythromycin resistances are conferred by multiple mechanisms (especially, in Gram-positive bacteria), coded by many different genes located within the bacterial genome or on plasmids, there are varied fitness costs associated with their presence (Rice, 1998). These fitness costs help determine the baseline prevalence of strains of bacteria harboring these resistance factors when competing for space and resources within ecological niches in the gut. Therefore, a longitudinal study was conducted to investigate the effects of in-feed copper, chlortetracycline, and tylosin – alone or in combination – on selection and co-selection of resistance strains of fecal enterococci.

(**Portions of this work was presented at the Annual meeting of the Missouri Valley Branch of the American Society for Microbiology, Manhattan, Kansas, USA, 12th to 14th April, 2012;

Third American Society for Microbiology Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, Aix-en-Provence, France, 26th to 29th June, 2012).

Materials and Methods

Animals, dietary treatments, experimental design, and fecal sampling schedule

The use of animals and the experimental procedures followed were approved by the Kansas State University Animal Care and Use Committee (IACUC # 2773). The study consisted of 240 weaned pigs (34 days old) with an average body weight (BW) of 7.0 kg (\pm 1 kg). The piglets were housed in two barns, each with 40 pens (13×10 cm) and only 24 pens (in each barn) divided into 4 quadrants with 5 piglets per pen (n=120 piglets per barn) were used. The pens were assigned randomly to six dietary treatment groups (4 pens/treatment in each barn): 1) a basal diet (16.5 mg/kg of feed of copper as copper sulfate, control group), 2) a basal diet supplemented with 125 mg/kg of feed of copper as copper sulfate (Cu group), 3) basal diet supplemented with chlortetracycline (CTC 50[®], Alpharma, Fort Lee, NJ) at 22 mg/kg BW, 4) basal diet supplemented with tylosin (Tylan 100[®], Elanco Animal Health, Greenfield, IN) at 22 mg/kg BW, 5) basal diet with both 125 mg/kg of feed of copper and chlortetracycline at 22 mg/kg BW (CuCTC group), and 6) basal diet with both 125 mg/kg of feed of copper and tylosin at 22 mg/kg BW (CuTyl group). The six dietary treatments were arranged as an incomplete factorial design; diets with copper, chlortetracycline, tylosin, and the 2-way combinations of copper with chlortetracycline or tylosin. The same basal premix was included in all experimental diets during the entire study period. Copper in the basal diet was from the trace mineral premix which was analyzed at the Ward Labs, Kearney, NE to verify copper concentration. Premix batch sheets were routinely audited to verify the each of the treatment inclusion rate. The basal premix was kept constant throughout the course of the feeding trial. Each pen had a wire-mesh floor that

allowed for 0.3 m² per piglet. Each pen was provided with a self-feeder containing four holes and a water nipple such that animals had *ad libitum* access to feed and water. Pens were randomly allocated to sequential blocks of six with subsequent blocks order established by the first block so as to ensure that each treatment pen was in contact with an equal number of other treatment pens. Initially, all piglets received basal diet as per NRC recommendations. The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements. Following a week of acclimatization (pre-treatment) to the basal diet, piglets were fed treatment diets for 4 weeks followed by a washout (post-treatment) phase of two weeks during which piglets were fed the basal diet with no supplemental copper or antibiotics. Rectal fecal samples were collected randomly from 3 piglets per pen on days 0, 7, 14, 21, 28, and 35 and were placed in individual Whirl-Pak (Nasco, Ft. Atkinson, WI) bags, kept in a cooler with ice packs and transported to the Molecular Epidemiology and Microbial Ecology laboratory of Kansas State University. A schematic representation of the study design and the sampling schedule is shown in Fig. 1.

Isolation and species identification of *Enterococcus*

All the laboratory microbiological procedures were approved by the Kansas State University's Institutional Biosafety Committee (IBC # 625 & 626). Unless otherwise mentioned all culture media used were from Difco (Becton and Dickson, Sparks, MD). Fecal samples (approximately 1 g) were diluted in phosphate-buffered saline (9 mL) and 50 µL of fecal suspension was spread-plated onto M-*Enterococcus* agar and incubated at 42° C for 24 h. Three putative colonies (pin-point red, pink or metallic red) were selected from the plate and streaked onto blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37° C for 24 h. The genus confirmation was by esculin hydrolysis determined by inoculating a single colony into 100

μL of Enterococcosel broth in a 96-well microtiter plate (Becton and Dickson, Franklin Lakes, NJ) and incubating at 37° C for 4 h. Three esculin positive isolates from each fecal sample were stored in protect beads (Cryo-Vac[®], Round Rock, TX) at -80°C until further use.

Species identification of enterococcal isolates was carried out using multiplex PCR that identifies *E. faecium*, *E. faecalis*, *E. gallinarum* and *E. casseliflavus* (Jackson et al., 2004). The ATCC strains of *E. faecium* (ATCC 19434), *E. faecalis* (ATCC 19433), *E. gallinarum* (ATCC 49579) and *E. casseliflavus* (ATCC 25788) served as positive controls for multiplex PCR. Superoxide dismutase (*sodA*) gene PCR and sequence analysis (Poyart et al., 2000) was carried out for further species confirmation. The protect beads were streaked onto blood agar plates and DNA was isolated by suspending a single colony in nuclease free water with Chelex[®] 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiled for 10 min. Master mixes, primers and running conditions for the multiplex PCR were as described by Jackson et al. (2004). The primers and PCR conditions for *sodA* sequence analysis were as described by Poyart et al. (2000). The primers used were supplied by Invitrogen Life Technologies (Carlsbad, CA).

PCR detection of *trcB*, *erm(B)*, and *tet(M)* genes

DNA was extracted as before. The *trcB* gene in enterococcal isolates was detected by the procedure described by Hasman et al. (2006). A *trcB*-positive *E. faecium* strain obtained from Denmark (7430162-6) served as the positive control. The primers and PCR conditions for detection of *erm(B)* and *tet(M)* genes were as per Amachawadi et al. (2010). *Enterococcus faecium* BAA-2127 strain served as the positive control for detection of *erm(B)* and *tet(M)* genes. The primers were supplied by Integrated DNA Technologies (IDT, Coralville, IA).

Copper, tetracycline, tylosin and vancomycin susceptibility determinations

The *tcrB*-positive isolates (n=372) and an equal number of *tcrB*-negative isolates (n=372), selected by matching treatments and dates of collection, were included for copper and antibiotic susceptibility determinations. The agar dilution method was employed to determine copper susceptibilities of enterococcal isolates (Hasman et al., 2006). The two *tcrB*-positive strains (BAA-2127 and BAA-2128) served as positive controls. Mueller Hinton agar plates were prepared with copper concentrations added as copper sulfate (Sigma-Aldrich, Saint Louis, MO) at 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 or 40 mM, adjusted to pH 7.0, and were used for copper susceptibility determinations. The plates were spot-inoculated in duplicates with 10 µl of culture (adjusted to McFarland turbidity standard no. 0.5; Key Scientific Products, Stamford, TX). Results were recorded as growth or no growth after incubation at 37°C for 48 h. The susceptibility determination was done in replicates and duplicates.

Antibiotic susceptibility determinations were done following the micro-broth dilution method (CLSI, 2008). Antibiotic stock solutions were prepared in sterile distilled water to obtain a concentration of 1,000 µg/mL based on potency of antibiotics. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 µg/mL. The bacterial inocula were prepared by diluting (1:100) cultures grown in 10 mL Mueller Hinton II broth for 6 h and the concentrations were adjusted to 0.5 McFarland turbidity standards. The antimicrobial susceptibilities were performed in 96-well microtiter plates (Becton and Dickinson, Franklin Lakes, NJ). Plates were incubated at 37°C for 24 h and results were recorded as growth or no growth. Each concentration of the antibiotic was duplicated in the micro titer plate and MIC determinations were repeated with different inocula preparations.

Determinations of antimicrobial susceptibility profile

In order to determine the potential association between copper resistance and resistance to other antibiotics, a subset of *tcpB*-positive isolates (n=50) and an equal number of design-matched *tcpB*-negative isolates (n=50) were used to determine the susceptibility to a number of other antimicrobial agents. Minimum inhibitory concentrations (MIC) were determined by the micro-broth dilution method according to CLSI. Individual bacterial colonies were mixed with demineralized water (Trek Diagnostic Systems, Cleveland, OH) to match 0.5 McFarland turbidity standards. A 50 μ L aliquot of the bacterial inoculum was added to Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH) and vortexed. Then, 100 μ L of the broth was dispensed into Gram-positive NARMS panel plates (CMV3AGPF, Trek Diagnostics Systems, Cleveland, OH) with the aid of the Sensititre[®] automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH). Plates were incubated for 18 h at 37°C. The breakpoints were recorded as resistant, intermediate or sensitive based on the CLSI guidelines (CLSI, 2008), then intermediate categories were recoded as susceptible. *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection, Manassas, VA) isolates served as reference quality control strains for MIC testing.

Statistical Analysis

Data were analyzed using STATA SE version 12.1 (Stata Corp, College Station, TX). Descriptive analysis comparing effects of treatments on the probability of resistance was carried out prior to multivariable analysis. The likelihood ratio chi-square test (LR χ^2) was performed to examine the differences among two levels of the same variable without adjusting for clustering by treatment or other confounding variables. The data were considered to be multilevel and

longitudinal in nature since barns were clustered within pens, pens were clustered within each treatment, animals were clustered within pens, and within-animal dependency existed because of repeated sampling during the study period. The unbalanced factorial design with repeated measures and subsequent mixed model analysis allowed us to compare responses at specific times, over time, and via the available interactions [copper and chlortetracycline, copper and tylosin, treatment and period].

The multivariable analysis was carried out using the XTMELOGIT (multi-level hierarchical logistic regression) procedure in Stata. First, the full factorial model was constructed for the effects of copper, chlortetracycline, tylosin, and the period on the prevalence of *tcrB* gene (binary outcome; present or absent). Then, the model was tested for the interaction terms of copper with chlortetracycline and tylosin. From the final model, marginal predictions were obtained for the proportion of enterococcal isolates positive for *tcrB* gene, which also were estimated with 95% confidence intervals. The marginal means were plotted using final predictions from the full factorial model for *tcrB* prevalence by treatment and period. The main effects of copper, chlortetracycline, tylosin, and period along with their 2-way and 3-way interaction terms were tested.

Both copper and antibiotic susceptibility MIC determinations also were analyzed using survival analysis. The Sensititre data for 16 antimicrobials was considered based on the resistant or susceptible enterococci, both as outcomes (binary) and as the multidrug resistance profiles. The results were cross tabulated with each of the treatment groups, and *tcrB* status, to compare the proportion of enterococcal isolates resistant to each of the antimicrobial agents. A non-parametric, Kruskal-Wallis equality of population rank test on the population median was performed to measure the overall closeness (based on population medians) of average ranks in

the individual isolates to the average of either the *tcrB*-positive or *tcrB*-negative isolates. Results were considered significant at $P < 0.05$.

Results

Prevalence of the *tcrB*, *erm(B)*, and *tet(M)* genes in fecal enterococci

A total of 2,592 enterococcal isolates consisting of 72 isolates (3 isolates per fecal sample, 3 piglets per pen, and 8 pens per treatment) per treatment group (control, copper, chlortetracycline, tylosin, copper and chlortetracycline, and copper and tylosin) and sampling day (days 0, 7, 14, 21, 28, and 35) were obtained. A total of 372 isolates were positive for the *tcrB* gene (372/2,592; 14.3%); of these, 331 isolates (89%) were *E. faecium* and 41 (11%) were *E. faecalis*. Among the treatment groups, chlortetracycline treated pigs exhibited the highest overall prevalence (79/432; 18.3%) of *tcrB*-positive enterococci, followed by copper and chlortetracycline (75/432; 17.4%), copper and tylosin (68/432; 15.7%), copper (52/432; 12.0%), tylosin (51/432; 11.8%), and control (47/432; 10.8%) (Table 1). Ignoring the significant ($P < 0.05$) interaction of treatment by period, the overall prevalence of the *tcrB* gene among fecal enterococci was significantly affected both by treatment ($P = 0.003$) and day of sampling ($P < 0.0001$). Interestingly, examined alone copper supplementation had no effect on the prevalence of *tcrB* gene ($P = 0.313$). Among the other treatments, chlortetracycline had a significant effect ($P < 0.0001$), whereas tylosin supplementation also had no effect on the prevalence of *tcrB*-positive enterococci ($P = 0.551$). Also, the interactions of copper with chlortetracycline ($P = 0.623$) or tylosin ($P = 0.958$) had no effect on the prevalence of *tcrB* gene across all treatment periods. Across all treatment groups, the prevalence by sampling day of *tcrB*-positive enterococci varied significantly from day 0 (38/432; 8.8%), day 7 (76/432; 17.6%), day 14 (69/432; 15.9%), day 21

(116/432; 26.9%), day 28 (53/432; 12.3%), and day 35 (20/432; 4.6%), respectively. The overall prevalence by 'period' (pre-treatment, treatment, and post-treatment) of *tcrB* was also significant ($P < 0.0001$) and it varied from 8.8% (38/432), 20.1% (261/1,296), and 8.5% (73/864) in pre-treatment, treatment, and post-treatment periods, respectively.

From the final model, marginal prediction probabilities were determined with 95% confidence intervals for the proportions of positive *tcrB* enterococcal isolates. Overall, the marginal mean predictions for *tcrB*-positive enterococci was highest in the copper and tylosin group followed by copper and chlortetracycline, tylosin, chlortetracycline, copper, and control groups (Fig. 2). The proportion of *tcrB*-positive enterococci was highest in the treatment period, as expected, when compared to both pre-treatment and post-treatment periods. All *tcrB*-positive isolates (n=372) and *tcrB*-negative (n=372) were positive for both *erm*(B) and *tet*(M) genes.

MICs of copper, tetracycline, tylosin, and vancomycin

All the *tcrB*-positive isolates were resistant to copper, with an MIC that ranged from 12 to 24 mM. The *tcrB*-negative isolates were susceptible to copper with an MIC ranging from 4 to 8 mM. The two positive reference strains BAA-2127 and BAA-2128 had MICs of 20 and 24 mM, respectively. The median copper MIC (18 mM) of *tcrB*-positive isolates was higher ($P < 0.0001$) than that of *tcrB*-negative isolates (6 mM). All *tcrB*-positive and *tcrB*-negative isolates, based on Clinical Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2008) were resistant to tetracycline (≥ 16 μ g/mL) and tylosin (≥ 8 μ g/mL). The median vancomycin MIC (0.78 μ g/ml) of *tcrB*-positive isolates was higher ($P < 0.0001$) than that of *tcrB*-negative isolates (0.39 μ g/ml). However, based on the CLSI breakpoint all the isolates were classified as susceptible to vancomycin (≤ 4 μ g/ml). The failure function graphs (Figs. 3 and 4) represent the cumulative susceptibility of *tcrB*-positive and *tcrB*-negative isolates to copper and vancomycin, respectively.

Susceptibilities to other antimicrobials

Overall MIC distribution and susceptibility or resistance profiles for the subset of *tcpB*-positive isolates (n=50) and an equal number of randomly selected and design-matched *tcpB*-negative isolates (n=50), to each of 16 antimicrobials, are shown in the form of a 'squashtogram' (Table 2). The highest frequency of resistance was recorded for lincomycin (100%), followed by tetracycline (95%), kanamycin (76%), tylosin (71%), erythromycin (69%), quinupristin/dalfopristin (61%), streptomycin (53%), and gentamicin (14%). All the isolates tested were susceptible to chloramphenicol, linezolid and vancomycin. The proportion of isolates resistant to each antimicrobial did not differ between *tcpB*-positive and *tcpB*-negative enterococcal isolates ($P > 0.05$). A majority of isolates (95%; 95/100) were resistant to three or more antimicrobials, and these were classified as multidrug resistant (MDR). More often, the MDR isolates were associated with ERY (erythromycin), TET (tetracycline), TYL (tylosin), STR (streptomycin), and SYN (quinupristin/dalfopristin) resistant phenotypes in both *tcpB*-positive and *tcpB*-negative isolates. The MDR phenotypes varied among the isolates, with one isolates resistant to 9 antimicrobials, 15 isolates to 8 antimicrobials, 22 isolates to 7 antimicrobials, 11 isolates to 6 antimicrobials, 25 isolates to 5 antimicrobials, 12 isolates to 4 antimicrobials, and 9 isolates to 3 antimicrobials. The descriptive analyses showed that, overall, copper ($P = 0.034$), tylosin ($P = 0.025$), and copper and tylosin ($P = 0.011$) treatment groups had a significant effect on the proportion of MDR isolates. With respect to sampling periods, both treatment ($P = 0.035$) and post-treatment ($P = 0.038$) periods showed significant effects on the proportion of MDR isolates. The significant treatment and sampling period interaction was seen with copper in the post-treatment period ($P < 0.0001$) and copper and tylosin in the treatment period ($P = 0.019$).

Discussion

In the present study, we investigated the effects of in-feed copper, a commonly used antibiotic alternative, alone or in combination with chlortetracycline and tylosin, on selection and co-selection of antimicrobial resistance in fecal enterococci, a common gut commensal in swine. The overall prevalence of *tcrB*-positive enterococcal isolates was low (14.3%; 372/2,592) in piglets fed diets supplemented with copper alone or in combination with antibiotics. The low prevalence is in contrast to our previous studies in which we observed a positive dose-response of copper on the prevalence of *tcrB*-positive enterococci in piglets. The lower prevalence may be due to the shorter duration of treatment period (3 weeks) compared to our previous studies in which animals were on a copper supplemented diet for 5 to 7 weeks (Amachawadi et al., 2010, 2011). It is possible that 3 weeks of treatment duration was not sufficient for copper to exert a major selection pressure on *tcrB*-positive enterococci.

Danish studies have reported a prevalence of up to 76 % (45/59) of *tcrB*-positive enterococci in pigs at the time of slaughter (Hasman and Aarestrup, 2002). The *tcrB* gene was detected in only two species of enterococci, *E. faecium* and *E. faecalis*; meanwhile, and the number of *tcrB*-positive *E. faecium* (331/372; 89%) was higher than the number of *tcrB*-positive *E. faecalis* (41/372; 11%). The predominance of *tcrB*-positive *E. faecium* is in accordance with Danish studies; however, the *tcrB* gene was also detected in *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* (Hasman and Aarestrup, 2002; Hasman et al., 2006). Our results are in agreement with a study from Australia where highest prevalence of *tcrB* gene was detected among *E. faecium* and *E. faecalis* isolates (Fard et al., 2011). Even among the randomly chosen *tcrB*-negative isolates (n=372), we did not detect species other than *E. faecium* and *E. faecalis*. Factors such as age of the animal, diet formulation, use of feed grade antimicrobials, bacterial isolation procedures, and

geographic location may play a role in enterococcal species distribution in pigs (Jackson et al., 2004).

The prevalence of *tcrB*-positive enterococci was lower in the copper supplemented group than in the piglets that received either chlortetracycline or tylosin. However, the prevalence was higher in those piglets that received both copper with chlortetracycline or tylosin than the single inclusion of either of the antibiotic, which suggests a possible additive effect of copper with antibiotics. The reason for the additive response is not known and may possibly occur because the antibacterial spectrum and mode of action of copper are different from that of the two antibiotics (Stahly et al., 1980).

Antibiotics and other alternatives like copper are extensively used in swine production to improve growth and nutrient utilization. It is generally hypothesized that the altered gut micro flora in response to in-feed usage leads to improvement in growth and efficiency of feed utilization. Additionally, antimicrobials also could act by inhibiting the growth of opportunistic gut pathogens in animals (Kim et al., 2012). Copper promotes growth of piglets independent of growth promotion by antibiotics (Cromwell, 1998). Chlortetracycline and tylosin are the most commonly used in-feed antibiotics for growth promotion at subtherapeutic doses in the swine industry (Dritz et al., 2002). Chlortetracycline with broad spectrum activity is used to treat or control respiratory and enteric bacterial infections. Tylosin, a macrolide antibiotic is used to treat or prevent swine dysentery, and other bacterial infections, including arthritis, ileitis, and erysipelas in swine (Dritz et al., 2002). Earlier studies have reported that dietary supplementation of copper, chlortetracycline, and tylosin alone increased the daily BW gain in piglets (Stahly et al., 1980). However, it is a common practice to supplement copper with antibiotics like chlortetracycline and tylosin (Dritz et al., 2002). The supplementation of these

antimicrobials in combination has been shown to increase feed intake, growth rate, and feed efficiency indicating that the effects of copper and antibiotics are additive (Stahly et al., 1980).

Consistent with our earlier findings (Amachawadi et al., 2011 & 2012), both *tcrB*-positive and *tcrB*-negative isolates were positive for macrolide [*erm*(B)] and tetracycline resistance [*tet*(M)] genes. This is in agreement with the findings of an abundance of tetracycline resistance genes in enterococci of the swine gut, even if not exposed to the antibiotic (Zhu et al., 2013). Because these piglets harbor higher amounts of antibiotic resistance determinants, any attempt to reduce the level of resistance to these antimicrobials in piglets is not likely to be effective. This may pose a potential public health concern since piglets that are fed chlortetracycline and tylosin have a greater chance of carrying tetracycline and macrolide resistant enterococci in their gut. Evidence from earlier studies suggests that *erm*(B) is most widely distributed macrolide resistance gene in piglets (Jackson et al., 2004; Patterson et al., 2007). The tetracycline [*tet*(M)] and macrolide [*erm*(B)] resistance determinants have been shown to reside on mobile genetic elements, such as plasmids and or transposons (Kak and Chow, 2002) which increases the chances for horizontal gene transfer of resistance determinants among gut commensals or pathogens.

More often, both heavy metal and antibiotic resistance determinants are carried on either conjugative or nonconjugative transposons (Tremblay et al., 2012). Therefore, there is a possibility that the selection pressure imposed by heavy metals in the feed may indirectly coselect for antibiotic resistance. Earlier studies from Denmark and Spain have reported the presence of transferable copper resistance in enterococci and its co-selection or linkage to macrolide and glycopeptide resistance genes on the same plasmid (Hasman and Aarestrup, 2002; Hasman et al., 2006). However, we were unable to assess the co-selection because both *tcrB*-

positive and *tcrB*-negative isolates were phenotypically and genotypically resistant to macrolide and tetracyclines. None of our *tcrB*-positive isolates carried glycopeptide resistance mediated by the *vanA* gene. The absence of glycopeptide resistance in enterococcal isolates of piglets is likely because avoparcin (a glycopeptide feed additive) or other related glycopeptide derivatives have never been used in the US swine industry (Coque et al., 1996). The vancomycin MIC values were higher for *tcrB*-positive isolates (0.78 µg/mL) than for *tcrB*-negative isolates (0.39 µg/mL). The reason for the difference is not known, possibly non-specific shared resistance mechanism(s) such as efflux pumps imparted by the *tcrB* gene is likely responsible for the difference in susceptibility to vancomycin (Baker-Austin et al., 2006).

Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria is of significant public health concern. Generally, multidrug resistant (MDR) bacteria are characterized based on resistance to multiple antimicrobials (Magiorakos et al., 2012). A gram-positive bacterium is considered MDR if it is resistant to three or more antimicrobial classes (Pillar et al., 2008). Enterococci, particularly *E. faecium* and *E. faecalis*, rank among the leading causes of multidrug resistant hospital-acquired bacterial infections in humans and stands second among the most commonly isolated organism in the US hospitals (Palmer & Gilmore, 2010). Enterococci are intrinsically resistant to a broad range of antimicrobial agents including routinely used antibiotics, disinfectants, and heavy metals. More often, hospital-adapted multidrug resistant strains are known to harbor antibiotic resistance determinants on multiple mobile genetic elements (plasmids and or transposons), pathogenicity islands, and capsule loci (Gilmore et al., 2013). Recently, certain lineages of enterococci have evolved with abundant mobile DNA, plasmids, transposons, and numerous phages encoding multiple antibiotic resistant determinants.

These lineages are implicated to cause nosocomial infection outbreaks around the world (Palmer & Gilmore, 2010).

The MDR isolates containing different antimicrobial resistant determinants are of major concern as this provides a great opportunity for bacteria to survive under different antimicrobial selection pressures. Consistent with our earlier studies, the MDR enterococci were found to be resistant to both macrolide and tetracycline antibiotics with *erm*(B) and *tet*(M) resistant determinants, respectively. More often, both these resistance genes were carried on conjugative transposons Tn916 and Tn916/Tn1545, which have a broad bacterial host range and are capable of horizontal gene transfer to a variety of gram-positive and gram-negative bacteria in the gastrointestinal tract consortia. These plasmids and transposons are of major concern since they harbor antibiotic resistant determinants and have a potential to spread between species (Clewell, 1995). The metals used as feed additives contribute to the complex mixture of selective pressures in these biological niches.

Understanding the impact of feed grade antimicrobials, including heavy metals, on the evolutionary and ecological versatility and flexibility of the gut microbial consortia is important to mitigate the problem of antibiotic resistance. There is limited information on the impact of antibiotic alternatives on antimicrobial resistance in food animals (Aarestrup et al., 2008). The results of this study show that supplementation of copper, either alone or in combination with chlortetracycline or tylosin, has an additive effect on the prevalence of transferable copper resistance gene among fecal enterococci of piglets. Further studies are needed to understand the effects of feed grade antimicrobials and their association with copper resistance gene in enterococci to better understand the ecology and epidemiology of multidrug resistant enterococci in piglets.

Acknowledgements

Financial support for this project was provided by the National Pork Board (NPB # 10-121) under the ‘Antimicrobial Use and Resistance’ category. We thank Tiffany Mainini, Debra Frey, Getahun Agga, Mathew McGowan, Joshua Springfield, Jasmine Walters, and Melody Phillips and the personnel at the KSU Swine Teaching and Research Unit for their help. We would like to thank and appreciate the help of Mal Hoover in preparation of these images.

Conflict of interest statement

The authors declare that there are no conflicts of interest relating to this work.

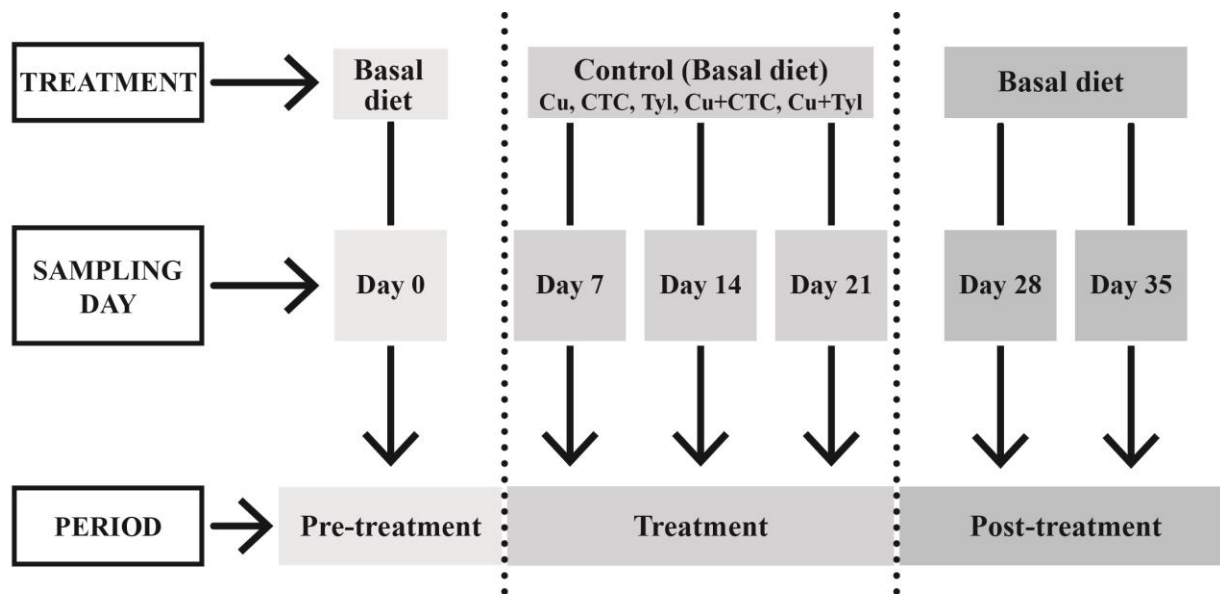


Figure 3.1: Schematic representation of the study design and sampling schedule

Cu = Copper, CTC = Chlortetracycline, Tyl = Tylosin, Cu + CTC = Copper and chlortetracycline, Cu + Tyl = Copper and tylosin

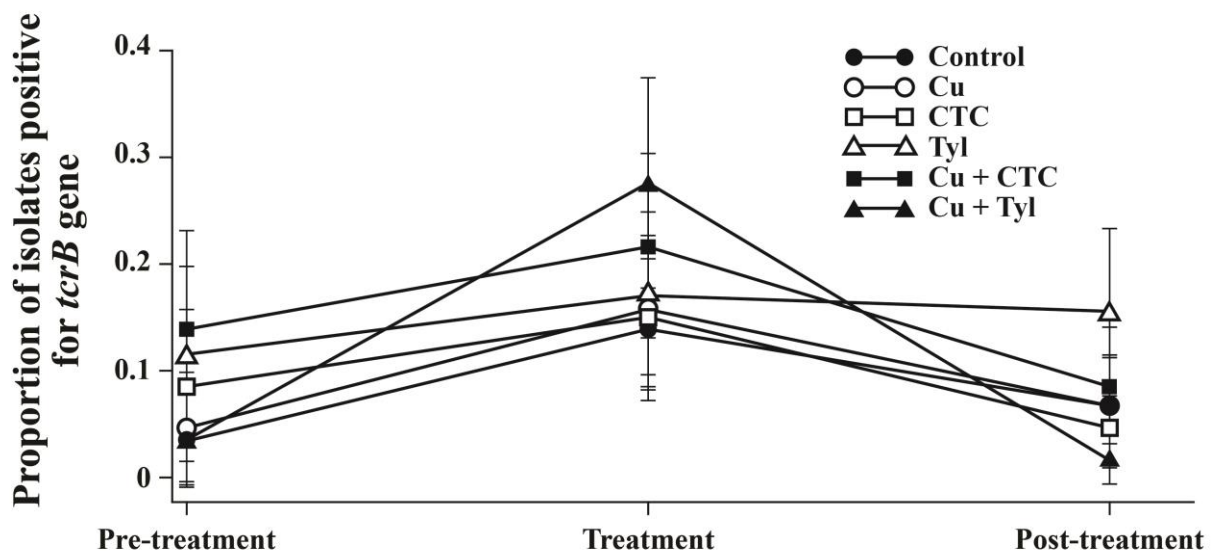


Figure 3.2: Marginal plot showing predicted probabilities with 95% confidence intervals for the prevalence of *tcrB*-positive enterococcal isolates for each treatment groups within each study period.

Control, Cu = Copper, CTC = Chlortetracycline, Tyl = Tylosin, Cu + CTC = Copper and chlortetracycline, Cu + Tyl = Copper and tylosin

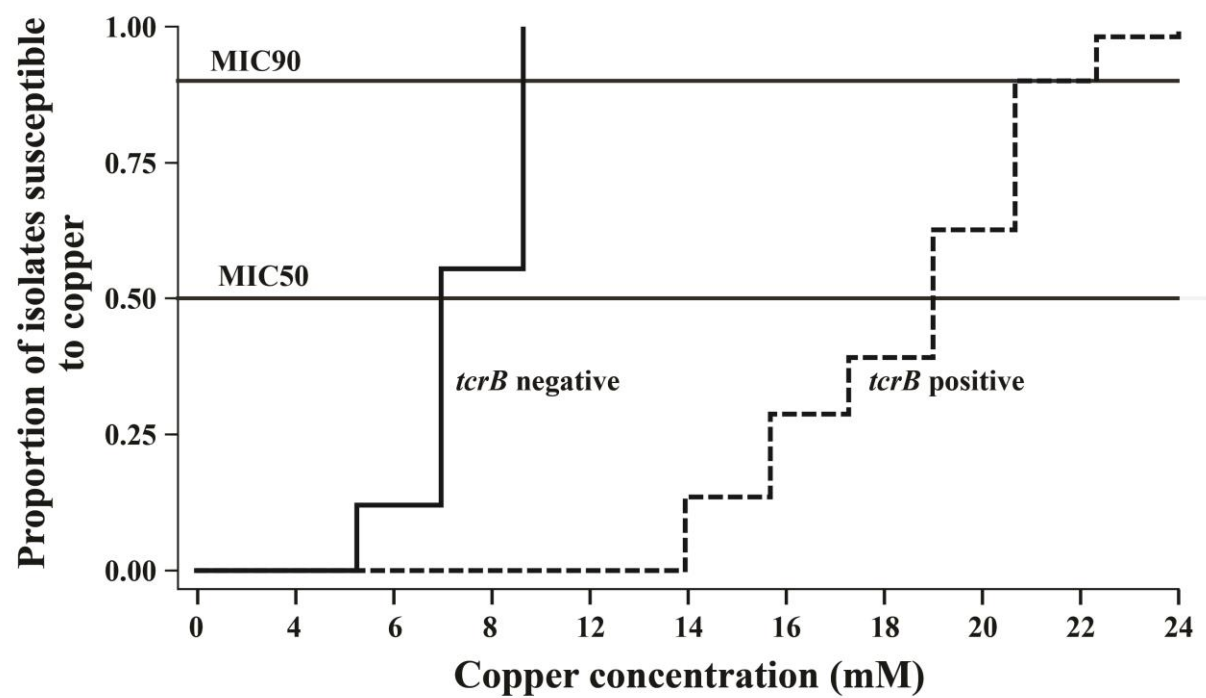


Figure 3.3: Failure function graph depicting cumulative susceptibilities of *tcrB*-positive and *tcrB*-negative isolates of fecal enterococci in relation to copper concentration

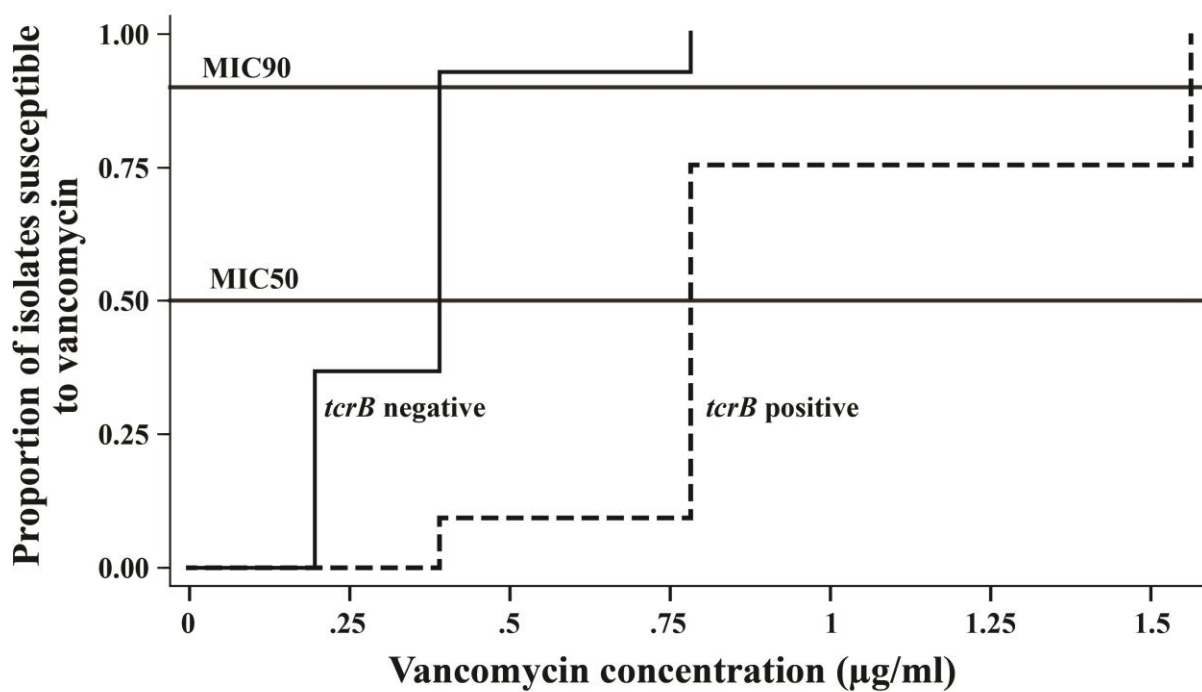


Figure 3.4: Failure function graph depicting cumulative susceptibilities of *tcrB*-positive and *tcrB*-negative isolates of fecal enterococci in relation to vancomycin concentration

Table 3.1: Prevalence of *tcrB*-positive enterococcal isolates in piglets fed diets with copper (Cu), chlortetracycline (CTC), and tylosin alone or in combination

		Number of <i>tcrB</i> -positive isolates/Number of isolates tested						Prevalence* (%)
Period**		Pre-treatment	Treatment				Post-treatment	
Sampling days		0	7	14	21	28	35	
Dietary treatments	Control	3/72	12/72	8/72	13/72	10/72	1/72	10.8
	Cu	4/72	10/72	12/72	15/72	8/72	3/72	12.0
	CTC	10/72	14/72	10/72	19/72	19/72	7/72	18.3
	Tyl	7/72	13/72	7/72	16/72	2/72	6/72	11.8
	Cu+CTC	11/72	9/72	11/72	30/72	11/72	3/72	17.4
	Cu+Tyl	3/72	18/72	21/72	23/72	3/72	0/72	15.7

Treatment groups: Control, Cu = Copper, CTC = Chlortetracycline, Tyl = Tylosin, Cu + CTC = Copper and chlortetracycline, Cu + Tyl = Copper and tylosin

**Effect of trial period was significant at $P < 0.001$

Table 3.2: The multidrug resistance profile of both *tcrB*-positive (n=50) and *tcrB*-negative (n=50) enterococcal isolates

Antimicrobials*	% Resistant	95% CI	Distribution of isolates (%) with minimum inhibitory concentrations in µg/mL																	
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Chloramphenicol	0	0-3.6*								2	32	64	2	0						
Ciprofloxacin	4	1.1-9.9				0	0	17	49	30	3	1								
Daptomycin	3	0.6-8.5					9	11	15	28	34	3	0							
Erythromycin	69	59-77.8					21	3	3	3	1	4	65							
Gentamicin	14	7.8-22.3													86	3	1	3	7	
Kanamycin	76	66.4-83.9													24	5	27	4	40	
Lincomycin	100	96.3-100							0	0	0	71	2	27						
Linezolid	0	0-3.6						2	12	85	1	0								
Nitrofurantoin	5	1.64-11.2								0	1	17	14	39	24	5				
Penicillin	3	0.6-8.51					7	7	12	20	43	8	3							
Quinupristin / dalfopristin	61	51.7-71.5						0	6	33	30	14	7	10						
Streptomycin	53	42.7-63.0																47	7	46
Tetracycline	95	88.7-98.3							2	0	2	1	1	2	92					
Tigecycline	1	0.02-5.4	0	0	35	62	2	1												
Tylosin tartarate	71	61-79.6					0	1	1	9	10	4	4	3	68					
Vancomycin	0	0-3.6*					6	47	14	11	17	5	0	0	0					

The whitened area indicates the range of each antimicrobial concentration tested and shaded grey areas fall outside the range of tested antimicrobial concentrations. The vertical bar indicates the CLSI or NARMS consensus breakpoints for resistance.

*One sided, 97.5% confidence interval (used only when the estimate was zero).

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CHAPTER 4 - Occurrence of the transferable copper resistance gene, *tcrB*, among fecal enterococci of U. S. feedlot cattle fed copper-supplemented diets

Introduction

Copper (Cu) is an essential micronutrient required for various biochemical functions in prokaryotic and eukaryotic cells (1). In livestock, copper requirements are met by inclusion of copper, generally as sulfate, as part of a multi-mineral supplement in the diet. Inclusion of copper in diets at elevated concentrations, particularly in cattle and swine, has growth promotional effects (2). In cattle, copper is included at concentrations of 10 to 100 mg/kg of diet to reduce mortality and morbidity in calves and for growth promotion in feedlot cattle (3). Copper in excess is very reactive and toxic to cells; therefore, intracellular Cu^{++} concentration is highly regulated (4). In biological systems, copper homeostasis is mediated by the expression of copper chaperone proteins, Cu^{++} transporting proteins, Cu^{++} transporting ATPases, and Cu^{++} chaperone for Cu-Zn superoxide dismutase, which help in the regulation of Cu uptake, intracellular transport, and export (1). A group of membrane spanning proteins called CPx-ATPases, encoded by a four-gene operon called *copYZAB*, regulate copper homeostasis in gram-positive bacteria, such as *Enterococcus hirae* (5). Acquired copper resistance has been reported both in gram-positive (6, 7) as well as in gram-negative bacteria (8). A transferable copper resistance gene, *tcrB*, in *Enterococcus faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* (6, 9) was first reported in Denmark. In Europe, the *tcrB* gene often is found on a

plasmid, which also carries *erm*(B) and *vanA* genes encoding resistance to macrolides and glycopeptides, respectively (6, 9). We have previously reported the occurrence and prevalence of the *tcrB* gene among fecal *E. faecium* and *E. faecalis* isolates of piglets fed elevated concentrations of copper in the diet (10, 11). The *tcrB* gene in fecal *E. faecium* and *E. faecalis* of piglets in the U.S. was on a plasmid which carried macrolide [*erm*(B)] and tetracycline [*tet*(M)] resistance genes, but no *vanA* genes. The potential genetic link between copper and antibiotic resistance is of interest because tylosin, a macrolide, and tetracyclines are widely used as feed additives in feedlot cattle (12). The present study was conducted to determine the occurrence of *tcrB* gene in fecal enterococcal isolates of cattle supplemented with elevated (100 mg/kg) levels of copper in the diet and to determine phenotypic susceptibilities to copper, tetracycline, tylosin, and vancomycin. Because enterococci, a common gut commensal of animals and humans (13, 14), are a frequent cause of nosocomial infections in humans, as well as sporadic infections in animals (15, 16), we also explored the presence of major virulence genes. Multi-locus variable number tandem repeat analysis (MLVA) was performed to determine the clonal relationship of the enterococcal isolates to the MLVA types (MT) reported in the global epidemiological database. Additionally, Southern blot hybridization was performed to show that *tcrB* was located on a plasmid and a conjugation assay was conducted to demonstrate the rate of transfer and co-transfer of *tcrB*, *erm*(B), and *tet*(M) genes.

(Portions of this work were presented at the Annual Meeting of the Missouri Valley Branch of the American Society for Microbiology, Manhattan, Kansas, USA, 12th to 14th April, 2012, and the Third American Society for Microbiology Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, Aix-en-Provence, France, 26th to 29th June, 2012).

Materials and Methods

The use of animals and the procedures employed were approved by the Kansas State University Animal Care and Use Committee.

Animals, study design, and sampling schedule

Crossbred yearling heifers (n=261) were blocked by initial body weight into heavy (330 ± 11 kg) and light (280 ± 16 kg) weight groups and assigned randomly to 24 pens with 10 or 11 heifers per pen. The pens were assigned, in a randomized complete block design, to a 2×2 factorial arrangement of treatments of dietary copper, normal (10 mg/kg of feed) or elevated (100 mg/kg of feed) and a commercial nutritional supplement (Linpro®, Oleet Processing Ltd., Regina, SK, Canada) at 0 or 10 % of the diet on a dry matter (DM) basis. The nutritional supplement was an extruded product of full-fat flaxseed and split field peas with added vitamins and minerals (22% crude protein; 23% fat) used as an energy and protein supplement in cattle diets. The basal diet included (on a DM basis) 35% dry-rolled corn grain, 35% wet corn gluten feed, 15% pelleted soybean hulls, 10% corn silage, and 5% minerals (1,000 mg of sodium, 1,500 mg of chlorine, 7,000 mg of calcium, 7,000 mg of potassium, 0.10 mg of cobalt, 0.6 mg of iodine, 0.25 mg of selenium, 60 mg of manganese, and 60 mg of zinc per kg of the feed), vitamins (2,200 IU of vitamin A and 22 IU of vitamin E per kg of feed), and antibiotics (300 mg/animal/d of monensin [Rumensin®, Elanco Animal Health, Greenfield, IN] and 90 mg/animal/d of tylosin [Tylan®, Elanco Animal Health]). Cattle were fed once daily and had *ad libitum* access to feed and water. Eight pen-floor fecal samples were collected with new plastic spoons from freshly defecated fecal pats from each pen (n=24 pens) on day 116 (a day before heifers in the heavy weight group were shipped for harvest) and from the remaining 12 pens

housing light weight heifers on day 131, which was a day before the shipment of the remaining lighter cattle for harvest. Spoons with feces were placed in individual Whirl-Pak[®] (Nasco, Ft. Atkinson, WI) bags, and transported in a cooler with ice packs to the Molecular Epidemiology and Microbial Ecology (ME²) laboratory of Kansas State University. A schematic representation of the study design and sampling schedule is shown in Fig. 1.

Isolation and speciation of *Enterococcus*

The culture media used in the study were from BD (Becton and Dickson, Sparks, MD). Approximately 1 g of each fecal sample was mixed in 10 mL of sterile phosphate-buffered saline and 50 µL of the suspension were spread-plated onto M-*Enterococcus* agar and incubated at 42°C for 24 h. Two putative colonies (pin-point red, pink or metallic red) were picked from each plate and streaked onto blood agar plates, and incubated overnight at 37° C for 24 h. An esculin hydrolysis test was conducted, for presumptive genus identification, by inoculating a single colony into 100 µL of Enterococcosel broth in a 96-well microtiter plate (Becton and Dickson, Franklin Lakes, NJ) and incubating at 37°C for 4 h. Esculin-positive isolates were stored in protect beads (Cryo-Vac[®], Round Rock, TX) at -80°C until further use. Species identification of enterococcal isolates was performed by multiplex PCR and superoxide dismutase (*sodA*) gene sequence analysis. The multiplex PCR identifies *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus* (17). DNA extraction was performed by suspending a single colony from the blood agar plate in nuclease-free water with Chelex[®] 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiling for 10 min. The ATCC strains of *E. faecium* (ATCC 19434), *E. faecalis* (ATCC 19433), *E. gallinarum* (ATCC 49579), and *E. casseliflavus* (ATCC 25788) were used as positive controls. Superoxide dismutase (*sodA*) gene PCR and gene sequence analysis was carried out for further confirmation of species (18).

PCR detections of *tcrB*, *erm(B)*, *tet(M)*, *vanA*, *vanB* and virulence genes

The DNA of enterococcal isolates were extracted as described above. All primers used in the study were supplied by Invitrogen Life Technologies (Carlsbad, CA). The PCR assay for *tcrB* was as described by Hasman et al. (9) and a *tcrB*-positive *E. faecium* isolate (7430275-4) obtained from those authors' laboratory in Denmark served as the positive control. The primers and PCR conditions for the detection of *erm(B)*, and *tet(M)* genes were according to Amachawadi et al. (10). *E. faecalis* MMH 594 (Dr. Lynn Hancock, Division of Biology, Kansas State University) and an *E. coli* strain harboring plasmid pFD 310 (19) served as positive controls for *erm(B)* and *tet(M)* genes, respectively. The *vanA* and *vanB* gene primers and PCR conditions were based on procedures described by Kariyama et al. (20). *Enterococcus faecium* (ATCC 51559) and *E. faecalis* (V583) were used as positive controls for *vanA* and *vanB* genes, respectively. A multiplex PCR was performed to identify *asaI* (aggregation substance), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *hyl* (hyaluronidase) genes (Vankerckhoven et al. (21). *Enterococcus faecalis* MMH594 again was used as a positive control. A subset of *tcrB*-negative isolates, equal to the number of *tcrB*-positive isolates (n=22; matched by pen, treatment, and date of sampling) was speciated and included for detection and statistical analysis of antibiotic resistance and virulence genes.

Copper and antibiotic susceptibility determinations

Copper susceptibilities of enterococcal isolates were determined by the agar dilution method (9). Two *tcrB*-positive strains from our previous study (BAA-2127 and BAA-2128; 10, 11) served as positive controls. The copper sulfate-Mueller Hinton agar plates were prepared with concentrations of copper sulfate (Fischer Scientific, Fair Lawn, NJ) of 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, or 40 mM. Bacterial inocula were prepared by growing isolates in Mueller Hinton II

broth for 5-6 h and turbidity adjusted to a McFarland standard of 0.5. The plates were spot inoculated with 20 μ L of bacterial inoculum and incubated for 36 to 48 h at 37° C to determine growth or no growth. The MICs of tetracycline, tylosin, and vancomycin (Sigma-Aldrich, St. Louis, MO) were determined by micro-broth dilution method as per CLSI guidelines (22). Antibiotic concentrations tested were based on the potency of the antibiotics. Stock solutions were prepared with sterile distilled water to obtain an initial concentration of 1,000 μ g/mL. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, and 0.098 μ g/mL. Bacterial cultures were grown in 10 mL of Mueller-Hinton II broth for 6 h and the inocula concentrations were adjusted to 0.5 McFarland turbidity standards. The susceptibility determinations for antibiotics were carried out using 96-well micro titer plates (Becton and Dickson) by incubating plates with the bacterial inocula at 37°C for 24 h and results were recorded as growth or no growth. MIC determinations were carried out on both *tcrB*-positive (n=22) and *tcrB*-negative (n=22) isolates.

Transferability of *tcrB* gene

Transferability of the *tcrB* gene from *tcrB*-positive to *tcrB*-negative strains via conjugation was determined by filter-mating procedure (23). *Enterococcus faecium* strain TX5034, resistant to tetracycline [positive for *tet*(M); MIC >100 μ g/mL] and susceptible to spectinomycin [MIC = 12.5 μ g/mL] and *E. faecalis* strain OG1SSp, resistant to spectinomycin [MIC >100 μ g/mL] and susceptible to tetracycline [negative for *tet*(M); MIC = 0.78 μ g/mL] were used as controls (these strains were provided by Dr. Barbara E. Murray, University of Texas-Houston Medical School). The *tcrB*-positive donor strains were resistant to tetracycline [positive for *tet*(M)] and susceptible to spectinomycin. The *tcrB*-negative recipient strains were resistant to spectinomycin and susceptible to tetracycline [negative for *tet*(M)]. Filter mating was carried out with donors and

recipients in a ratio of 1:10. Overnight cultures of donors (0.5 mL) and recipients (4.5 mL) were mixed followed by cell collection using 0.22 µm nitrocellulose membrane filter. The strains were selected on brain heart infusion (BHI) agar for the *tcrB*-positive (donor: 40 µg/mL of tetracycline), *tcrB*-negative (recipient: 500 µg/mL of spectinomycin), and the transconjugants using both tetracycline and spectinomycin at those concentrations. The donor and recipient strains were grown on BHI agar plates containing 40 µg/mL of tetracycline and 500 µg/mL of spectinomycin, respectively. The transfer frequency was expressed as the number of CFU transconjugants per recipient CFU. The resultant transconjugants were also tested for *tcrB*, *erm(B)*, and *tet(M)* genes by PCR and their susceptibilities to copper by using the procedures described above.

Southern blot hybridization

Southern blot hybridization was performed on three *tcrB*-positive *E. faecium* strains as per the procedure described in Amachawadi et al. (11). The procedure was performed using digoxigenin (DIG) High Prime labeling and detection starter kit II (Roche Diagnostics, Indianapolis, IN).

Multiple-locus variable number tandem repeat analysis (MLVA) of *tcrB*-positive enterococci

MLVA, a typing scheme based on six different tandem repeat loci for *E. faecium*, was performed as described by Top et al. (24). Six variable number tandem loci (VNTR), VNTR-1, VNTR-2, VNTR-7, VNTR-8, VNTR-9, VNTR-10, were used for genotyping of *tcrB*-positive enterococcal isolates. The alleles were defined based on the amplicon size difference at each locus. The MLVA types (MT) were assigned using the MLVA database at

<http://www.umcutrecht.nl/subsite/MLVA/Method/>. The population snapshot for MLVA types were performed using eBURST analysis at <http://eburst.mlst.net>.

Nucleotide sequence accession number

The partial nucleotide sequence of one *tcrB*-positive *E. faecium* (strain 11A) was deposited in the NCBI GenBank database (Accession number JN660793).

Statistical analyses

The statistical analysis was performed using STATA v.12.1 (StataCorp, College Station, TX). Bivariate descriptive statistics of gene or phenotype prevalence by diet type were assessed prior to multivariable analysis. The Likelihood ratio Chi-square test was performed to statistically assess unadjusted differences in prevalence proportion among two variables or between two genotypes [*tcrB* vs. *erm*(B), *tcrB* vs. *tet*(M), *erm*(B) vs. *tet*(M)]. Thereafter, the multivariable adjusted effects of important variables such as block, copper supplementation, commercial nutritional supplement (Linpro[®]), and sampling days on the prevalence of *tcrB*, *erm*(B), and *tet*(M) genes in enterococci also were assessed. The association of each variable with prevalence was tested using mixed (random and fixed) effects logistic regression. Pen was considered as the experimental unit. The data were considered multilevel and longitudinal in nature since some of the pens were sampled twice, animals were clustered within each pen, multiple isolates within each fecal sample were analyzed, and there existed also the potential presence of within-animal dependencies because of the possibility of repeated sampling of the same animal's feces throughout the study period. The data were analyzed for each of the main effects of copper supplementation, commercial nutritional supplement (Linpro[®]), blocking (by body weight), and the sampling days on the prevalence of *tcrB* gene first, and then the interaction terms among remaining main effects. Copper and antibiotic MIC susceptibility data were

analyzed using a log₂MIC-transformed and adapted survival analysis and the MIC was expressed and compared as the median (MIC₅₀) after a log rank assumption for normality (25). The differences in transfer frequency (number of transconjugants per recipient) of *tcrB* gene for both intra- and inter-species conjugation were assessed using the two-sample t test following a normality assessment using the Shapiro-Wilk normality test. The transfer frequencies were also tested for equality of variance (variance comparison test) and independence was assumed.

Results

Prevalence of *tcrB*, *erm*(B), *tet*(M), and virulence genes among fecal enterococci

A total of 576 enterococcal isolates, comprising 288 isolates in each of the two main trial groups, control or copper-supplemented, from fecal samples collected on days 116 and 131, were obtained. Twenty-two (3.8%) of the 576 isolates were positive for the *tcrB* gene. Of the 22 *tcrB*-positive isolates, 2 (2/288; 0.7%) were from cattle fed the basal diet with a normal concentration of copper (10 mg/kg), while 20 (20/288; 6.9%) were from cattle fed the basal diet supplemented with a higher concentration (100 mg/kg) of copper (Table 1). The overall prevalence of *tcrB*-positive enterococcal isolates was higher ($P < 0.001$) in the copper-supplemented group than in the control group (i.e., 6.9 vs. 0.7%). The prevalence of *tcrB* gene also was affected by sampling period ($P = 0.03$), and the initial blocking of heifers by body weight ($P = 0.03$). The Linpro[®] supplementation had no effect ($P = 1.0$) on the overall prevalence of *tcrB* gene. Based on multiplex PCR and *sodA* gene sequence analyses, all *tcrB*-positive (n=22) and *tcrB*-negative (n=22) isolates (selected by matching pen, date of sampling, and treatments) were *E. faecium*. All *tcrB*-positive (n=22) and *tcrB*-negative (n=22) isolates were positive for *erm*(B) and *tet*(M) and negative for *vanA* and *vanB* genes. The prevalence of *erm*(B) and *tet*(M) genes or multi-gene types (*tcrB*, *erm*(B) and *tet*(M) alone or in combination)

is shown in Table 1. Forty-one of the 288 isolates (14.2%) of the control group and fifty-six of the 288 isolates (19.4%) of the copper group were negative for *tcrB*, *erm(B)*, and *tet(M)* genes. The overall prevalence (control and copper groups) of *erm(B)*, *tet(M)* and *erm(B)* and *tet(M)* combinations in enterococcal isolates were 53.3%, 62.3%, and 36.2% respectively (Table 1). Both *tcrB*-positive (n=22) and *tcrB*-negative (n=22) isolates were negative for *asaI* (aggregation substance), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *hyl* (hyaluronidase) genes.

Phenotypic susceptibilities to copper, tetracycline, tylosin, and vancomycin

All *tcrB*-positive isolates grew on MH agar containing copper at a concentration of 20 or 24 mM. However, the *tcrB*-negative isolates did not grow on MH agar containing copper at a concentration of > 8 mM. The two positive reference strains BAA-2127 and BAA-2128 had MIC's of 20 and 24 mM, respectively. The median copper MIC of *tcrB*-positive isolates (22 mM) was higher ($P < 0.001$) than that of the *tcrB*-negative (4 mM) isolates (Fig. 2). The failure-function graph (Fig. 2) represents the cumulative susceptibility of both *tcrB*-positive and – negative isolates. Both *tcrB*-positive and -negative isolates were resistant (based on CLSI breakpoint; ≥ 16 $\mu\text{g/mL}$) to tetracycline with a median MIC of >100 $\mu\text{g/mL}$ and 37.5 $\mu\text{g/mL}$, respectively. The median tetracycline MIC of *tcrB*-positive isolates was significantly higher ($P < 0.001$) than that of the *tcrB*-negative isolates. All *tcrB*-positive isolates were resistant (based on CLSI breakpoint; ≥ 32 $\mu\text{g/mL}$) to tylosin with a median MIC of 50 $\mu\text{g/mL}$ and all *tcrB*-negative isolates were susceptible (based on CLSI breakpoint; ≤ 8 $\mu\text{g/mL}$) to tylosin with a median MIC of 1.6 $\mu\text{g/mL}$, respectively ($P < 0.001$). Both *tcrB*-positive and -negative isolates were susceptible (based on CLSI breakpoint; ≤ 4 $\mu\text{g/mL}$) to vancomycin though the median MIC

of *tcrB*-positive (0.39 µg/mL) was significantly higher ($P < 0.001$) than that of *tcrB*-negative (0.2 µg/mL) isolates. The median MIC results are shown in Table 2.

Transferability of *tcrB* gene

All 22 *tcrB*-positive *E. faecium* isolates were used to demonstrate both intraspecies (*E. faecium*) and interspecies (*E. faecalis*) transferability of *tcrB* gene by conjugation. The mean transfer frequencies of 22 isolates for both intra- and inter-species conjugations were 1.7×10^{-5} and 2.0×10^{-5} , respectively. The conjugation frequency was higher in inter-species conjugation when compared to intra-species ($P = 0.02$). The transconjugants were positive for *tcrB*, *erm*(B), and *tet*(M) genes and phenotypically resistant to copper, tylosin, and tetracycline. The median copper MIC of the transconjugants was 20 mM.

Southern hybridization

Hybridization of DNA from *tcrB*-positive *E. faecium* isolates with *tcrB* or *erm*(B) gene probes yielded distinct bands of ~175-180 kbp size (Fig. 3). The *tcrB*-negative isolates showed bands ranging from 150-170 kbp in size. However, probing of *tcrB*-negative isolates with *tcrB* gene showed no hybridization.

Multiple-locus variable number tandem repeat analysis (MLVA)

The MLVA procedure revealed 20 novel MLVA types (MTs) as shown in Fig. 4. Among 22 *tcrB*-positive isolates, two isolates were clonally related to the existing MT 7 and MT 190 in the database. The other 20 *tcrB*-positive isolates with unassigned MT's were submitted to the curator (Dr. J. Top, University of Utrecht, Netherlands) for sequence types and cluster assignment and also to update the global epidemiological database of *E. faecium*. The MT 449 isolate was closely related to the predominant hospital-adapted clone MT 12, which had been isolated from blood and feces of hospitalized patients. On eBURST, clustering of eight of our

isolates suggests that they share one or more alleles at loci with MT 12 and MT 1, which were from hospitalized patients or clinics in the European Union. Fourteen of our *tcrB*-positive isolates were clonally different (437, 438, 440, 441, 442, 444, 445, 446, 447, 450, 452, 454, 455, and 456) and did not share alleles at any loci with existing MTs.

Discussion

This study, designed primarily to evaluate the effects of feeding an elevated concentration of copper (125 mg/kg of the feed) and a commercial energy and protein supplement (Linpro[®]) on cattle performance, was utilized to investigate the effects of feeding elevated concentrations of copper on the prevalence of *tcrB*-mediated, copper resistant enterococci in feedlot cattle feces. In the US, cattle diets are supplemented with in-feed antimicrobials for growth promotion, to increase feed efficiency, to decrease respiratory infections, or to reduce the prevalence of liver abscesses at slaughter (26). Most in-feed antimicrobials are known to alter the gut microbial flora, thereby reducing fermentation loss of nutrients and suppressing pathogens (27). There is concern that gut bacterial commensals and pathogens in food-producing animals develop and propagate resistance to antibiotics and thereafter contaminate food products, providing a direct pathway for transmission to humans. Therefore, alternatives to antibiotics, including feeding of heavy metals and particularly copper and zinc, have been employed as feed additives in food animal production (2).

In the present study, cattle diets were supplemented with a commercial energy and protein supplement (Linpro[®]) to evaluate its performance benefits. This factor was a nuisance parameter for our study purposes, and as expected, the commercial supplement had absolutely no effect on the prevalence of *tcrB*-mediated copper resistant fecal enterococci ($P = 1.0$). In cattle fed a

higher concentration of copper, only a small proportion (6.9%; 20/288) of fecal enterococcal isolates harbored the *tcrB* gene and these isolates also were phenotypically resistant to copper. However, the higher prevalence of *tcrB* gene in cattle supplemented with copper compared with the control group (6.9% vs. 0.7%) suggests the exertion of selection pressures of feeding elevated level of copper. Similar low prevalence (4.6% and 11.9%) were reported for enterococcal isolates from piglets fed an elevated level (125 mg/kg) of copper in two studies (10, 11). In contrast, Danish studies have reported a prevalence of 76% of copper resistant enterococci in pig feces obtained at the time of slaughter (6), perhaps because of higher levels (175 to 250 mg/kg) of copper and supplementation for a longer period of time during their production cycle, starting from birth to slaughter.

All *tcrB*-positive enterococcal isolates in our study were *E. faecium*, which is in contrast to studies from Denmark and Australia that have reported the occurrence of the *tcrB* gene not only in *E. faecium* but also in *E. faecalis*, *E. gallinarum*, *E. durans*, *E. casseliflavus*, and *E. mundtii* (9, 28). Among *Enterococcus* species, *E. faecium* and *E. faecalis* most commonly colonize the gut in humans and animals (29). In our study, there were no *E. faecalis* isolates among the 22 *tcrB*-positive enterococci. Because only a limited number (n=22) of *tcrB*-negative isolates were speciated, occurrence of other species in cattle feces cannot be ruled out; in fact, it is more likely than not. Fluckey et al. (30), in a study on the species distribution of *Enterococcus* in cattle in the US, have reported *E. faecium*, *E. faecalis* and *E. durans* as the dominant species. Factors such as age of the animal, diet, type and level of supplement used in the diet, in-feed antibiotics, the geographical location and even isolation methods employed are likely to have an impact on species distribution (17).

In Denmark, the copper resistance in enterococci was shown to co-select both macrolide and glycopeptide resistance (6). In our study, all 22 *tcrB*-positive isolates contained the *erm*(B) gene and were phenotypically resistant to tylosin; this is not surprising since the diet included tylosin, a macrolide that is routinely included in feedlot cattle diets in the US for the prevention of liver abscesses (12). The *erm*(B) gene is the most common macrolide resistance genetic determinant present in animal and human enterococcal isolates (31). Because all *tcrB*-negative isolates were also positive for *erm*(B) gene, an assessment of co-selection could not be made in our study (12). Perhaps the prevalence of *erm*(B) in all the isolates tested is reflective of the selection pressure applied over a long period with the wide spread use of tylosin, a macrolide widely used to control liver abscesses in feedlot cattle (12). All *tcrB*-positive isolates from our study also contained the *tet*(M) gene, which is in agreement with our earlier studies that showed all *tcrB*-positive *E. faecium* and *E. faecalis* isolates of piglets to contain the *tet*(M) gene (10, 11). Unlike for *erm*(B), there were *tcrB*-negative isolates that also were negative for *tet*(M). Both *erm*(B) and *tet*(M) genes are carried on conjugative transposons, Tn916 and Tn916/Tn1545, which have a broad bacterial host range and a strong capacity for horizontal gene transfer among gut commensals (32). Our *tcrB*-positive isolates did not contain either *vanA* or *vanB* genes and were phenotypically susceptible to vancomycin. Although susceptible based on CLSI breakpoint, the vancomycin MIC values were significantly ($P < 0.001$) higher for *tcrB*-positive isolates than the *tcrB*-negative isolates. This suggests a non-specific function, such as an efflux pump used for copper homeostasis, might also aid in ridding bacterial cells of vancomycin, though not at an MIC sufficient to confer resistance. The prevalence of vancomycin resistant enterococci in Europe is likely because of the historical use of avoparcin, a glycopeptide. Avoparcin was once applied extensively in livestock feed in Europe for growth promotion in broiler chickens, pigs,

calves and beef cattle (33). The absence of *vanA* or *vanB* genes in our study is not surprising since avoparcin was never approved for use in animal agriculture in the US (34) and an FDA prohibition on extra-label use of glycopeptides in animal agriculture has been in place since 1997 (35).

Southern blot hybridization revealed that the *tcrB* gene is located on a ~180 kb plasmid. In our previous study in nursery piglets, the *tcrB* gene was on a conjugative mobilizable plasmid of size ~194 kb (11). Earlier studies from Europe have reported the presence of *tcrB*, *erm(B)*, and *vanA* genes on a 175-kb plasmid in an *E. faecium* isolate from a pig (6). Generally, megaplasms are known to harbor antibiotic and heavy metal resistance genes (36). Conjugation systems involving plasmids and transposons are abundant in the genus *Enterococcus* and contribute to the dissemination of both antibiotic resistance determinants as well as virulence factors. The intra- and inter-species transferability of *tcrB* gene in our study is in agreement with our earlier studies with pig isolates (10, 11). Interestingly, the conjugation frequency was higher in the inter-species assay, possibly because of the production of pheromones (peptide signal) by the recipient *E. faecalis* isolate strains, which are specific for a particular plasmid or a group of related plasmids (37, 38). In this pheromone-inducible plasmid transfer, the recipient bacterium excretes the peptide pheromone molecule into the medium, where it can diffuse to a donor cell thus facilitating transfer of antibiotic resistance determinants. The sex pheromones have also been described in *E. faecium*; more often, they are associated with vancomycin resistance (38). However, we detected a lower intra-species conjugation frequency rate compared to inter-species conjugation, which may be due to the absence of vancomycin resistance gene determinants in our *tcrB*-positive isolates.

Enterococci have become major nosocomial pathogens in humans and often are associated with multiple antibiotic resistant infections of blood stream, urinary tract, and surgical wounds (39). Virulence in enterococci is associated with several genes, particularly those that encode for aggregation substance (*asaI*), gelatinase (*gelE*), cytolysin (*cylA*), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*) (40, 21). Studies have shown that *esp* is the major virulence gene in *E. faecium*, followed by *asaI*, *gelE*, and *hyl* genes (41, 21). None of the *tcrB*-positive (n=22) or *tcrB*-negative (n=22) isolates in our study carried any of the virulence genes tested. However, the evolution and propagation of antibiotic resistance involve a complex network consisting of multilevel populations and variable selection pressures. Both ecology and evolution play a vital role in linking antibiotic resistance and bacterial virulence (42, 43).

All 22 *tcrB*-positive isolates possessed diverse MLVA types (MT) suggesting a genetically diverse and heterogeneous population in cattle. The majority of newly assigned MTs were clonally related in single and or double alleles at loci with clinically isolated human isolates. Note that this does not imply direction or causation. Order of entry into libraries is typically driven by various biases, such as the more intensively sampled clinical isolates. Twenty of the 22 strains were novel MLVA types. The MT 451 isolate was closely related to the predominant clone MT 112, which was isolated from blood and feces of hospitalized patients in Germany and in feces of chickens in the Netherlands. The MT 190 isolate was closely related to MT 325 (as per the MLVA database). The genetic diversity among the *E. faecium* isolates may be attributable to their propensity for genetic exchange coupled with a high rate of recombination (44). No doubt, as broader studies into populations of bacteria outside of clinical realms enter databases, a clearer picture will emerge.

In summary, the feeding of elevated dietary copper levels resulted in an increased prevalence of *tcrB*-mediated copper-resistant fecal enterococci in feedlot cattle. The *tcrB* gene was on a plasmid that can be transferred by conjugation, which is strongly suggestive of potential transferability of the gene under selection pressure. The finding of strong associations between copper resistance and other antibiotic (tetracycline and tylosin) resistance determinants may be significant because of the propensity of enterococci to transfer resistance genes to other bacteria in the gut when placed under selection pressure. Further studies are needed to investigate the effects of feeding antibiotics and their association with copper resistance gene in enterococci to better understand the potential co-selection and molecular epidemiology of multidrug resistant enterococci in cattle.

Acknowledgements

Authors would like to thank the personnel at the Kansas State University Beef Cattle Research Center for their help with the sampling process. We also thank Tiffany Mainini, Melody Phillips, and Zac Paddock for their help with the sampling. We would like to thank and appreciate the help of Mal Hoover in preparation of these images. We also extend our thanks to Dr. Anuradha Ghosh (Dr. Zurek's laboratory) for her technical support with the MLVA.

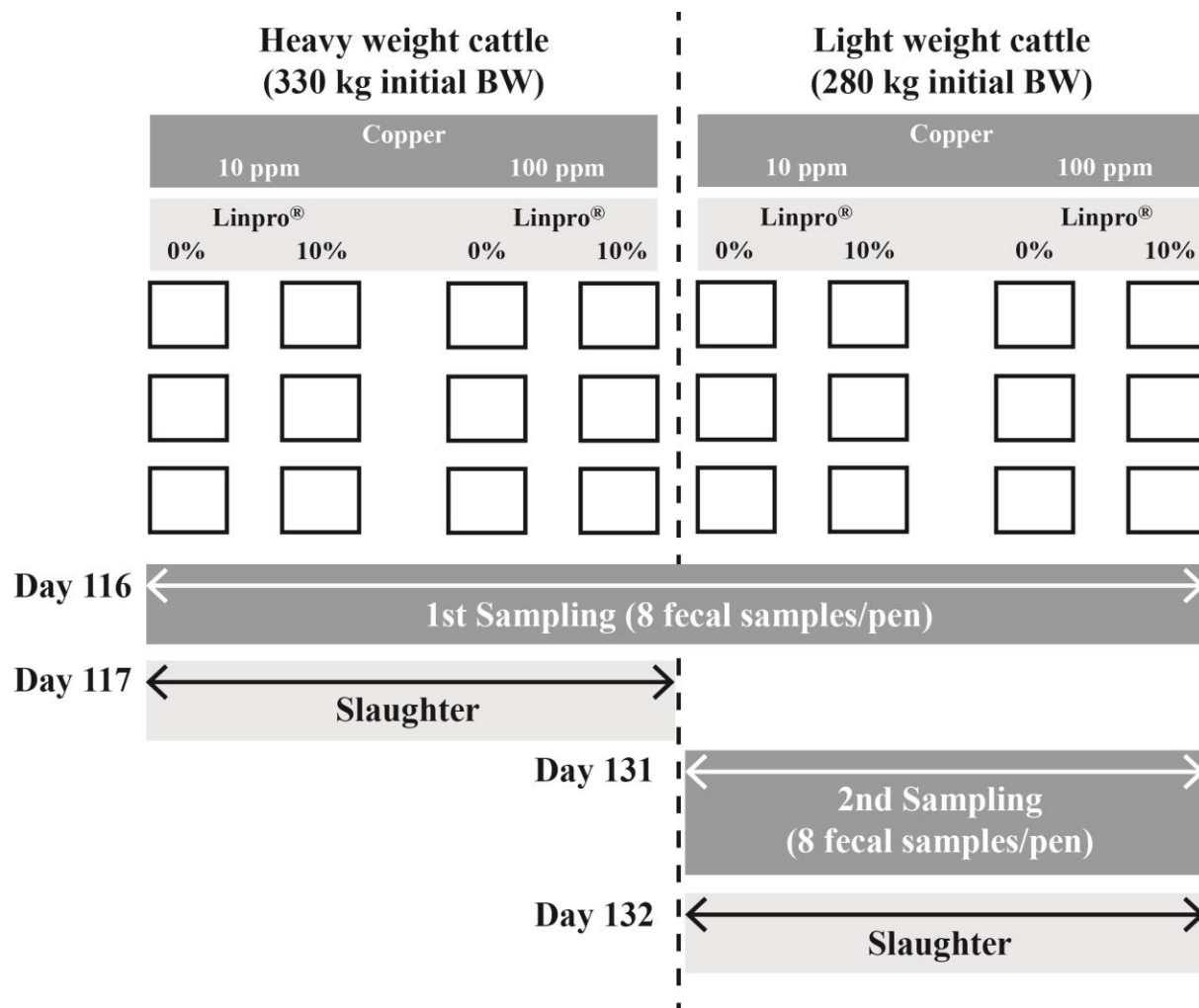


Figure 4.1: Schematic representation of the study design. *Linpro® is a commercial feed supplement containing an extruded blend of flax seed and field peas with added vitamins and minerals

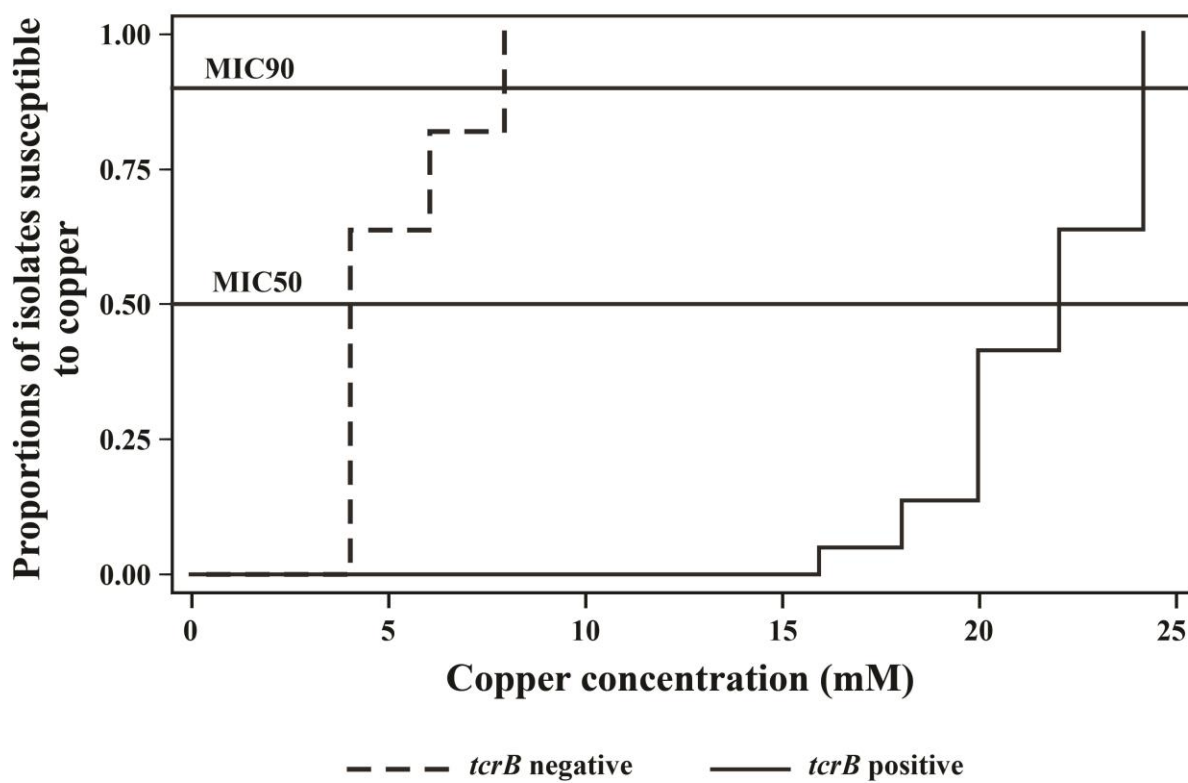


Figure 4.2: Failure function graph showing cumulative susceptibilities of *tcrB*-positive and *tcrB*-negative isolates to increasing copper concentrations

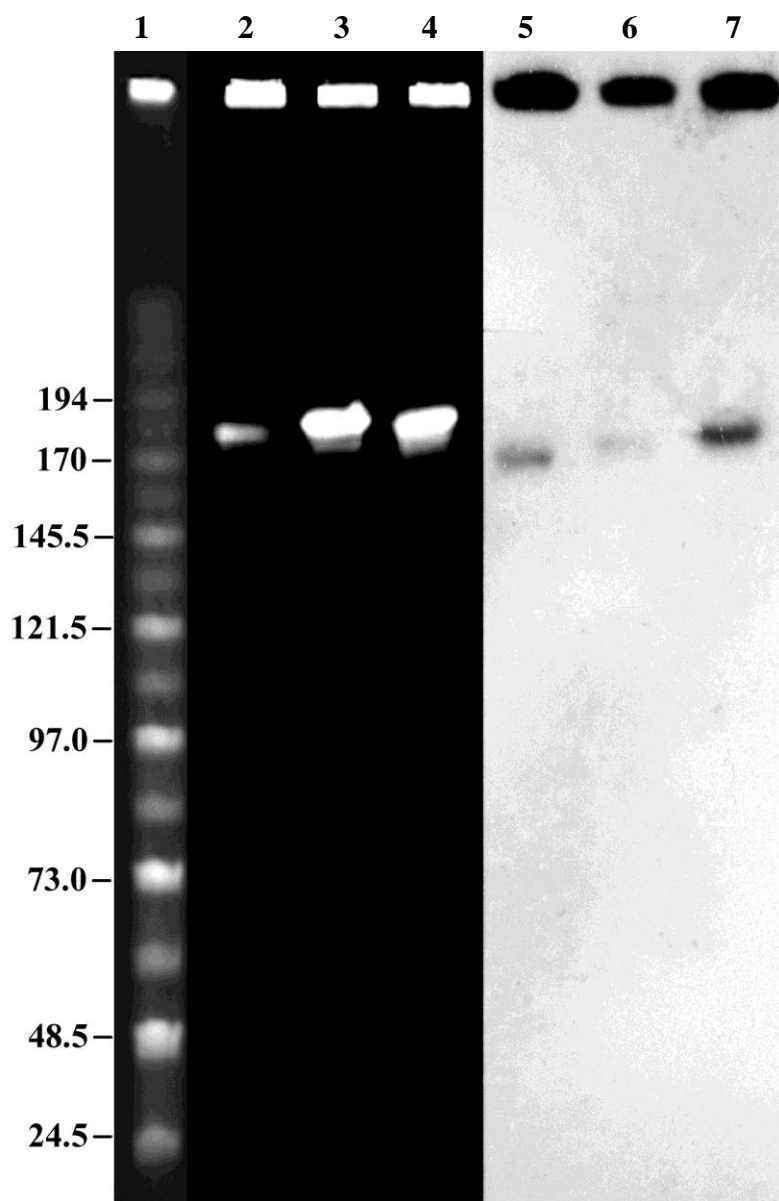


Figure 4.3: Southern blot hybridization of *tcrB* probe with S1 nuclease-digested genomic DNA of *tcrB*-positive enterococcal isolates. Lane 1: midrange PFG marker II; Lanes 2, 3 and 4: S1 nuclease-digested genomic DNA of *E.faecium* isolates, Lanes 5, 6, and 7: hybridization with *tcrB* probe

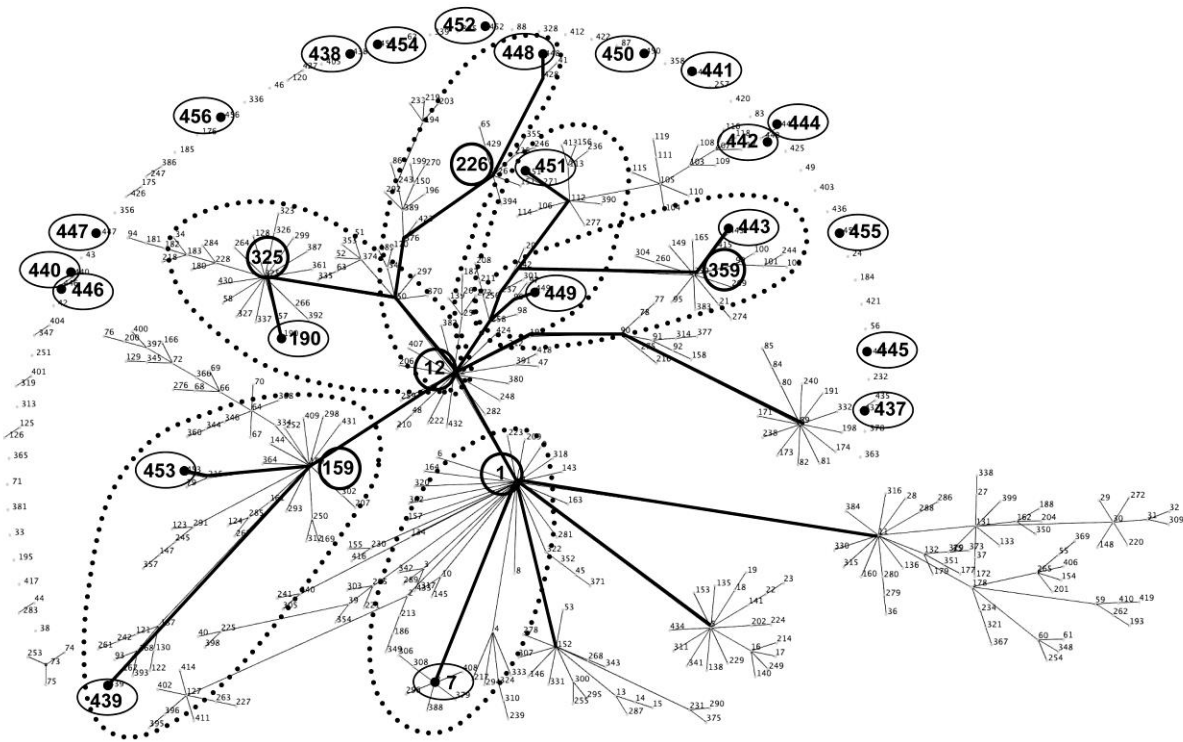


Figure 4.4: eBURST-based population snapshot of *Enterococcus faecium* on 455 available MT's (MLVA types) in *Enterococcus faecium* MLVA (Multiple-Locus Variable number tandem repeat analysis) database (<http://www.umcutrecht.nl/subsite/MLVA>). eBURST-clustering of twenty two MLVA analysis types (MTs) of *E. faecium* isolates from feedlot cattle (in bold and enclosed in ovals). Each MT represents one node with a difference in one locus. The dotted circles indicate MTs that are closely related to the existing predominant clones in the database (solid circles).

1 **Table 4.1:** Prevalence of copper resistance, *tcrB*, and antibiotic resistance, *erm*(B) and *tet*(M) genes among fecal enterococci in cattle
2 fed diets supplemented with or without copper at elevated concentrations

3

Treatment group and copper concentration in the diet, mg/kg	Sampling days	No. of enterococcal isolates	*No. of enterococcal isolates positive for (%):					
			None	<i>tcrB</i>	<i>erm</i> (B)	<i>tet</i> (M)	<i>erm</i> (B) + <i>tet</i> (M)	<i>tcrB</i> + <i>erm</i> (B) + <i>tet</i> (M)
Control, 10	116	192	31	2	116	134	91	2
	132	96	10	0	65	62	41	0
Total		288	41	2	181	196	132	2
			(14.2%)	(0.7%)	(62.8%)	(68%)	(45.8%)	(0.7%)
Copper, 100	116	192	41	14	82	106	51	14
	132	96	15	6	44	57	26	6
Total		288	56	20	126	163	77	20
			(19.4%)	(6.9%)	(43.7%)	(56.6%)	(26.7%)	(6.9%)

Table 4.2: Median minimum inhibitory concentrations of antimicrobial compounds for fecal enterococcal isolates with or without *tcrB*

Enterococcal isolates	Copper	Tetracycline	Tylosin	Vancomycin
	(mM)	(µg/mL)	(µg/mL)	(µg/mL)
<i>tcrB</i> -positive (n=22)	22*	100*	50*	0.39*
<i>tcrB</i> -negative (n=22)	4	37.5	1.6	0.20

*Significantly different from *tcrB*-negative isolates at $P < 0.05$

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CHAPTER 5 - Effects of in-feed copper and tylosin supplementations on copper and antibiotics resistance in fecal enterococci of feedlot cattle

Introduction

Feed grade antimicrobials are supplemented in feedlot cattle diets to promote growth, improve feed efficiency and to reduce the incidence of respiratory infections, foot rot, and liver abscesses (Nagaraja, 1995; Butaye et al., 2003). The use of antimicrobial feed additives is controversial because of the emergence and potential dissemination of antimicrobial resistance and subsequent risk to human health (Marshall and Levy, 2011). Therefore, alternatives to conventional antimicrobials, such as heavy metals, are being used to achieve improved animal performance (Thacker, 2013). Copper (Cu), a heavy metal, is supplemented in cattle diets at levels higher than physiological requirements to promote growth and improve feed efficiency (NRC, 2000). Copper, generally as copper sulfate, is supplemented in the diet at a concentration of 10-100 mg/kg (NRC, 2000). Copper is an essential micronutrient, required for several enzymes, cofactors, and metalloproteins in biological systems. Copper at high concentrations have antimicrobial activity, primarily due the production of free hydroxyl radicals by copper ions that block functional groups of proteins, enzymes, inhibit or alter synthesis of nucleic acids, and modify bacterial cell wall synthesis (Gant et al., 2007). Bacteria utilize several mechanisms to regulate intracellular copper concentration. A transferable copper resistance gene, *tcrB*, which confers resistance to copper has been reported in *Enterococcus faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* isolated from swine and cattle (Hasman and

Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2010 & 2011). The *tcrB* gene was on a plasmid, which also carried *vanA* (Hasman and Aarestrup, 2002; Hasman et al., 2006), *erm(B)* (Hasman et al., 2006; Amachawadi et al., 2010 & 2011), or *tet(M)* (Amachawadi et al., 2010 & 2011) genes, encoding resistance to glycopeptides, macrolides, and tetracyclines, respectively. Because antibiotic resistance genes are co-located with *tcrB* on the same plasmid, it is possible that feeding elevated level of copper in the diet may co-select for enterococci resistant to macrolides in the absence of antibiotic selection pressure. The potential genetic link between copper and antibiotic resistance is of interest because tylosin, a macrolide, and tetracyclines are widely used as feed additives in feedlot cattle. Tylosin is included in the feed to reduce the incidence of liver abscesses (Nagaraja and Chengappa, 1998) and tetracyclines (chlortetracycline or oxytetracycline) are used for improved growth rate and feed efficiency, to prevent liver abscesses, to treat bacterial enteritis, and for the control of respiratory disease associated with transportation stress (Nagaraja, 1995).

In a recent study, we have observed that feeding a diet with elevated (100 mg/kg of feed) level of copper increased the fecal prevalence *tcrB*-positive enterococci (Amachawadi et al., 2013). Because of the genetic link between *tcrB* with *erm(B)* and *tet(M)* genes, we hypothesize that copper supplementation may exert selection pressure for enterococci to become resistant to macrolides and tetracyclines. Therefore, a longitudinal study was conducted in cattle to investigate whether feed supplementation of copper, at elevated level, co-selects for macrolide resistance in fecal enterococci in the absence of tylosin. Because of the high propensity of enterococci to acquire and transfer antimicrobial resistance determinants (horizontal gene transfer) and of their clinical significance in causing nosocomial infections in humans (Hegstad

et al., 2010), we also investigated the resistance of the enterococcal isolates to other antibiotics and determined the presence of virulence genes.

Materials and Methods

The use of animals and the procedures employed were approved by the Kansas State University Animal Care and Use Committee (IACUC # 2914).

Study design and sampling schedule

Eighty crossbred steers (body weight = 568.8 ± 55 kg) were blocked by body weight and randomly allocated to four treatments and housed in individual concrete floor pens in four barns. Each barn consisted of 20 pens of individual animals with a water facility shared by two adjacent pens. The study design consisted of 2×2 factorial arrangement of supplemental copper, included as copper sulfate (10 or 100 mg/kg of feed) and tylosin (0 or 10 mg/kg of feed; Tylan[®], Elanco Animal Health, Greenfield, IN) in the diet. Individual pens in each of the four barns were randomly allocated to the four treatments. Cattle received a high-grain diet and were fed once daily with *ad libitum* access to feed and water. The basal diet included (on a dry matter basis) 54% steam-flaked corn, 35% wet corn gluten feed, 6% wheat straw, and 2.2% minerals (0.1 mg of cobalt, 10 mg of copper, 0.6 mg of iodine, 60 mg of manganese, 0.25 mg of selenium, and 60 mg of zinc per kg of the feed), vitamins (4,535 IU of vitamin A and 22 IU of vitamin E per kg of feed), and monensin (300 mg/animal/d; Rumensin[®], Elanco Animal Health). Approximately 20 g of pen floor fecal samples (n = 80) were collected with plastic spoons from freshly defecated fecal pats from each pen on days 0, 7, 14, 21, and 28 of feeding treatment diets. Spoons with feces were placed in individual Whirl-Pak[®] (Nasco, Ft. Atkinson, WI) bags and transported in a

cooler with ice packs to the laboratory. A schematic representation of the study design and sampling schedule is shown in Fig. 1.

Bacteriological procedures

All the laboratory microbiological procedures were approved by the Kansas State University's Institutional Biosafety Committee (IBC # 625 & 626). All culture media used in the study were from BD (Becton and Dickson, Sparks, MD). *Enterococcus* species were isolated by diluting 1 g of fecal sample in 10 mL of sterile phosphate-buffered saline and spread-plating 50 μ L of the fecal suspension onto M-*Enterococcus* agar. After 24 h of incubation at 42° C, two putative colonies (pin-point red and metallic pink) were picked from each plate and replated onto blood agar plates for overnight incubation at 37° C. Presumptive genus identification of the isolates were based on esculin hydrolysis, and esculin-positive isolates were stored in protect beads (Cryo-Vac[®], Key Scientific Products, Round Rock, TX) at -80° C for further use.

DNA was extracted from enterococcal isolates by suspending a single colony from the blood agar plate in nuclease-free water with Chelex[®] 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiling for 10 min. Species identification was performed by multiplex PCR (Jackson et al., 2004), which was further confirmed by superoxide dismutase (*sodA*) gene sequence analysis (Poyart et al., 2000). The multiplex PCR identifies four different *Enterococcus* spp. such as *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus*. The ATCC strains of *E. casseliflavus* (ATCC 25788), *E. faecalis* (ATCC 19433), *E. faecium* (ATCC 19434), and *E. gallinarum* (ATCC 49579) were used as positive controls.

PCR detections of *tcrB*, *erm(B)*, *tet(M)*, and virulence genes

The DNA from enterococcal isolates was extracted as before and subjected to PCR assays for detections of *tcrB*, *erm(B)*, *tet(M)*, and virulence (*asaI*, *gelE*, *cylA*, *esp*, and *hyl*) genes

(Amachawadi et al., 2013). Primers used in the study were supplied by Invitrogen Life Technologies (Carlsbad, CA).

Susceptibility determinations for copper, tylosin and tetracycline

Enterococcal isolates positive for *tcrB* (n=37; *tet*(M) and *erm*(B) positive) and a specified subset of *tcrB*-, *tet*(M)-, and *erm*(B)-negative isolates (n=40; matched as best as possible by treatment) were subjected to susceptibility determinations for copper, tylosin, and tetracycline. Copper susceptibilities were determined by the agar dilution method (Hasman et al., 2006). Briefly, Mueller-Hinton agar plates containing 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, or 40 mM of copper sulfate (Fischer Scientific, Fair Lawn, NJ) were prepared and spot inoculated with 20 μ L of bacterial inoculum adjusted to a McFarland turbidity standard of 0.5. The plates were incubated for 36 to 48 h at 37° C to determine growth or no growth. Two *tcrB*-positive strains from our previous study (BAA-2127 and BAA-2128; Amachawadi et al., 2013) served as positive controls.

The minimum inhibitory concentrations (MIC) for tylosin and tetracycline (Sigma-Aldrich, St. Louis, MO) were determined by the micro-broth dilution method as per CLSI guidelines (CLSI, 2008). Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, and 0.098 μ g/mL. Bacterial cultures were grown in 10 mL of Mueller-Hinton II broth for 6 h and the inocula concentrations were adjusted to 0.5 McFarland turbidity standards. The susceptibility determinations for antibiotics were carried out in 96-well micro titer plates (Becton and Dickson); plates inoculated with the bacterial inocula were incubated at 37° C for 24 h and results recorded as growth or no growth for each well. The MIC of ≥ 16 μ g/ml and ≥ 32 μ g/ml were considered as resistant breakpoints for tetracycline and tylosin, respectively.

Susceptibility determinations to other antibiotics

All *tcpB*-positive isolates (n=37) and the same subset of *tcpB*-negative isolates (n=40) were subjected to susceptibility determination for a more complete array of antibiotics using NARMS (National Antimicrobial Resistance Monitoring System) Gram-positive panel plates (CMV3AGPF, Trek Diagnostics Systems, Cleveland, OH) with the aid of the Sensititre[®] automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH). The list of 16 antimicrobials tested, their concentration ranges used, and breakpoints for susceptibility classification according to NARMS, are shown in Table 1. Appropriate ATCC (American Type Culture Collection, Manassas, VA) quality control strains; *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were used as reference standards for susceptibility testing. The bacterial suspension was prepared by mixing an individual colony with demineralized water (Trek Diagnostic Systems, Cleveland, OH) to obtain 0.5 McFarland turbidity standards (Key Scientific Products, Stamford, TX). A 50 µL aliquot of the bacterial inoculum was added to cation-adjusted Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH) and vortexed. Then, 100 µL of the broth was dispensed into Gram-positive NARMS panel plates and incubated for 18 h at 37° C. The MIC for each isolate was recorded and classified as resistant, intermediate or sensitive based on the Clinical Laboratory Standards Institute (CLSI, 2008) guidelines. For our analytical purposes, intermediate strains were reclassified as susceptible.

Statistical Analysis

Data were analyzed using STATA v.12.1 (StataCorp, College Station, TX). Bivariate descriptive statistics of gene or susceptibility phenotype prevalence by diet type were assessed

prior to multivariable analysis. Unadjusted differences in prevalence proportions among two variables [*tcrB* vs. *erm*(B), *tcrB* vs. *tet*(M), *erm*(B) vs. *tet*(M)] were assessed using the Likelihood ratio chi-square test. Thereafter, the multivariable adjusted effects of copper and tylosin supplementation (alone or in combination) and sampling days on the prevalence of *tcrB*, *erm*(B), and *tet*(M) genes among enterococci were assessed.

The association of each variable with the probability of a positive result (genotype or phenotype) was tested using mixed (random and fixed) effects logistic regression and also multi-level hierarchical logistic regression. Animal was considered as the experimental unit. The data were considered multilevel and longitudinal in nature because of the repeated sampling of animals and multiple isolates within each fecal sample. A full factorial model was built including the copper, tylosin, and sampling day to test their interaction term to assess its impact on the binary outcome (present or absent) of *tcrB*, *erm*(B), and *tet*(M) genes. Then, the main effects were tested individually to determine the effects of copper, tylosin, and sampling day on the prevalence of *tcrB*, *erm*(B), and *tet*(M) genes. From the final model, marginal adjusted predicted probabilities were obtained for the proportion of enterococcal isolates positive for *tcrB*, *erm*(B), and *tet*(M) gene; these were estimated along with their 95% confidence intervals.

Additional analyses include the following. From all possible combinations of the three resistance genes (binary response variables, and assuming they are truly random) a new ‘nominal’ variable called ‘multi-gene’ type was created. Data were then analyzed using multinomial logistic regression for the main effects of copper and tylosin supplementation (alone or in combination) and the sampling days on the prevalence of ‘multi-gene’ types. Copper, tylosin, and tetracycline MIC data were log₂-transformed and analyzed using survival analysis, accounting for right-censored growth beyond maximum antibiotic concentration. The MICs

were expressed and compared as the median (MIC₅₀) after assuming for normality (Stegeman et al., 2006). The MIC data, further categorized as resistant or susceptible enterococci to 16 antimicrobials were considered as both binary outcomes and as multidrug resistance profiles. The results were cross tabulated with each of the treatment groups and interactions (and by sampling day) and by *tcrB* presence to compare the proportion of enterococcal isolates resistant to each of the antimicrobial agents. A Likelihood ratio chi-square test was performed to assess the relationship between multidrug resistance profile and the treatments/days and *tcrB*-presence. Results were considered significant at a *P*-value of < 0.05.

Results

Prevalence of *tcrB*, *erm*(B), *tet*(M), and virulence genes in fecal enterococci

A total of 800 enterococcal isolates, comprising 40 isolates selected from each of the four treatment groups (control, copper, tylosin, and copper and tylosin) and five sampling days (0, 7, 14, 21, and 28) were analyzed further. Thirty-seven isolates (4.6%; 37/800) were positive for the *tcrB* gene and all of these were speciated as *E. faecium*. Of the 37 *tcrB*-positive isolates, 4 (4/200; 2.0%), 9 (9/200; 4.5%), 7 (7/200; 3.5%) and 17 (17/200; 8.5%) were from control, copper, tylosin, and copper and tylosin groups, respectively (Fig. 2). The prevalence of *tcrB*-positive enterococci was significantly affected by treatments (*P* = 0.02); however, sampling day had no effect (*P* = 0.26) and the treatment and sampling day interaction likewise was not significant (*P* = 0.4). Copper (*P* = 0.01), tylosin (*P* = 0.04), and copper and tylosin (*P* = 0.007) treatment groups each exhibited higher prevalence of *tcrB* gene than the control group.

The overall prevalence of the *erm*(B) gene among enterococcal isolates was 46.8% (375/800) and the treatment and sampling day interaction was significant (*P* < 0.001); copper (*P* = 0.002), tylosin (*P* = 0.03), and copper and tylosin (*P* = 0.001) supplementations significantly increased

the prevalence of *erm*(B)-positive enterococci. Overall, the prevalence was significantly affected both by the treatment ($P = 0.001$) and the sample day main effects ($P < 0.0001$). The overall prevalence of *tet*(M) gene among enterococcal isolates was 57.5% (460/800) and the treatment and sampling day interaction was significant ($P = 0.05$). Among main effects, the prevalence was significantly affected by sampling day ($P = 0.003$) but not by the treatment ($P = 0.34$). None of the 800 isolates examined had *tcrB* alone, or *tcrB* gene with *erm*(B) or *tet*(M) separately.

All *tcrB*-positive isolates were positive for both *erm*(B) and *tet*(M) genes. Therefore, the prevalence of all three resistance genes [*tcrB*, *erm*(B), *tet*(M)] together was the same as the prevalence of *tcrB* gene alone (2.0%, 4.5%, 3.5%, and 8.5% for control, copper, tylosin, and copper and tylosin groups, respectively; Table 2). A number of isolates had *erm*(B) and *tet*(M) together in the absence of *tcrB* and the prevalence of this combination was not affected by the treatments (Table 2). The overall prevalence of virulence genes was 2 (2/800; 0.3%) for each of *esp* (enterococcal surface protein) and *hyl* (hyaluronidase), 3 (3/800; 0.4%) of *cyla* (cytolysin), 7 (7/800; 0.9%) of *asaI* (aggregation substance) and 25 (25/800; 3.1%) of *gelE* (gelatinase E). None of the *tcrB*-positive ($n=37$) isolates in our study carried any of the virulence genes tested. The treatments, sampling day, and their interaction terms did not have an effect on the prevalence of virulence genes ($P > 0.05$) among enterococci.

Phenotypic susceptibilities to copper, tylosin, and tetracycline

All *tcrB*-positive isolates grew on MH agar containing copper at a concentration of 20 or 24 mM. However, the *tcrB*-negative isolates did not grow with copper at a concentration of > 8 mM. The two positive reference strains BAA-2127 and BAA-2128 exhibited MIC values of 20 and 24 mM, respectively. The median copper MIC of *tcrB*-positive isolates (20 mM) was higher ($P < 0.0001$) than that of the *tcrB*-negative (4 mM) isolates. A failure function graph

representing the cumulative susceptibilities of *tcxB*-positive and *tcxB*-negative isolates to copper is shown in Fig. 3. All *tcxB*-positive isolates, which also contained *erm*(B), were resistant (≥ 32 $\mu\text{g/ml}$, based on CLSI breakpoint) to tylosin with a median MIC of 50 $\mu\text{g/ml}$ and all *tcxB*-, *tet*(M)-, and *erm*(B)- negative isolates were susceptible (≤ 8 $\mu\text{g/ml}$, based on CLSI breakpoint) to tylosin with a median MIC of 1.6 $\mu\text{g/ml}$ ($P < 0.0001$). The failure function graph that depicts the cumulative susceptibilities of *tcxB*-positive and *tcxB*-, *tet*(M)-, and *erm*(B)- negative isolates [n=40] to tylosin are shown in Fig. 4. Interestingly, both sets of *tcxB*-positive (n=37) and *tcxB*-, *tet*(M)-, and *erm*(B)- negative [n=40] isolates were resistant (≥ 16 $\mu\text{g/ml}$), based on CLSI breakpoint, to tetracycline. However, the median tetracycline MIC of *tcxB*-positive isolates (>100 $\mu\text{g/ml}$) was significantly higher ($P < 0.0001$) than that of the *tcxB*-negative isolates (37.5 $\mu\text{g/ml}$).

Susceptibilities of enterococci to other antimicrobials

Overall MIC distributions and resistance profiles of enterococcal isolates (n= 77; 37 *tcxB*-positive isolates and 40 *tcxB*-, *tet*(M)-, and *erm*(B)-negative isolates) to each of 16 antimicrobials tested are shown in the form of a 'squashtogram' (Table 3). All isolates tested were susceptible to nitrofurantoin, tigecycline, and vancomycin. The MIC₅₀ and MIC₉₀ values, as well as the proportions of *tcxB*-positive (n=37) and *tcxB*-negative (n=40) isolates resistant to each antimicrobial are shown in Table 4. The proportions of isolates resistant to any of the antimicrobials did not differ between *tcxB*-positive and *tcxB*-negative enterococcal isolates ($P > 0.05$). The highest proportion of resistant isolates were with lincomycin (98.7%), followed by tetracyclines (89.6%), erythromycin (84.4%), tylosin (83.1%), kanamycin (81.8%), quinupristin/dalfopristin (75.3%), and streptomycin (50.6%). The majority of the isolates (93.5%; 72/77) were resistant to three or more antimicrobials, and were therefore classified as

multidrug resistant (MDR; Pillar et al., 2008). The MDR phenotypes varied among the isolates, with two isolates each resistant to 11(2.6%) and 12 (2.6%) antibiotics, respectively, and 60 (93.5%) isolates resistant to 5 or more antimicrobials, inclusive of the first four. Most often, the MDR isolates were associated with lincomycin, erythromycin, tetracycline, tylosin, streptomycin, and quinupristin/dalfopristin resistant phenotypes in both *tcrB*-positive and *tcrB*-negative isolates.

Discussion

In the present study, we investigated the effects of copper and tylosin supplementation, alone or in combination, on the prevalence of *tcrB* and *erm(B)* genes in fecal enterococci of feedlot cattle. Feed grade antimicrobials are used in 80% of the US feedlots for growth promotion or prophylaxis (McEwen and Fedorka-Cray, 2002). However, the concern is that bacterial gut commensals and pathogens in food animals develop resistance to antimicrobials, which may get disseminated via food chain. Therefore, alternatives to antimicrobials, including heavy metals and particularly copper and zinc, have been used as feed additives in food animal production (Hasman et al., 2006). We have reported that supplementing cattle diets with elevated (100 mg/kg) levels of copper increased the prevalence *tcrB*-positive enterococci (Amachawadi et al., 2013). Interestingly, *tcrB*-positive isolates also contained *erm(B)* and *tet(M)* genes and were phenotypically resistant to erythromycin and tetracycline. The genetic linkage between genes conferring resistance to metals and other antibiotics suggests maintenance of antibiotic resistance genes even in the absence of selective pressure (Singer et al., 2006).

The prevalence of *tcrB*-positive enterococci was higher among cattle fed a higher concentration of copper (copper and copper + tylosin groups; 26/400; 6.5%) compared to groups (control and tylosin group; 11/400; 2.7%) that did not receive higher concentration of copper ($P = 0.01$). This prevalence of *tcrB*-positive enterococci is in agreement with our earlier study, in which we reported that 6.9% (20/288) of fecal enterococcal isolates of cattle were positive for *tcrB* (Amachawadi et al., 2013). Similar low prevalence of *tcrB*-positive enterococci were recorded from cattle (16 %) in Denmark (Hasman and Aarestrup, 2002). The prevalence of *tcrB*-positive enterococci was also higher among cattle fed tylosin (tylosin and copper + tylosin groups; 24/400; 6.0%) compared to groups that did not receive tylosin (control and copper groups; 13/400; 3.2%). The prevalence of *tcrB*-positive enterococci was higher in cattle fed diets supplemented with both copper and tylosin (17/200; 8.5%) than either of the diet alone (copper, 9/200; 4.5% and tylosin, 7/200; 3.5%). This suggests that copper and tylosin in combination exert an additive selection pressure for *tcrB*-positive enterococci. Similar to *tcrB*, the prevalence of *erm(B)* positive enterococci was higher among cattle fed a higher concentration of copper (copper and copper + tylosin groups; 208/400; 52%) compared to groups that did not receive higher concentration of copper (control and tylosin group; 167/400; 41.7%). The prevalence of *erm(B)* positive enterococci was also higher ($P = 0.001$) among cattle fed tylosin (tylosin and copper + tylosin groups; 201/400; 50.2%) compared to groups that did not receive tylosin (control and copper groups; 174/400; 43.5%). This suggests that the feeding of elevated dietary copper and tylosin alone or in combination resulted in an increased prevalence of macrolide resistance [*erm(B)*] among fecal enterococci in feedlot cattle. All *tcrB*-positive enterococcal isolates in our study were *E. faecium*, which is in agreement with our earlier study in cattle (Amachawadi et al., 2013). Earlier studies from Denmark and Australia have reported the

occurrence of *tcrB* gene in many *Enterococcus* spp. such as *E. faecium*, *E. faecalis*, *E. durans*, *E. gallinarum*, *E. casseliflavus* and *E. mundtii* (Hasman et al., 2006; Fard et al., 2011). All enterococci, both *tcrB*-positive (n=37) and *tcrB*-negative (n=40) isolates that were speciated, were *E. faecium*. Because only limited number of *tcrB*-negative isolates was speciated, we cannot rule out the possibility of finding other enterococcal species in cattle feces. Fluckey et al. (2009) have reported *E. faecium*, *E. faecalis*, and *E. durans* as the dominant enterococcal species in feces of cattle.

Enterococci have become major nosocomial pathogens in humans and often are associated with multiple antibiotic resistant infections of blood stream, urinary tract, and surgical wounds (Palmer et al., 2010). Antibiotic resistance along with the expression of virulence traits is necessary for the bacterium to become more virulent (Johnson, 1994). Virulence in enterococci is associated with several genes, particularly those that encode for aggregation substance (*asaI*), gelatinase (*gelE*), cytolysin (*cylA*), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*) (Hancock & Gilmore, 2002). None of the *tcrB*-positive (n=37) isolates in our study carried any of the virulence genes tested, which is in agreement with our earlier study (Amachawadi et al., 2013). In our study, the prevalence of virulence genes was low among the fecal enterococcal isolates of cattle. Studies have shown that *esp* is the major virulence gene in *E. faecium*, followed by *asaI*, *gelE*, and *hyl* genes (Eaton & Gasson, 2002; Vankerckhoven et al., 2004). This is in agreement with the some of the earlier studies where they recorded lower prevalence of virulence genes in *E. faecium* and *E. faecalis* isolated from food animal (Franz et al., 2001; Eaton & Gasson, 2002).

Antimicrobial resistance, particularly multi-resistance, is common among *Enterococcus* spp., because of their ability to acquire antimicrobial resistance genes (Murray, 1990). Majority of the

tested *tcrB*-positive (n=37) and *tcrB*-, *tet*(M)-, and *erm*(B)-negative (n=40) isolates were multidrug resistant (93.5%; 72/77). This is in agreement with the earlier study in feedlot cattle, where fecal enterococcal isolates (*E. faecium*) were resistant to 5-7 antimicrobials (Edrington et al., 2009). Increased levels of resistance to tetracyclines and macrolides (erythromycin and tylosin) among *E. faecium* isolates of feedlot cattle suggest possible selection pressure placed on food producing animals by the use of these antimicrobials for growth promotion and therapeutic purposes (Anderson et al., 2008). The abundance of tetracycline resistance determinant among *E. faecium* isolates is more common than other gut bacteria because the acquired tetracycline resistance is more prevalent in enterococci (Anderson et al., 2008). The high degree of macrolide resistance in this study may be related to the feeding of tylosin for the control of liver abscesses. Not surprisingly, high degree of resistance to erythromycin (84.4%), and tylosin (83.1%) was noticed among both *tcrB*-positive and *tcrB*-negative *E. faecium* isolates.

Broth microdilution assay from our study yielded MIC of 1.6 µg/ml for tylosin among *tcrB*-negative isolates. This might be attributed to the differences in the approach, antibiotic preparation (concentration, potency), and changes in resistance endpoints which tend to shift over time. Therefore, careful attention must be exercised while interpreting results from broth microdilution assay (Hillard et al., 2005). Whereas, the sensititre procedure yielded a different results with respect to macrolide resistance, which is suggestive of presence of macrolide resistance determinants other than *erm*(B) gene among enterococci. Macrolide resistance in enterococci is mediated by *erm* genes via target modification due to ribosomal methylase gene encoded by *ermA*, *erm*(B), and *ermC* genes. The other resistance mechanism is through efflux pump system mediated by the membrane bound efflux proteins encoded by *mef* (A/E) and *msr* genes (Singh et al., 2001). It has been reported that macrolide resistance mediated by non-*erm*

genes generally express lower resistance to erythromycin. Considerable differences in the MICs were also reported in macrolides (erythromycin, tylosin) among enterococcal isolates (Jaglic., 2012) due to mutation in innate genes, which is seen in 1-4% of the macrolide resistant enterococci (Roberts, 2004). Both, *tcrB*-positive and *tcrB*-negative isolates were resistant to tetracycline. This might be due to the presence of other tetracycline resistance [*tet*(L), *tet*(O), *tet*(K), and *tet*(S)] determinants (Aarestrup et al., 2000; Wilcks et al., 2005). However, screening for these genes is beyond the scope of present study. In our study, we screened for the most common tetracycline resistant gene, *tet*(M), among enterococcal isolates. This co-selection might also be attributed to the co-localization of both macrolide [*erm*(B)], and tetracycline [*tet*(M)] resistance determinants on a conjugative mobile plasmid and or transposon (Clewell, 1995). Enterococci have natural transfer mechanisms via plasmids and transposons and can harbor resistance to multiple antimicrobials. We have reported the co-localization of *tcrB* gene with *erm*(B), and *tet*(M) genes on a mobilizable plasmid from earlier studies in pigs and cattle (Amachawadi et al., 2011 & 2013). More often, co-selection among antimicrobial resistance determinants is an obvious trait where the antimicrobial resistance is genetically linked to the other antibiotics (Chen et al., 2008) or even heavy metals (Hasman and Aarestrup, 2002). These determinants are located mainly on mobile genetic elements such as plasmids and or transposons, which ensure their spread by horizontal gene transfer. In majority of the cases, these plasmids are conjugative with broad host range (Martinez, 2009). If carrying genetic elements conferring resistance is not a burden, reducing the use of antimicrobial agents alone may not decrease the prevalence of antibiotic resistance bacteria within the gut (Gillespie, 2001). Diversity among the multidrug resistant enterococci suggests that no single genotype is favored in a particular environment. However, no specific patterns of multidrug resistance were readily apparent in our

study. Multiple drug resistant strains of *E. faecium* are more often associated with hospital acquired infections due to transmission of these strains to humans and their ability for horizontal gene transfer (Leberton et al., 2013).

The extent of antimicrobial resistance in enterococci should be monitored to fully assess the role of feed grade antimicrobials in the selection and dissemination of resistance in feedlot cattle. Although many enterococci in our study were multidrug resistant, they did not show resistance to some of the critically important antimicrobials such as vancomycin, and a low incidence of resistance to chloramphenicol, ciprofloxacin, daptomycin, and penicillin. These results indicate that the fecal enterococci of feedlot cattle were susceptible to clinically relevant antimicrobials. In summary, the feeding of elevated dietary copper and tylosin alone or in combination resulted in an increased prevalence of *tcrB* and *erm(B)* mediated copper and tylosin resistant fecal enterococci in feedlot cattle. This is suggestive of co-selection of both copper and macrolide resistance determinants due to the selection pressure from copper and or tylosin supplementation. Further studies are warranted to investigate the factors involved in maintenance and dissemination of these resistance determinants and their co-selection mechanism in relation to feed grade antimicrobials usage in feedlot cattle. Therefore, monitoring antimicrobial resistance in enterococci of food animal origin is important for preserving the therapeutic value and efficacy of the antimicrobials.

Acknowledgements

Financial support for this project was provided by the College of Veterinary Medicine, Kansas State University under the 'Mentored Clinical, Applied, or Translational Research (MCAT Research Award # 2012). Authors would like to thank the personnel at the Kansas State University Beef Cattle Research Center for their help with the sampling process. We also thank Tiffany Mainini, Melody Phillips, Lia Locatelli, and Flavia van Cleef for their help with the sampling. We would to like to thank and appreciate the help of Mal Hoover in preparation of these images.

Table 5.1: Prevalence of copper (*tcrB*), tylosin (*erm*[B]) and tetracycline (*tet*[M]) resistance genes alone or in combinations in fecal *Enterococcus faecium* isolates of feedlot cattle fed diets supplemented with or without copper and or tylosin

Genes	No. of isolates (%) positive (n=200)			
	Control	Copper	Tylosin	Copper and Tylosin
None	53 (26.5)	46 (23.0)	48 (24.0)	46 (23.0)
<i>tcrB</i> alone	0	0	0	0
<i>erm</i> (B) alone	23 (11.5)	46 (23.0)	34 (17.0)	44 (22.0)
<i>tet</i> (M) alone	78 (39.0)	49 (24.5)	54 (27.0)	51 (25.5)
<i>tcrB</i> + <i>erm</i> (B)	0	0	0	0
<i>tcrB</i> + <i>tet</i> (M)	0	0	0	0
<i>tcrB</i> + <i>erm</i> (B) + <i>tet</i> (M)	4 (2.0)	9 (4.5)	7 (3.5)	17 (8.5)
<i>erm</i> (B) + <i>tet</i> (M)	42 (21.0)	50 (25.0)	57 (28.5)	42 (21.0)

Table 5.2: National antimicrobial resistance monitoring system gram positive bacterial panel (CVM3AGPF): antimicrobials, tested concentrations, and their resistant breakpoints

Antimicrobials	Concentrations (µg/ml)	Breakpoints (µg/ml)	
		Resistant	Susceptible
Chloramphenicol	2 - 32	≥ 32	≤ 8
Ciprofloxacin	0.12 - 4	≥ 4	≤ 1
Daptomycin	0.25 - 16	N/A ¹	≤ 4
Erythromycin	0.25 - 8	≥ 8	≤ 0.5
Gentamicin	128 – 1024	> 500	≤ 500
Kanamycin	128 - 1024	≥ 1024	≤ 512
Lincomycin	1 - 8	≥ 8	≤ 2
Linezolid	0.5 - 8	≥ 8	≤ 2
Nitrofurantoin	2 - 64	≥ 128	≤ 32
Penicillin	0.25 - 16	≥ 16	≤ 8
Quinupristin / dalfopristin	0.5 - 32	≥ 4	≤ 1
Streptomycin	512 - 2048	> 1000	≤ 1000
Tetracycline	1 - 32	≥ 16	≤ 4
Tigecycline	0.015 - 0.5	N/A ²	≤ 0.25
Tylosin tartrate	0.25 - 32	≥ 32	≤ 8
Vancomycin	0.25 - 32	≥ 32	≤ 4

N/A = Not applicable

¹For daptomycin, only susceptible breakpoint (≤ 4 µg/ml) has been established. The isolates with an MIC ≥ 8 µg/ml are considered as resistant

²For tigecycline, only susceptible breakpoint (≤ 0.25 µg/ml) has been established. The isolates with an MIC ≥ 0.5 µg/ml are considered as resistant

1 **Table 5.3:** Antimicrobial susceptibilities of *Enterococcus faecium* (n-77; 37 *trb*-positive and 40 *trb*-negative) strains isolated from
2 feces of feedlot cattle fed diets supplemented with or without copper and or tylosin

Antimicrobials	% Resistant	95% CI	Distribution of isolates (%) with minimum inhibitory concentrations in µg/mL																			
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048		
Chloramphenicol	1.3	0.03-7										9	67	0	1							
Ciprofloxacin	3.9	0.8-10.9	4	0	0	1	2	18	27	22	3											
Daptomycin	2.6	0.03-9					3	4	10	8	41	9	2									
Erythromycin	84.4	74.3-91.6					7	0	4	1	0	65										
Gentamicin	13	6.4-22.5					1	3	0	0	0	0	0	0	0	63	2	1	7			
Kanamycin	81.8	71.3-89.6										4	0	0	0	10	14	13	36			
Lincomycin	98.7	92.9-99.9								1	0	0	74	0	2							
Linezolid	12.9	0.03-7								3	69	4	1									
Nitrofurantoin	6.5	2.1-14.5											3	8	9	34	23					
Penicillin	6.5	2.1-14.5					3	18	17	11	23	0	5									
Quinupristin / Dalfopristin													27	9	7	15						
Streptomycin	50.6	39-62.2														4	0	0	0	34	2	37
Tetracycline	89.6	80.5-95.4								4	0	4	0	0	69							
Tigecycline	0	0-4.6*				6	43	27	1	0												
Tylosin tartrate	83.1	72.8-90.6							1	5	5	2	0	0	64							
Vancomycin	0	0-4.6*							2	41	22	8	4	0								

3

4 The whitened area indicates the range of each antimicrobial dilution tested and shaded grey areas fall outside the range of tested
5 antimicrobial concentrations. The vertical bar indicates the CLSI (Clinical and Laboratory Standard Institute) or NARMS (National
6 antimicrobial resistance monitoring system) consensus breakpoints for resistance.

7 *One sided, 97.5% confidence interval (used only when the estimate was zero)

Table 5.4: Antimicrobial susceptibilities and minimum inhibitory concentrations (MIC) of *tcrB*-positive and *tcrB*-negative *Enterococcus faecium* strains isolated from feces of feedlot cattle fed diets supplemented with or without copper and or tylosin.

Antimicrobials	<i>tcrB</i> -positive (n=37)			<i>tcrB</i> -negative (n=40)		
	Resistant (%)	MIC ₅₀	MIC ₉₀	Resistant (%)	MIC ₅₀	MIC ₉₀
Chloramphenicol	0	8	8	1 (2.5%)	8	8
Ciprofloxacin	2 (5.4)	1	2	1 (2.5%)	1	2
Daptomycin	0	4	8	2 (5.0)	4	8
Erythromycin	34(91.9)	8	8	31(77.5)	8	8
Gentamicin	3(8.1)	128	2048	7(17.5)	128	1024
Kanamycin	30(81.1)	512	1024	33(82.5)	1024	1024
Lincomycin	37(100)	8	8	39(97.5)	8	8
Linezolid	0	2	2	1(2.5)	2	3.8
Nitrofurantoin	3(8.1)	32	64	2(5.0)	32	64
Penicillin	3(8.1)	1	6.4	2(5.0)	2	4
Quinupristin / Dalfopristin	33(89.2)	4	32	25(62.5)	4	32
Streptomycin	12(32.4)	512	2048	27(67.5)	2048	2048
Tetracycline	33(89.2)	32	32	36(90.0)	32	32
Tigecycline	0	0.25	0.25	0	0.12	0.25
Tylosin tartrate	34(91.9)	32	32	30(75)	32	32
Vancomycin	0	1	2	0	2	7.6

Treatment groups	Copper										
	10 mg/kg					100 mg/kg					
	Tylosin					Tylosin					
	0 mg/kg (1)	100 mg/kg (2)				0 mg/kg (3)	100 mg/kg (4)				
Pen and treatment assignments	Barn A	2	2	3	2	1	1	2	4	2	3
		4	1	3	1	3	4	1	4	4	3
	Barn B	1	2	1	4	4	3	3	3	2	4
		2	4	3	4	1	1	2	3	1	2
	Barn C	4	3	1	4	1	4	2	3	2	2
		3	2	4	4	3	1	1	2	1	3
	Barn D	2	4	4	1	4	3	1	2	1	2
		3	2	4	1	3	3	3	1	2	4
Sample collection days	0	7	14	21	28						

Figure 5.1: Schematic representation of the study design and sampling schedule

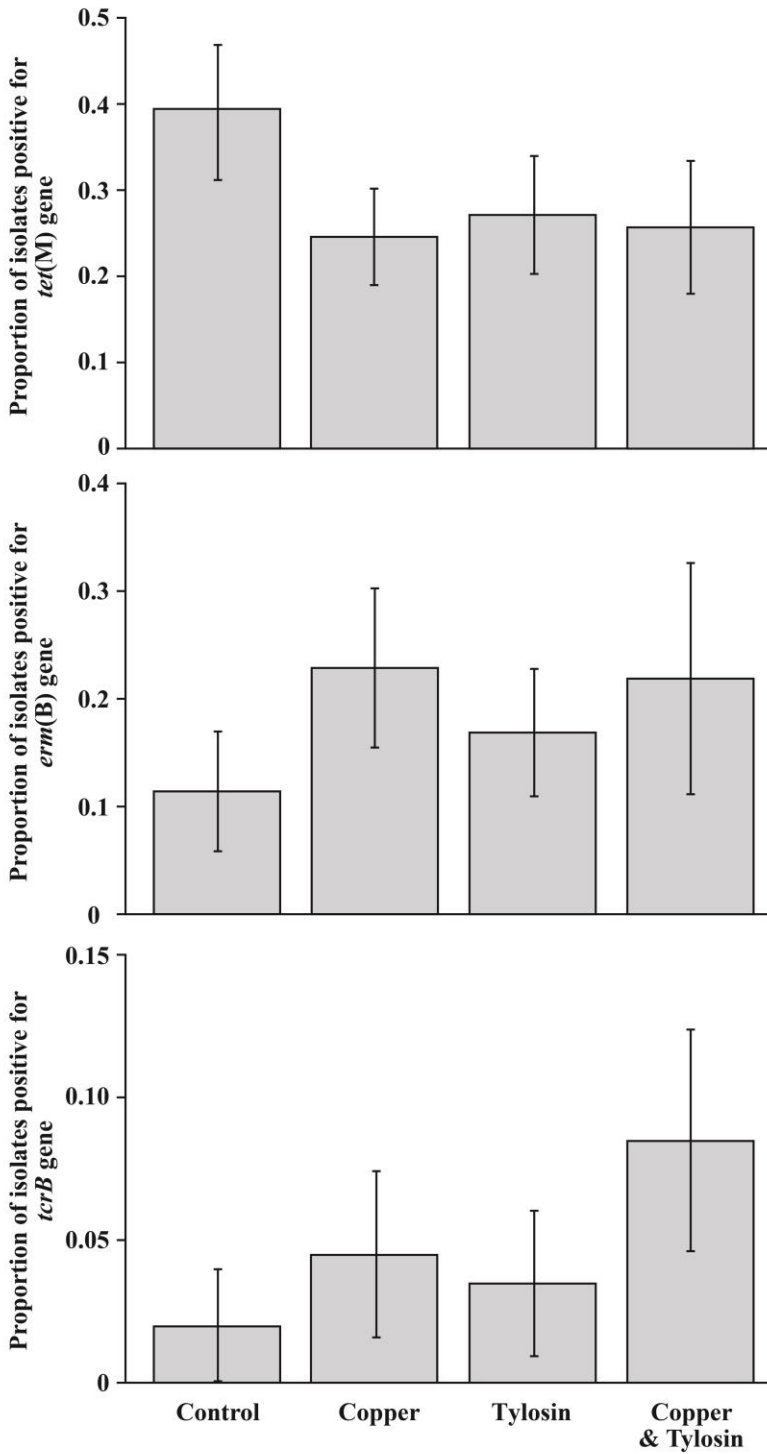


Figure 5.2: Prevalence of *tcrB*, *erm(B)*, and *tet(M)* enterococcal isolates for each treatment groups showing predicted prevalence with 95% confidence intervals. These results are across all treatment groups and sampling days and include co-resistant strains.

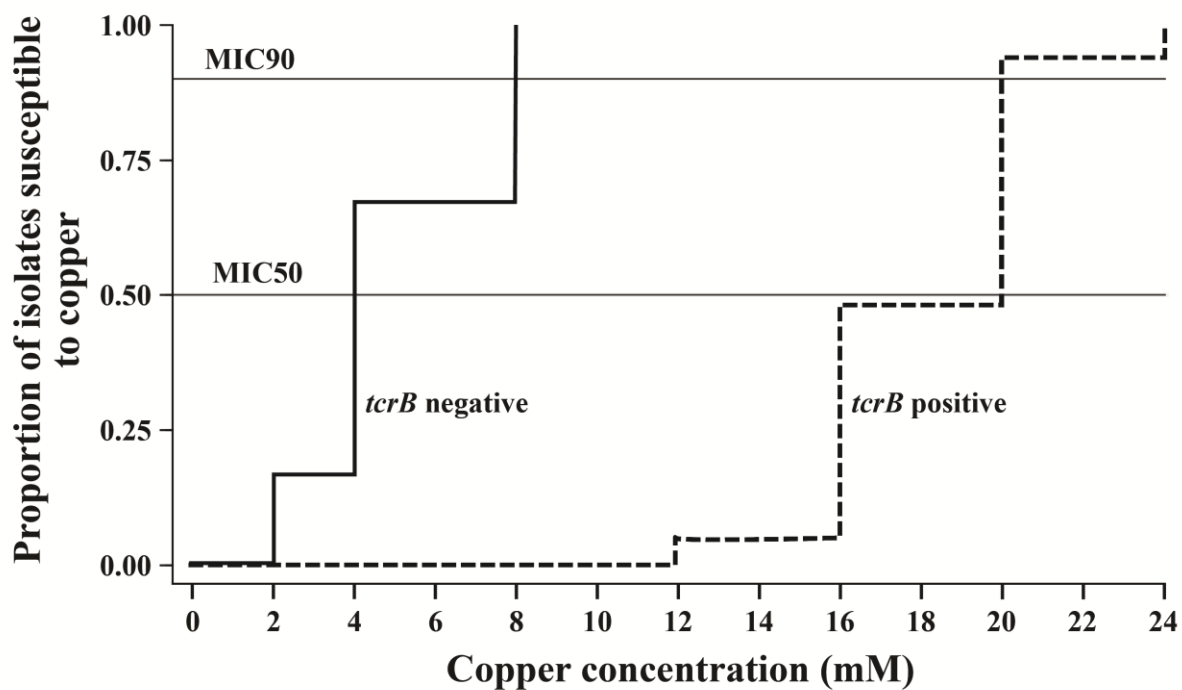


Figure 5.3: Failure function graph depicting cumulative susceptibilities of *tcrB*-positive [*erm*(B) and *tet*(M) positive] and *tcrB*-negative [*erm*(B) and *tet*(M) negative] fecal enterococci isolates in relation to copper concentration.

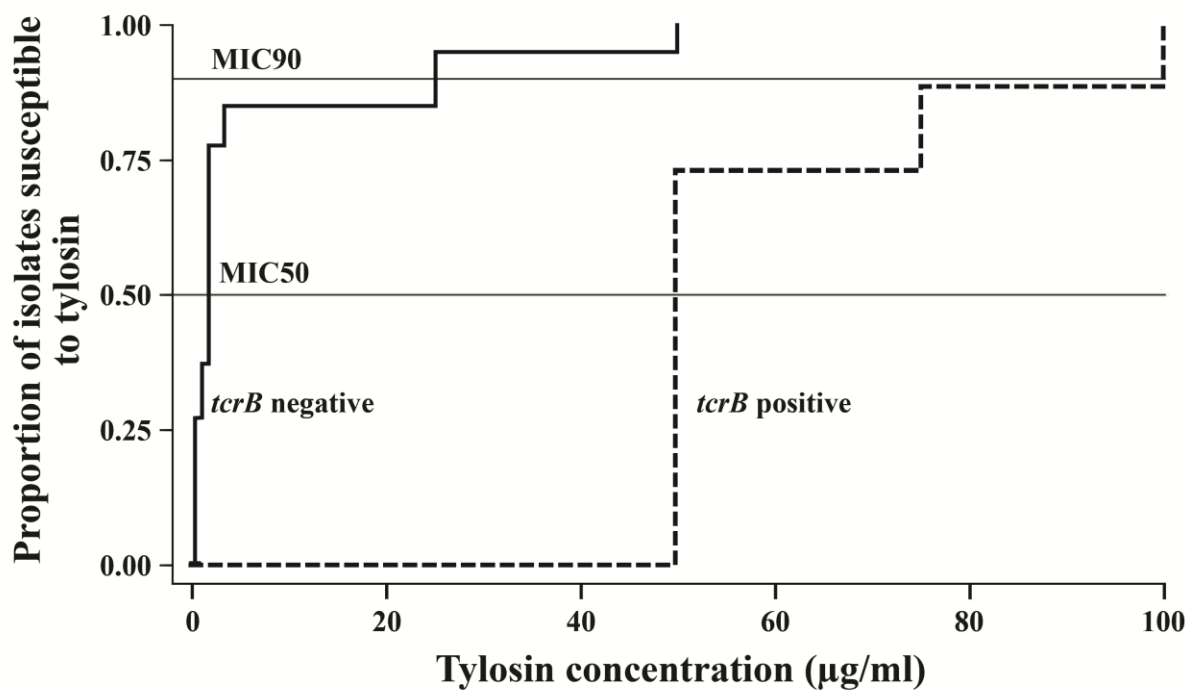


Figure 5.4: Failure function graph depicting cumulative susceptibilities of *tcrB*-positive [*erm*(B) and *tet*(M) positive] and *tcrB*-negative [*erm*(B) and *tet*(M) negative] isolates of fecal enterococci in relation to tylosin concentration.

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CHAPTER 6 - Molecular epidemiology of vancomycin-resistant *Enterococcus faecium* isolated from a semi-closed and integrated Human and Swine population

Introduction

Vancomycin, a glycopeptide antibiotic, is the drug of choice for treating enterococcal infections caused by multidrug resistant Gram positive bacteria (Cetinkaya et al., 2000). The therapeutic use of vancomycin in U.S. hospitals has contributed to the emergence and dramatic expansion of vancomycin resistant enterococci (VRE) over the past three decades (Edmond et al., 1995; Kirst et al., 1998). Vancomycin resistance among *Enterococcus* species is a major public health concern in human clinical settings since the rate of occurrence of VREs is expected to increase during the onset of disease (Harwood et al., 2001). In the U.S., VRE are extremely rare in animals and as a source of community infections in humans. The likely reason is that avoparcin, a glycopeptide growth promoter closely related to vancomycin, was never approved for use in animals in the U.S. Only recently, an occurrence of VRE in swine was reported in the U.S., unlike in Europe where its presence was well documented during the 1990s (Bager et al., 1997; Donabedian et al., 2010). Generally, there are five major recognized phenotypes of vancomycin resistance: VanA, VanB, VanC, VanD, and VanE. Both VanA and VanB types of resistance have been implicated in hospital acquired infections. Among all the species of this genus, *Enterococcus faecium* and *E. faecalis* are the most commonly reported agents associated with human disease (Friden et al., 1993; Cetinkaya et al., 2000). Based on the phenotypic and genotypic characteristics of the bacteria, *E. faecium* is considered to be the most host-adapted

organism with different ecovars and host-specific groups (Willems et al., 2000). In the United States, the emergence of *E. faecium* as a nosocomial pathogen started with an increase in resistance to ampicillin in the 1980s followed by emerging resistance to vancomycin in the 1990s leading to an increase in vancomycin resistant *E. faecium* (Perlada et al., 1997; Coque et al., 2005).

Molecular epidemiological studies and population structure analyses indicate that the emergence of *E. faecium* and *E. faecalis* as major nosocomial pathogens has resulted from the evolutionary development of specific lineages, or clonal complexes, harboring antimicrobial resistance determinants (Willems et al., 2005; Galloway-Pena et al., 2009). More often, these outbreaks involve clonal dissemination of strains, not only within the hospitals but also between hospitals (Moreno et al., 1995). Most of the VRE causing human infections have been identified as *E. faecium* of the clonal complex CC17, which seems to have played a major role in the emergence of vancomycin resistance (Valdezate et al., 2009). Studies relating to investigating potential barriers to resistance in community-based and agricultural strains of VREs, and also to understanding evolutionary dynamics in the dissemination of VREs in humans and animals, are sparse. We hypothesized that there are differences in the molecular epidemiology of VRE that help to explain this geographical disparity and that understanding these differences is essential for maintaining low levels of VRE in community and agricultural settings in the U.S. Here, we compared VREs from both human-derived waste water and swine fecal samples to explore possible differences in resistance determinant carriage and to elucidate the genetic and clonal differences that help to address the global epidemiological status of each level of genetic aggregation. The purpose of this study was to characterize VRE, and to determine the

emergence of any major clonal complexes isolated from human waste water effluents and swine fecal samples within an integrated semi-closed agri-food system in Texas.

Materials and Methods

Study population, experimental design, and sampling. The study consisted of both humans and swine populations arranged in a semi-closed vertically integrated agri-food system at eighteen geographically separated farm and non-farm sites in Texas (Scott et al., 2005; Poole et al., 2005; Alali et al., 2008). A schematic representation of sampling design, consisting of both human and swine group-level cohorts, is given in Fig.1. Humans were housed in 19 geographical units with 12 of the units having co-located swine operations; one location was co-located with a swine slaughter house facility. Based on occupational exposure, the human populations consisted of both “swine worker”, who are involved both in swine rearing operations and slaughter facilities, and “non-worker” cohorts,. Importantly, the human population at the intake to the system included only non-workers. All humans were fed in a common eating area specific to each cohort with centrally prepared meals. Multiple representative human waste water samples (n=1,315) were collected at each unit location monthly by trained facility staff. The wastewater manholes were selected for sampling if they were determined to be draining a lavatory facility for each of the appropriate occupational housing cohorts. In addition, influent samples from the appropriate wastewater treatment plant were collected each month.

The swine population consisted of farrow-to-finisher and grower-to-finisher facilities distributed among the 12 swine units. There was no or little movement of swine into the system with no movement of live swine out of the system since all the pigs were slaughtered and consumed within the system. The swine moved vertically within the system from farrowing

units and nurseries to grower and finisher barns, and finally to slaughter, where the pork was processed and fed back to the human population within the system. Fresh floor fecal samples (n=486) and the influent samples from the barn wash were collected by a swine specialist veterinarian. The collected samples were shipped on ice to the USDA-ARS-laboratory in College Station, TX for further analyses. The total number of samples screened in the present study was purposively selected from the samples which were shipped to the Microbial Ecology and Molecular Epidemiology (ME²) Laboratory repository, Kansas State University. All the fecal samples were processed in duplicates.

Isolation of enterococci. Unless otherwise mentioned all culture media used were from Difco (Becton and Dickson, Sparks, MD). The fecal and waste water samples were processed by diluting 1 g of the sample in 10 ml of Enterococcoseal broth and incubating them at 37°C for 24 h. The enriched fecal suspension was spread-plated @ 50 µl onto M-*Enterococcus* agar containing 20 µg/ml of vancomycin. The plates were incubated for 24 h-36 h at 42°C. All isolates were tested for esculin hydrolysis by inoculating them into 100 µl Enterococcosel broth in a 96 well micro titer plate (Becton and Dickinson, Franklin Lakes, NJ) and incubating at 37° C for 4 h. Isolates were stored in Cryocare protect beads (Key Scientific Products Inc. Stamford, TX) for future use.

PCR for the detection of VRE's. The DNA was extracted by suspending a single colony in nuclease-free water with Chelex[®] 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiling it for 10 min. The vancomycin resistance genes were detected according to the procedure described by Kariyama et al. (2000). *Enterococcus faecium* (ATCC 51559) and *E. faecalis* (V583) were used as positive controls for the screening of *vanA*, and *vanB* genes, respectively.

Speciation of enterococci. Species identification of the enterococcal isolates was carried out by a multiplex PCR that identifies *E. faecium*, *E. faecalis*, *E. gallinarum* and *E. casseliflavus* (Jackson et al., 2004). The DNA was extracted by suspending a single colony in nuclease-free water with Chelex[®] 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiling it for 10 min. The ATCC strains of *E. faecium* (ATCC 19434), *E. faecalis* (ATCC 19433), *E. gallinarum* (ATCC 49579) and *E. casseliflavus* (ATCC 25788) served as positive controls. Master mixes, primers and running conditions for the multiplex PCR were as described by Jackson et al. (2004). Speciation was further confirmed by superoxide dismutase (*sodA*) gene sequence analysis (Poyart et al., 2000).

PCR detection of *erm*(B) and *tet*(M) genes. The DNA from enterococcal isolates was extracted as before and subjected to PCR assays for detections of *erm*(B), and *tet*(M) genes (Amachawadi et al., 2013). Primers used in the study were supplied by Invitrogen Life Technologies (Carlsbad, CA).

Detection of virulence genes. Virulence genes detection was done on the vancomycin resistant enterococcal isolates, by multiplex PCR that identifies *asaI* (aggregation substance), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *hyl* (hyaluronidase) genes. The DNA template was prepared using aforesaid procedure. *Enterococcus faecalis* MMH594 (Dr. Lynn Hancock, Dept. of Biology, Kansas State University) was used as a positive control. Master mixes, primers and running conditions for the multiplex PCR were as described by Vankerckhoven et al. (2004).

Antibiotics susceptibility determinations. The MICs of ampicillin, erythromycin, fusidic acid, rifampicin, and vancomycin (Sigma-Aldrich, St. Louis, MO) were determined by micro-broth dilution method as per CLSI guidelines (CLSI, 2013). Antibiotic concentrations

tested were based on the potency of the antibiotics. Stock solutions were prepared with sterile distilled water to obtain an initial concentration of 1,000 µg/mL. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, and 0.098 µg/ mL. Bacterial cultures grown in 10 mL of Mueller-Hinton II broth for 6 h and the inocula concentrations were adjusted to 0.5 McFarland turbidity standards. The susceptibility determinations for antibiotics were carried out using 96-well micro titer plates (Becton and Dickson) by incubating plates with the bacterial inocula at 37°C for 24 h and results were recorded as growth or no growth. MIC determinations were carried out on both human (n=62) and swine (n=8) VRE isolates.

Transferability of *vanA* genes. A conjugation assay was performed using the filter mating procedure (Tendolkar et al., 2006) to demonstrate the transferability of the *vanA* gene from *vanA*-positive *E. faecium* isolates to *vanA*-negative *E. faecium* and *E. faecalis* isolates. The interspecies conjugation was performed with five isolates each of *vanA*-positive *E. faecium*. The *vanA*-positive donor strains (human and swine VREs, *E. faecium*) were resistant to erythromycin (MIC > 100 µg/ml) and susceptible to rifampicin (MIC = 12.5 µg/ml) and fusidic acid (MIC = 12.5 µg/ml). The recipient strains [*E. faecium* (BM 4105 RF) and *E. faecalis* (JH2-2)] were resistant to rifampicin and fusidic acid (MIC > 100 µg/ml), and susceptible to vancomycin and erythromycin (MIC = 0.78 µg/ml). The resultant transconjugants were selected on BHI agar plates containing rifampicin, fusidic acid, and erythromycin. The transconjugants were tested for *vanA* and *erm(B)* genes by PCR and their susceptibility to copper was determined as described above. The transfer frequency for each isolate was calculated as the number of transconjugants per recipient CFU.

Determination of antimicrobial susceptibility for vancomycin resistant

enterococci. Antimicrobial susceptibility testing was performed with a Sensititre automated system, according to the manufacturer's instructions (Trek Diagnostic Systems, Cleveland, OH) using a custom gram-positive panel (CMV3AGPF, Trek Diagnostics Systems) designed by the National Antimicrobial Resistance Monitoring System (NARMS). In order to determine the potential association between vancomycin resistance and resistance to other antimicrobial agents, both human waste water VREs (n=62) and swine VREs (n=8) were used. Minimum inhibitory concentrations (MIC) were determined by the micro-broth dilution method according to CLSI. Individual bacterial colonies were mixed with demineralized water (Trek Diagnostic Systems, Cleveland, OH) to match 0.5 McFarland turbidity standards. A 50 µL aliquot of the bacterial inoculum was added to Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH) and vortexed. Then, 100 µL of the broth was dispensed into Gram-positive NARMS panel plates with the aid of the Sensititre[®] automated inoculation delivery system (Trek Diagnostics Systems). Plates were incubated for 18 h at 37°C. The breakpoints were recorded as resistant, intermediate or sensitive based on the CLSI guidelines (CLSI, 2013), with intermediate strains reclassified as susceptible for statistical analyses. *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection, Manassas, VA) isolates served as reference quality control strains for MIC testing. The list of 16 antimicrobials tested, their concentration ranges used, breakpoints according to NARMS, and their classification based on World Health Organization (WHO, 2012) criteria, are shown in Table 2.

Multiple-locus variable number tandem repeat analysis (MLVA) of *vanA*-

positive enterococci. MLVA, a typing scheme based on six different tandem repeat loci for *E.*

faecium, was performed with both human and swine VRE isolates, as per the procedures described by Top et al. (2004). Six variable number tandem loci (VNTR), VNTR-1, VNTR-2, VNTR-7, VNTR-8, VNTR-9, VNTR-10, were used for genotyping of *vanA*-positive enterococcal isolates. The MLVA types (MTs) were assigned based on the sequence results using the MLVA database at <http://www.umcutrecht.nl/subsite/MLVA/Method/>. The sequence analyses for MLVA were performed using ClustalW and ST/CC, and assigned using eBURSTv3 analysis (Feil et al., 2004) at <http://eburst.mlst.net> with ST/CCs already available in the MLVA *E. faecium* database.

Multilocus sequence typing of *vanA*-positive enterococci. Multilocus sequence typing (MLST) was performed to determine the evolutionary relationship between the isolates as per a previously described procedure (Homan et al., 2002). MLST was performed targeting seven housekeeping genes of *E. faecium* (*adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purk*, and *pstS*). MLST on all 70 VRE isolates was performed at the Massachusetts Eye and Ear Infirmary, Harvard Medical School. The eBURST analyses were conducted as stated above in the MLVA procedure to assign a sequence type (ST) to each isolate according to its allelic profile (Feil et al., 2004) and then to assign to a clonal complex (CC).

Statistical Analysis. The statistical analysis was performed using STATA v.12.1 (StataCorp, College Station, TX). Bivariate descriptive statistics of gene or phenotype prevalence cross-tabulated by sample type, production stage, unit, and location were assessed prior to multivariable analysis. Unadjusted differences in prevalence proportions between variable levels were assessed using the Likelihood ratio chi-square test. The antibiotic MIC susceptibility data were analyzed using a log₂MIC-transformed and adapted survival analysis and the MIC was expressed and compared as the median (MIC₅₀) after a log rank assumption for

normality (Kasstele et al., 2012). The phenotypic data for 16 antimicrobials were considered based on the resistant or susceptible enterococci, both as outcomes (binary) and as the multidrug resistance profiles. The results were cross-tabulated with each of the treatment groups, and *vanA* gene prevalence to compare the proportion of enterococcal isolates resistant to each of the antimicrobial agent. The differences in transfer frequency (number of transconjugants per recipient) of *vanA* gene for both intra- and inter-species conjugation were assessed using the two-sample t test following a normality assessment using the Shapiro-Wilk test. The transfer frequencies were also tested for equality of variance (variance comparison test) and independence was assumed. Results were considered significant at a *P*-value of < 0.05.

Results

Prevalence of VREs, *erm*(B), *tet*(M), speciation, and virulence genes

Human waste water samples: A total of 1,315 waste water samples were processed across 19 geographical units consisting of 5 different sample types; worker, non-worker, mix, hospital, and employee cohorts. A total of 566 enterococcal isolates were obtained (566/1,315; 43%), which were used for further analyses. Sixty two of the 566 (10.9%) enterococcal isolates were positive for the presence of VRE (Table 1), with one isolate positive for both *vanA* and *vanB* genes. All the VRE isolates were *E. faecium*. All the VRE isolates also were positive for *erm*(B) and *tet*(M) genes. All VRE isolates (n=62) tested were negative for *asaI* (aggregation substance), *gelE* (gelatinase), *hyl* (hyaluronidase) and *cylA* (cytolysin) genes; however, all were positive for the *esp* (enterococcal surface protein) gene.

Swine fecal samples: A total of 616 esculin positive isolates (63.3%; 616/972) were obtained. A total of 8 VRE isolates (*vanA* gene) were obtained fecal samples were processed

from 13 geographical units representing 8 swine production types nursery, grower and finisher, farrowing, breeding, boars, holding pen, and sick pig cohorts were examined (Table 1). None of the isolates were positive for the *vanB* gene. Based on multiplex PCR all the VRE isolates were *E. faecium*. All the VRE isolates also were positive for *erm(B)*, *tet(M)*, and *esp* genes.

Multidrug resistant profile of VREs

The overall MIC distributions and susceptibility or resistance profiles of vancomycin resistant *E. faecium* isolates by host (human waste water versus swine fecal samples) to each of 16 antimicrobials, along with their MIC₅₀ and MIC₉₀ values, and proportion resistant are shown in Table 3. The proportion of VRE isolates resistant to any of the other antimicrobials did not differ between human and swine VRE isolates ($P > 0.05$). Among the human VREs, 50% of them were resistant to ten or more antimicrobials. The vast majority of the human VRE isolates (98.4%; 61/62) were resistant to three or more antimicrobials, and thus were classified as multidrug resistant (MDR; Pillar et al., 2008). Among both human and swine VRE isolates, the highest proportion of resistance was to lincomycin, tetracycline, tylosin, erythromycin, penicillin, ciprofloxacin, kanamycin, and gentamicin. All the swine VRE isolates were multidrug resistant (100%) and were resistant to either eight (n=1), nine (n=1), or ten (n=6) antimicrobials. The prevalence of multidrug resistant phenotypes among VREs from human wastewater (n=62) and swine feces (n=8) are depicted by human occupational cohorts and swine production stages in Tables 4 and 5, respectively.

Transferability of *vanA* gene

Ten vancomycin resistant *E. faecium* isolates (5 human waste water VRE and 5 swine VRE) were used to demonstrate both intraspecies (*E. faecium*) and interspecies (*E. faecalis*) transferability of the *vanA* gene by conjugation. The mean transfer frequencies for both intra-

and inter-species conjugations were 1.8×10^{-5} and 2.0×10^{-5} , respectively. The conjugation frequency did not differ between inter-species and intra-species ($P = 0.36$). The transconjugants were positive for *vanA*, and *erm(B)* genes and phenotypically resistant to vancomycin and erythromycin, respectively. The median vancomycin MIC of the transconjugants was 37.5 µg/ml.

Multiple-locus variable number tandem repeat analysis (MLVA)

The MLVA procedure revealed many novel MLVA types (MTs) among human and swine VRE isolates, as shown in Fig. 2. Among 62 human VREs, three isolates were clonally related to existing MT 81, MT89 and MT 172 in the database. Eight swine VRE isolates were clustered among seven novel MTs. The VRE isolates with unassigned MT's were submitted to the curator (Dr. J. Top, University of Utrecht, Netherlands) for sequence types and cluster assignment and also to update the global epidemiological database of *E. faecium*. On eBURST, clustering of three of the human VRE isolates shared one or more than one alleles at loci (MTs 81, 89, and 172) which were from hospitalized patients or clinics in the European Union. The rest of the human VRE isolates were clonally different and did not share any alleles at loci with existing MTs. The eight VRE isolates were also clonally different and did not share any alleles at loci with existing MTs in the global database or with the human VRE isolates.

Multilocus sequence typing analysis (MLST)

The MLST sequence analyses revealed two novel sequence types among 8 swine VRE isolates. Six swine VRE isolates were belonged to the sequence type, ST16 and ST132, which are subgroup founders with their origins being clonal complex 17 (CC17). This clonal complex is largely associated with hospital outbreaks and nosocomial infections around the world, and is characterized by ampicillin and quinolone resistance and with the presence of the *esp* gene.

Discussion

Studies relating to the prevalence of VREs among swine or food animal agriculture are sparse in the United States. Hence, this study was undertaken to study the potential barriers to resistance in community based and agricultural strains and to understand the evolutionary dynamics of the VREs in humans and animals. Therefore, this well characterized study population, with regards to swine and swine product exposure of humans, provided a unique opportunity to test intractable hypotheses regarding the potential risk of multidrug resistant enterococci, coupled with vancomycin resistance, among humans and swine. Our study is the second report on the occurrence of VREs from swine that were never given glycopeptides in the United States. Importantly, these samples were collected in 2004, fully 5 years before the reports of VRE in show pigs emerged from Michigan (Donebadian et al...). In the present study, we compared VRE isolates of swine and their affiliated occupational cohorts of human. In this study population, there was a limited movement of both swine and humans in and out of the integrated system, and all pork products were processed at the single slaughter plant with the products being fed to humans within the system. The role of different reservoirs in the spread of vancomycin resistant enterococci (VRE) remains unclear.

There is clear evidence linking the use of avoparcin, a glycopeptide antibiotic in farm animals in Europe, and high levels of VREs in farm animals which subsequently caused colonization in the human community (Bager et al., 1997). However, the first description of vancomycin resistant enterococci was based on isolates from show pigs in several Michigan counties, United States (Donabedian et al., 2010); importantly, in the absence of selective pressure from avoparcin or other related glycopeptide antibiotics.

It has been suggested that the rapid increase of VRE in human hospitals in the United States has been due to a 5-10 fold higher use of vancomycin in the U.S. compared to other European countries, such as France, Italy, Germany, and The Netherlands (Kirst et al., 1998; Bonten et al., 2001). In the United States, the emergence of *E. faecium* as a nosocomial pathogen started with an increase in resistance to ampicillin in the 1980s, followed by resistance to vancomycin in the 1990s, leading to an increase in vancomycin resistant *E. faecium* (Perlada et al., 1997; Coque et al., 2005).

Environmental factors such as those found in farms, communities or hospitals, impose selective pressures, which likely play a significant role in determining the characteristics of VREs (Martinez and Baquero, 2002; Willems et al., 2000). More often, horizontal transmission of vancomycin resistance among enterococci strains is the common route of dissemination, since the *vanA* gene cluster resides on a mobile genetic element, Tn1546. Earlier studies have demonstrated the transmission of the *vanA* gene between animals and humans (Aarestrup et al., 1996; Willems et al., 1999; Woodford et al., 1998). These determinants are located mainly on mobile genetic elements such as plasmids or transposons, which ensure their spread by horizontal gene transfer. In the majority of cases, these plasmids are conjugative and exhibit a broad host range in nature (Martinez, 2009). If carrying genetic elements conferring resistance is not a fitness burden, reducing the use of antimicrobial agents alone may not decrease the prevalence of antibiotic resistance bacteria within the gut (Gillespie, 2001).

Studies from Denmark have showed that the majority of the VREs from pigs also can be co-selected for tetracycline resistance, which is highly plausible due to the extensive use of tetracycline in animal agriculture (Aarestrup et al., 2000; Teuber, 2001). The predominant MDR phenotype among these VRE isolates suggests that certain clones carrying antibiotic resistance

determinants are more prevalent in waste water treatment plants versus swine fecal samples. Besides animal movements in the global market, the selective pressures imparted from the use of antimicrobial alternatives (e.g., heavy metals such as copper), feed grade antimicrobials (tetracyclines, macrolides), and other antibiotics (β -lactams) may have contributed to the persistence and dissemination of VRE among swine farms in Europe and also facilitated the horizontal transfer of resistance determinants via conjugative plasmids or transposons to other enterococcal hosts (Freitas et al., 2011).

The presence of high-level aminoglycoside resistance (e.g., gentamicin, kanamycin, and streptomycin) among our VRE isolates is cause for concern as these antimicrobial agents have been classified as ‘critically important’ by the World Health Organization (WHO Report, 2012). A study from Portugal (Araujo et al., 2010) has reported up to 75% resistance to gentamicin and streptomycin among vancomycin resistant *E. faecium* isolates from wastewater and sludge. Previous studies have also reported the prevalence of high-level of aminoglycoside resistant enterococci among VRE isolates of wastewater samples (Talebi et al., 2008; Araujo et al., 2010; Rosenbern Goldstein et al., 2014). A high rate of erythromycin resistance was also reported among our VRE isolates. This is in agreement with the study from Talebi et al. (2008), where they reported high rate of concomitant resistance to erythromycin and gentamicin in vancomycin resistant *E. faecium* isolates of sewage sludge and humans.

All the VRE isolates of human waste water and swine feces from our study are resistant to tetracycline [*tet*(M)] and macrolide [*erm*(B)] genes. This is in agreement with the earlier study from Michigan, where all the swine VRE isolates (n=6) shown to carry *tet*(M) and *erm*(B) in addition to *vanA* in the same plasmid (Freitas et al., 2011), indicating that co-selection might have played a role in their survival and persistence in the absence of glycopeptide use. There is a

strong evidence from our earlier studies suggesting the presence of acquired copper resistance (*tcrB*), macrolide [*erm*(B)], and tetracycline [*tet*(M)] resistance genes on a mobilizable plasmid among *E. faecium* isolates of swine in the U.S. (Amachawadi et al., 2011). The abundance of at least one tetracycline resistance determinant among *E. faecium* isolates is more common than for other gut bacteria since; that is, the acquired tetracycline resistance is more prevalent in enterococci (Anderson et al., 2008).

MLVA analyses revealed an extensive genetic diversity among our VRE isolates. Both human and swine VRE isolates were more diverse and heterogeneous, with an absence of predominant MLVA type or MT. The majority of newly assigned MTs were clonally related in single or double loci with the already existing global epidemiological database. Note that this does not imply direction or causation. Order of entry into these MLVA libraries is typically driven by necessity for which clinically derived isolates tend to have an advantage. Fifty-nine of the 62 human VREs and all the 8 swine VREs were novel MLVA types. The genetic diversity among the *E. faecium* isolates may be attributable to their propensity for genetic exchange coupled with a high rate of recombination (Willems et al., 2009).

MLST analyses revealed the presence of predominant clonal complex, CC17 among swine VRE isolates. In our study, all the swine VRE were belonged to the sequence type, ST16 and ST132, which were responsible for hospital outbreaks and nosocomial infections around the world with ampicillin resistance and presence of *esp* gene (MLST database). To our knowledge, this is the first report of VRE from swine from the United States with CC17 clonal type. A specific clonal complex, CC17, has been responsible for worldwide dissemination among hospitals, including the United States (Ko et al., 2005; Leavis et al., 2004). Recent molecular epidemiological studies have revealed global expansion of this clone, CC17, containing a

putative pathogenicity island with *esp* gene (enterococcal surface protein); this was found to be more prevalent among clinical isolates (Top et al., 2008). Recently, the same CC17 clone was reported from swine in Portugal (Freitas et al., 2009). Therefore, it is of utmost importance to investigate the prevalence of this major clonal complex and factors responsible for its spread to continue to assess the extent to which these strains have acquired vancomycin resistance (Gordoncillo et al., 2013). No doubt, as broader studies into populations of bacteria outside of clinical realms enter these databases, a clearer picture will emerge. Diversity among the multidrug resistant enterococci suggests that no single genotype is favored in a particular study environment. Multiple drug resistant strains of *E. faecium* are more often associated with hospital acquired infections due to transmission of these strains to humans and their ability for horizontal gene transfer. Future studies are warranted to study the global epidemiology to understand the spread and evolution of clones via mobile genetic elements encoding vancomycin resistance and to overcome the potential barriers between human and swine hosts.

Acknowledgements

Financial support for this project was provided by the College of Veterinary Medicine, Kansas State University under the ‘Mentored Clinical, Applied, or Translational Research (MCAT Research Award # 2011). We would like to thank and appreciate the help of Mal Hoover in preparation of these images. We would also like to thank Jose Saavedra (Laboratory Technician, Dr. Gilmore’s Research group, Harvard Medical School) for his support with the MLST. We also extend our thanks to Dr. Anuradha Ghosh (Dr. Zurek’s laboratory) for her technical support with the MLVA.

Table 6.1: Total number of samples screened selectively for the prevalence of vancomycin-resistant *Enterococcus faecium* by host species

Host species / genes	Number positive/Number tested		Prevalence (%)
	<i>vanA</i>	<i>vanB</i>	
Human wastewater	62/1315	1/1315	4.7
Swine	8/972	0	0.8

Table 6.2 : National antimicrobial resistance monitoring system gram positive bacterial panel (CMV3AGPF): antimicrobials, WHO classification, tested concentrations, and their resistant breakpoints

Antimicrobials	WHO Classification of Antimicrobials	Concentrations (µg/ml)	Breakpoints (µg/ml)
Chloramphenicol (CHL)	Highly Important	2 - 32	≥ 32
Ciprofloxacin (CIP)	Critically Important	0.12 - 4	≥ 4
Daptomycin (DAP)	Critically Important	0.25 - 16	N/A ¹
Erythromycin (ERY)	Critically Important	0.25 - 8	≥ 8
Gentamicin (GEN)	Critically Important	128 – 1024	> 500
Kanamycin (KAN)	Critically Important	128 - 1024	≥1024
Lincomycin (LIN)	Highly Important	1 - 8	≥ 8
Linezolid (LZD)	Critically Important	0.5 - 8	≥ 8
Nitrofurantoin (NIT)	Important	2 - 64	≥ 128
Penicillin (PEN)	Critically Important	0.25 - 16	≥ 16
Quinupristin/dalfopristin (SYN)	Highly Important	0.5 - 32	≥ 4
Streptomycin (STR)	Critically Important	512 - 2048	>1000
Tetracycline (TET)	Highly Important	1 - 32	≥ 16
Tigecycline (TGC)	Critically Important	0.015 - 0.5	N/A ²
Tylosin tartrate (TYL)	Critically Important	0.25 - 32	≥ 32
Vancomycin (VAN)	Critically Important	0.25 - 32	≥ 32

N/A = Not applicable

¹For daptomycin, only susceptible breakpoint (≤ 4 µg/ml) has been established. The isolates with an MIC ≥ 8 µg/ml are considered as resistant

²For tigecycline, only susceptible breakpoint (≤ 0.25 µg/ml) has been established. The isolates with an MIC ≥ 0.5 µg/ml are considered as resistant

Table 6.3: Antimicrobial susceptibilities and minimum inhibitory concentrations (MIC) of Vancomycin-resistant *Enterococcus faecium* (VREs) strains isolated from human waste water and swine fecal samples

Antimicrobials	Human VREs (n=62)				Swine VREs (n=8)			
	Resistant (%)	95% CI	MIC ₅₀	MIC ₉₀	Resistant (%)	95% CI	MIC ₅₀	MIC ₉₀
Chloramphenicol	14.5	6.8-25.7	4	4	0	0-37*	4	4
Ciprofloxacin	80.6	68.6-89.5	4	4	87.5	47.3-99.6	4	4
Daptomycin	3.2	0.4-11.1	2	4	12.5	0.3-52.6	4	8
Erythromycin	75.8	63.2-85.7	7	7	100	63-100*	7	7
Gentamicin	56.4	43.2-69.0	4	4	75	35-96.8	4	4
Kanamycin	72.6	59.7-83.1	5	5	100	63-100*	5	5
Lincomycin	96.7	88.8-99.6	4	4	100	63-100*	4	4
Linezolid	9.7	3.6-19.8	2	3	0	0-37*	2	2
Nitrofurantoin	11.7	9.2-29.5	3	5	0	0-37*	3	3
Penicillin	74.2	61.5-84.4	6	6	100	63-100*	6	6
Quinupristin / Dalfopristin			1	6		0.3-52.6	1	3
Streptomycin	42.0	29.5-55.1			12.5			
Tetracycline	66.13	53-77.6	4	4	100	63-100*	4	4
Tigecycline	80.6	68.6-89.5	2	6	100	63-100*	2	6
Tylosin tartrate	1.6	0.04-8.6	0.06	0.12	0	0-37*	0.06	0.06
Vancomycin	74.2	61.5-84.4	6	6	100	63-100*	6	6
	100	94.2-100*	4	4	100	63-100*	4	4

*One sided, 97.5% confidence interval

Table 6.4: Prevalence of multidrug resistant phenotypes among Vancomycin-resistant *Enterococcus faecium* (VREs; n=62) strains isolated from human waste water across their vocation groups

Vocation groups	MDR Phenotypes	No. of resistant isolates
Employee	CIP-ERY-PEN-SYN-TET-VAN	1
	CHL-LZD-LIN-SYN-VAN	1
Mix	CIP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1
	CIP-ERY- KAN-LIN-NIT-PEN-STR-TET-TYL-VAN	1
	CIP-ERY-KAN-LIN-NIT-PEN-STR-TYL-VAN	1
	CIP-ERY-LIN-NIT-PEN-TET-VAN	1
	LIN-PEN-SYN-TET-TYL-VAN	1
	LIN-SYN-VAN	1
	CHL-CIP-ERY-GEN-KAN-LIN-NIT-PEN-STR-TET-VAN	1
	CHL-CIP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1
	CHL-CIP-ERY-GEN-KAN-LZD-LIN-NIT-STR-SYN-TET-TYL-VAN	1
	CHL-CIP-ERY-GEN-KAN-LZD-LIN-STR-SYN-TET-TYL-VAN	1
	CHL-LIN-SYN-TET-TGC-VAN	1
	CHL-LIN-SYN-TET-VAN	1
	CHL-LZD-LIN-SYN-TYL-VAN	1
	CIP-DAP-ERY-GEN-KAN-LIN-PEN-STR-SYN-TET-TYL-VAN	1
	CIP-DAP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1
	CIP-ERY-GEN-KAN-LIN-NIT-PEN-STR-TET-TYL-VAN	1
	CIP-ERY-GEN-KAN-LIN-PEN-STR-SYN-TET-TYL-VAN	12
	CIP-ERY-GEN-KAN-LIN-NIT-PEN-STR-SYN-TET-TYL-VAN	1
Non-worker	CIP-ERY-KAN-LIN-NIT-PEN-STR-TYL-VAN	1
	CIP-ERY-KAN-LIN-NIT-PEN-SYN-TET-TYL-VAN	2
	CIP-ERY-KAN-LIN-PEN-STR-TET-TYL-VAN	2
	CIP-ERY-KAN-NIT-PEN-TET-VAN	1
	CIP-GEN-LIN-PEN-VAN	1
	CIP-GEN-LIN-SYN-TET-TYL-VAN	1
	CIP-LIN-SYN-TET-VAN	2
	ERY-KAN-LIN-PEN-STR-SYN-TET-TYL-VAN	1
	ERY-KAN-LIN-SYN-VAN	1
	LIN-SYN-VAN	1
	LIN-VAN	1
	CHL-CIP-ERY-KAN-LIN-PEN-STR-TET-TYL-VAN	1
	CIP-ERY-GEN-KAN-LIN-PEN-STR-SYN-TET-TYL-VAN	1
Worker	CIP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	9
	CIP-LZD-LIN-SYN-TET-VAN	1
	LIN-SYN-VAN	1
	LZD-LIN-SYN-VAN	1

*Please refer Table 6.2 for three letter antibiotic observations

Table 6.5: Prevalence of multidrug resistant phenotypes among Vancomycin-resistant *Enterococcus faecium* (VREs; n=8) strains isolated from swine feces across their production groups

Production groups	MDR Phenotypes	No. of resistant isolates
Nursery	ERY-KAN-LIN-PEN-STR-SYN-TET-TYL-VAN	1
	CIP-DAP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1
Farrowing	CIP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1
Grower/Finisher	CIP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	4
Holding pens	CIP-DAP-ERY-GEN-KAN-LIN-PEN-STR-TET-TYL-VAN	1

*Please refer Table 6.2 for three letter antibiotic observations

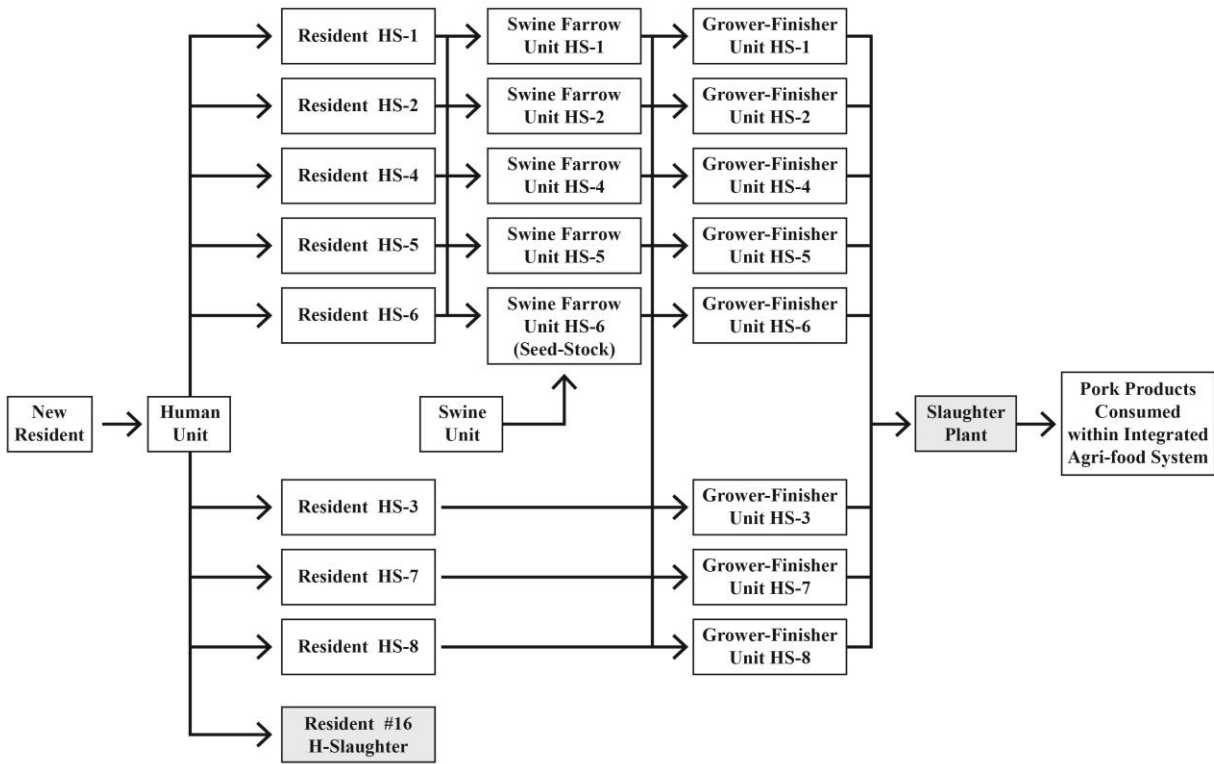


Figure 6.1: Schematic diagram illustrating unit locations with human populations and the flow of swine units (farrowing, grower-finisher) through the integrated agri-food system (Adapted from Alali et al., 2008)

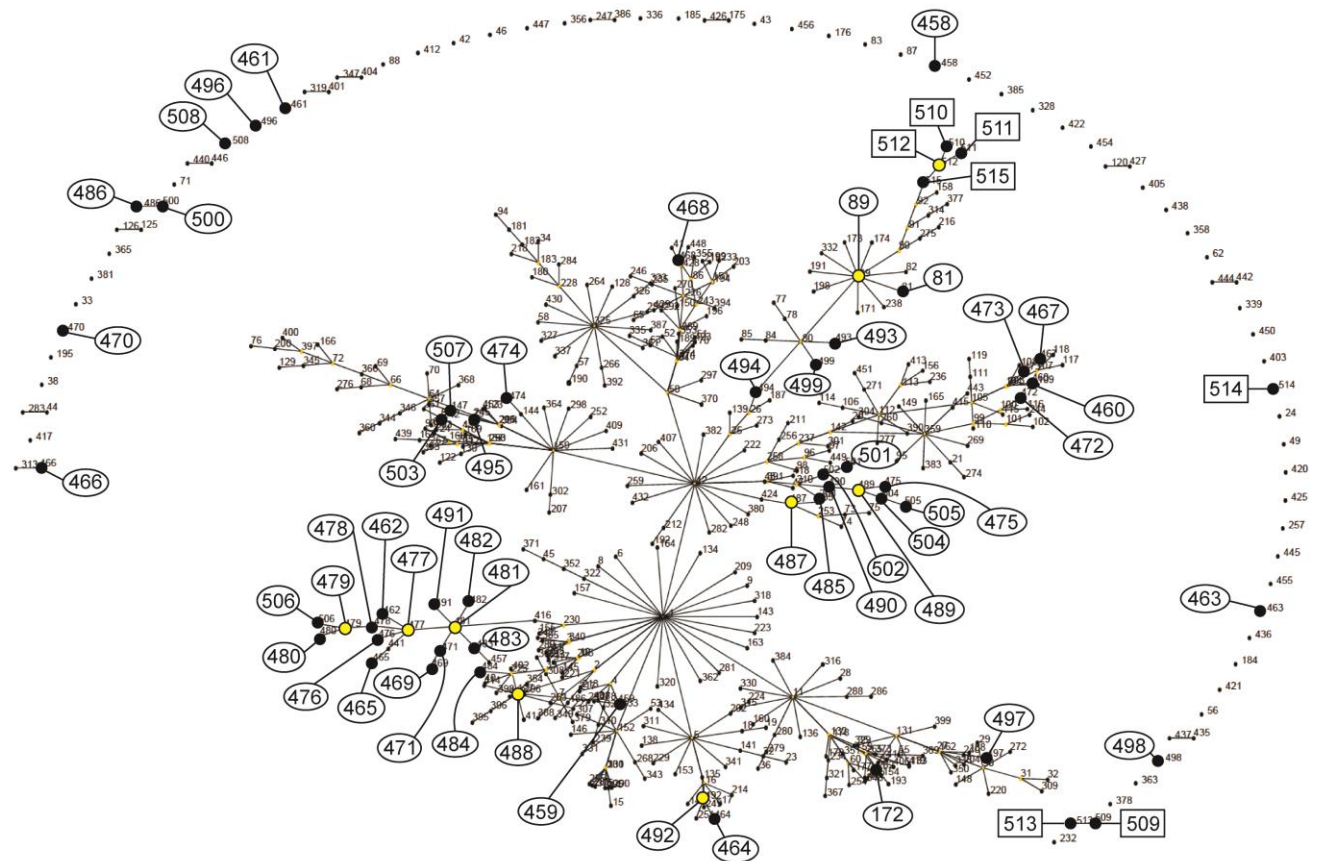


Figure 6.2: eBURST-based population snapshot of vancomycin resistant *Enterococcus faecium* on 515 available MT's (MLVA types) in the *Enterococcus faecium* MLVA (Multiple-Locus Variable number tandem repeat analysis) database (<http://www.umcutrecht.nl/subsite/MLVA>). eBURST-clustering of 70 MLVA analysis types (MTs) of vancomycin resistant *E. faecium* isolates from human wastewater (n=62; in ovals) and swine (n=8; in rectangles). Each MT represents one node with a difference in one locus



Figure 6.3: eBURST-based population snapshot of vancomycin resistant *Enterococcus faecium* isolated from swine feces (n=8) with all the available 887 ST's (sequence types) in the *Enterococcus faecium* MLST (Multilocus sequence typing) database (<http://www.efaecium.mlst.net/>). eBURST- analyses revealed two major STs (ST16 and ST132; subgroup founder), both belonged to the clonal complex, CC17 (founder).

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CHAPTER 7 - Summary and Conclusions

Antimicrobial resistance (AMR) is a serious and growing public health concern. Both prophylactic and growth promotion uses of antimicrobials have come under increasing scrutiny in recent years because of concerns over antimicrobial resistance (AMR) among pathogenic bacteria and the public health consequences of same. Animal agriculture has been blamed both for the emergence, dissemination, propagation, and persistence of antimicrobial-resistant bacteria. Measures to control the emergence, amplification and dissemination of antibiotic resistance have had limited success, in spite of the restricted use of many antibiotics. This suggests that there are components impacting the evolution, dissemination and persistence of antibiotic resistance, which have not yet been identified. There is, therefore, a need for research to develop mitigation strategies that will reduce AMR at a faster pace.

Intervention strategies to reduce the level of antimicrobial resistance should be directed towards reducing resistance drivers among food producing animals. Because of concerns about emergence and dissemination of resistance, alternatives to antibiotics as growth promoters are employed in animal feeding. Heavy metals, particularly copper and zinc, are currently being used extensively in food animal agriculture. The specific mechanisms by which these antimicrobial alternatives act as growth promoters are unclear. Apart from their beneficial effects, these antimicrobials are known to negatively affect the gut environment, potentially giving rise to immune and metabolic deficiencies, and may also lead to lasting changes in commensal microorganisms. Enterococci are members of the gastrointestinal tract consortia of both animals and humans, known to acquire resistance to heavy metals and other feed grade

antimicrobials. The genus, *Enterococcus*, has gained attention because of the emergence of multidrug resistant strains, thus becoming important pathogens and ranking as the second most common cause of nosocomial infections worldwide.

High concentrations of copper, fed as copper sulfate, are often used to increase growth rates in swine. Bacteria exposed to copper may acquire resistance, and in *Enterococcus faecium* and *E. faecalis*, a plasmid-borne transferable copper resistance (*tcrB*) gene that confers copper resistance has been reported. In Europe, the plasmid also carries macrolide [*erm*(B)] and glycopeptide (*vanA*) antibiotic resistance genes, suggesting a potential linkage of copper resistance to antibiotic resistance. Also, the transferability of *tcrB* from *tcrB*-positive to *tcrB*-negative enterococci strains was determined. We have also showed that the *tcrB* gene is carried on a transferable plasmid and provide evidence for interspecies co-transfer of *tcrB* and *erm*(B) genes in enterococci. In our study, all *tcrB*-positive isolates contained the *erm*(B) gene and were phenotypically resistant to erythromycin. Enterococcal isolates in our studies were also positive for *tet*(M) and phenotypically resistant to tetracyclines.

The potential link between *tcrB* and antimicrobial resistance genes and the propensity of enterococci to transfer *tcrB* to other strains raises the possibility that copper supplementation may exert selection pressure for antimicrobial resistant enterococci. Because tetracycline resistance is provided in so many different ways, coded by many different genes located in a variety of locations within the bacterial genome and on plasmids, there are varied fitness costs associated with their presence. These fitness costs help determine the baseline prevalence of strains of bacteria harboring these resistance factors when comparing these to strains competing for space and resources within ecological niches in the gut. Therefore, a longitudinal study was conducted in piglets to investigate the effects of in-feed copper, chlortetracycline, and tylosin,

alone or in combination, on selection and co-selection of resistance strains of fecal enterococci (**Chapter 3**). The overall prevalence of *tcrB*-positive enterococci was 14.3% (372/2,592). Among the 372 *tcrB*-positive isolates, 331 were *E. faecium* and 41 were *E. faecalis*. All *tcrB*-positive isolates contained both *erm*(B) and *tet*(M) genes; in pigs, so did all of the other enterococci we examined. The median MIC of copper for *tcrB*-negative and *tcrB*-positive enterococci was 6 and 18 mM, respectively. The majorities of isolates (95/100) were resistant to multiple antimicrobials, and were classified as multidrug resistant (MDR). The supplementation of copper, in combination with chlortetracycline or tylosin, appeared to have an additive effect on the prevalence of *tcrB* gene among fecal enterococci of piglets. Understanding the impact of feed grade antimicrobials, including heavy metals, on the evolutionary and ecological versatility and flexibility of the gut microbial consortia is important to mitigate the problem of antibiotic resistance. There is limited information on the impact of antibiotic alternatives on antimicrobial resistance in food animals.

Since the prevalence of the *tcrB* gene had not yet been reported in cattle, we conducted a study in feedlot cattle to investigate the effects of feeding copper on the prevalence of copper resistant enterococci in cattle feces. The present study was conducted to determine the occurrence of *tcrB* gene in fecal enterococcal isolates of cattle supplemented with elevated (100 mg/kg) levels of copper in the diet and to determine phenotypic susceptibilities to copper, tetracycline, tylosin, and vancomycin (**Chapter 4**). A total of 22 isolates, each identified as *E. faecium*, were positive for *tcrB*, with an overall prevalence of 3.8% (22/576). The prevalence was higher among the cattle fed diets supplemented with copper (6.9%; 95% CI: 0.050-0.102) compared to normal copper or control diets (0.7%; 95% CI: -0.00-0.02). The *tcrB*-positive isolates always contained both *erm*(B) and *tet*(M) genes; however, among *tcrB*-negative isolates

the phenotype varied. Median copper MICs for *tcrB*-positive and *tcrB*-negative enterococci were 22 mM and 4 mM, respectively. The transferability of the *tcrB* gene from *tcrB*-positive strains to *tcrB*-negative strains was demonstrated via a filter mating assay. Multi-locus variable number tandem repeat analysis revealed a genetically diverse and heterogeneous population of enterococci. The finding of a strong association between the copper resistance gene and other antibiotic (tetracycline and tylosin) resistance determinants may be significant because enterococci remain potential pathogens and have the propensity to transfer resistance genes to other bacteria in the gut. In summary, (**Chapter 4**) the feeding of elevated dietary copper levels resulted in an increased prevalence of *tcrB*-mediated copper-resistant fecal enterococci in feedlot cattle. The *tcrB* gene was on a mobilizable plasmid, which is strongly suggestive of potential transferability of the gene under selection pressure. The finding of the strong association between copper resistance and other antibiotic (tetracycline and tylosin) resistance determinants may be significant because of the propensity of enterococci to transfer resistance genes to other bacteria in the gut when placed under selection pressure. Further studies are needed to investigate the effects of feeding antibiotics and their association with copper resistance gene in enterococci to better understand the potential co-selection and molecular epidemiology of multidrug resistant enterococci in cattle.

The high propensity of enterococci to acquire, express and transfer antimicrobial resistance determinants (horizontal gene transfer) enhances their ability to sustain antibiotic selection pressure, and therefore, they have become important opportunistic and nosocomial pathogens in humans. Because antibiotic resistance genes are co-located with *tcrB* on the same plasmid, it is possible that feeding elevated level copper in the diet may co-select for enterococci resistant to antibiotics in the absence of antibiotic selection pressure. The presence of both antibiotic and

heavy metal resistance genes may help in the plasmid maintenance in environments with no selection pressure of antibiotics. Because of the potential link between *tcrB* with *erm*(B), we hypothesize that copper supplementation may exert selection pressure for enterococci to become resistant to macrolides (**Chapter 5**). The prevalence of *tcrB*-positive enterococci was also higher among cattle fed tylosin (tylosin and copper + tylosin groups; 24/400; 6.0%) compared to groups that did not receive tylosin (control and copper groups; 13/400; 3.2%). The prevalence of *tcrB*-positive enterococci was higher in cattle fed diets supplemented with both copper and tylosin (17/200; 8.5%) than either of the dietary supplements alone (copper, 9/200; 4.5% and tylosin, 7/200; 3.5%). This suggests that copper and tylosin in combination exert an additive selection pressure for *tcrB*-positive enterococci.

Similar to *tcrB*, the prevalence of *erm*(B) positive enterococci was higher among cattle fed a higher concentration of copper (copper and copper + tylosin groups; 208/400; 52%) compared to groups that did not receive higher concentration of copper (control and tylosin group; 167/400; 41.7%). The prevalence of *erm*(B) positive enterococci was also higher among cattle fed tylosin (tylosin and copper + tylosin groups; 201/400; 50.2%) compared to groups that did not receive tylosin (control and copper groups; 174/400; 43.5%). This suggests that the feeding of elevated dietary copper and tylosin alone or in combination resulted in an increased prevalence of macrolide resistance [*erm*(B)] among fecal enterococci in feedlot cattle. In **Chapter 5**, we explored the possibility that feeding of elevated dietary copper and tylosin alone, or in combination, resulted in an increased prevalence of *tcrB* and *erm*(B) mediated copper and tylosin resistant fecal enterococci in feedlot cattle. This is suggestive of potential co-selection of both copper and macrolide resistance determinants due to selection pressures from copper and or tylosin supplementation. Future studies are warranted to investigate the factors involved in

maintenance and dissemination of these resistance determinants and their co-selection mechanism in relation to feed grade antimicrobials usage in feedlot cattle. Therefore, monitoring antimicrobial resistance in enterococci of food animal origin is important for preserving the therapeutic value and efficacy of the antimicrobials.

Vancomycin-resistant enterococci (VRE) have emerged as important nosocomial human pathogens in the United States. Horizontal transmission of vancomycin resistance among enterococci is largely attributed to the genetic determinant *vanA*, which typically resides on the mobile genetic element Tn1546 in conjugative plasmids. We earlier reported for the first time non-clinical human VRE isolated from community wastewater in a semi-closed agri-food system in Texas. The purpose of the present study was to characterize the virulence genes in enterococci that are often associated with *vanA* type resistance, perform multilocus sequence typing for across host comparisons, and to determine the transferability of the *vanA* gene to host-specific or non-host-specific susceptible enterococcal isolates via conjugation and comparison these traits with vancomycin-susceptible *E. faecium* (VSE) isolates. In the US, VRE are rare in animals and unlikely as a source of community infections in humans. The reason is that avoparcin, a glycopeptide growth promoter closely related to vancomycin, was never approved for use in animals in the U.S. Only recently, for the first time, has the occurrence of VRE in swine been reported (Donabedian et al., 2010). We hypothesize that there are differences in molecular characteristics of VRE to explain this geographical disparity and that understanding these differences is essential for maintaining low levels of VRE in community and agricultural settings in the US (**Chapter 6**). In the present study, we compared VRE isolates of swine and their affiliated occupational cohorts of human. In this study population, there was a limited movement of both swine and humans, integrated system, and that all pork products were

processed at the single slaughter plant and the products were being fed to human within the system. Our study is the second report on the occurrence of VREs from swine populations that had not previously been exposed to glycopeptides in the United States. All the swine VRE isolates belonged to a specific clonal complex, CC17, which has been responsible for global wide dissemination among human hospitals, including in the United States. All the VRE isolates of human waste water and swine feces from our study were resistant to tetracycline [*tet*(M)] and macrolide [*erm*(B)] genes. Diversity among the multidrug resistant enterococci suggests that no single genotype is favored in a particular study environment. Multiple drug resistant strains of *E. faecium* are more often associated with hospital acquired infections due to transmission of these strains to humans and their ability for horizontal gene transfer. Future studies are warranted to study the global epidemiology to understand the spread and evolution of clones via mobile genetic elements encoding vancomycin resistance and to overcome the potential barriers between human and swine hosts.

The use of antibiotics has resulted in an increased efficiency of livestock production and provided a means of producing a low cost, high quality product to deliver to consumers. Antibiotic use in swine and cattle production has benefits in all stages of production, the most important effect being improvements in growth performance. While a mode of action has yet to be elucidated, it is likely that many factors contribute to the growth promotion effect of subtherapeutic antibiotics. Subtherapeutic use of antibiotics is under increased scrutiny due to the increasing prevalence of antibiotic-resistant bacteria. Research has definitively shown that bacteria have developed resistance to a wide variety of antibiotics and it is unlikely that resistance will decrease with the current production practices firmly in place. Concerns of antibiotic resistance in pathogenic and commensal enteric bacteria, as well as in the environment,

are well-documented. There is also evidence that resistance to some antibiotics will not decrease even with the removal of antibiotics from livestock feeds. Research on the prevalence or causes of resistant bacteria in livestock production are probably less important than finding ways to reduce or eliminate resistance, while still providing producers with a means to sustain high levels of production. Decisions on future uses of antibiotics in animal agriculture must be made with attention to sound, scientific research taking into consideration not only the risks of antibiotic-resistance, but the health benefits that antibiotics provide to the animal and the safety of products consumed.

The use of antimicrobial compounds in food animal production provides demonstrated benefits, including improved animal health, higher production, and reduction of foodborne pathogens. The effect of these antimicrobials on the susceptibility of both commensal and food-borne bacterial species is heavily debated and has potentially important ramifications for food safety. The issues surrounding antibiotic resistance in bacteria of food animal origin are complex and of high relevance to industries, consumers, and health care providers. Again, it is generally accepted that any antimicrobial use will provide a selective pressure for antimicrobial resistance. While the use of antibiotics in livestock is known to select for resistant bacteria, and impact of these organisms and the resistance genes they carry are not clearly understood. In contrast to Europe, regulatory agencies in the United States and other countries have not imposed total bans on the nontherapeutic growth promoting use of antibiotics (though in the United States, a voluntary withdrawal period of 3 years was set in place in December of 2013). In United States, newer restrictions have focused on the withdrawal of antibiotics deemed critical for use in humans.

It is likely that both risks and benefits are realized in the use of antibiotics for food animal production. Given that there is an increasing prevalence of antimicrobial resistance and that this resistance has clinical implications, there is a critical need for effective mitigation efforts. Such actions will require collaborative efforts by several partners, including the farming, veterinary, medical, and public health communities. There is a need for enhanced surveillance for evaluating and directing the use of antimicrobials in animals. Because in-feed antimicrobials provide a continuous selective pressure, it is not unreasonable to believe they have an effect on the susceptibility of bacteria with which they associate. Finding replacements for antimicrobial growth promoters and management changes will play a key role in maintaining animal productivity in their absence. It is unlikely that any single replacement will be found that will prove to be economically viable. Also, we need to develop a better understanding of how bacteria acquire resistance, the importance of mutated strains, plasmids, integrons, and clonality. Trace amounts of antibiotics and or heavy metals could act as selection pressures for maintenance and co-transfer of antibiotic resistance genes. Acquisition of antibiotic resistances through mechanisms of selective mutations and lateral gene transfer may be acting in concert with other natural mechanisms of genetic adaptations among diverse range of bacteria. In the end, the sheer complexity of the problem makes progress seem daunting, which is why it is important to always return to the mantra of resistance: “in all cases, less use is probably better.”

Appendix A - Cumulative susceptibilities of *tcrB*-negative and *tcrB*-positive enterococcal isolates to copper

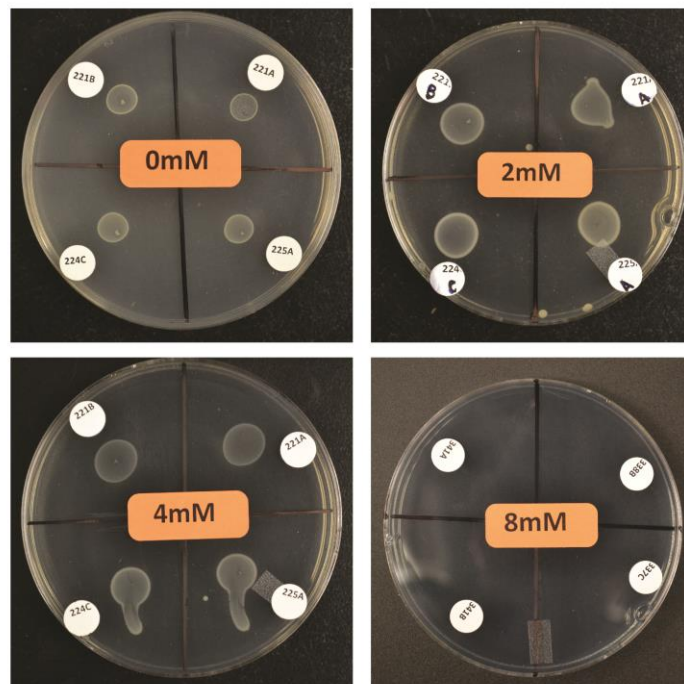


Figure A.1: Cumulative susceptibilities of *tcrB*-negative enterococcal isolates to copper

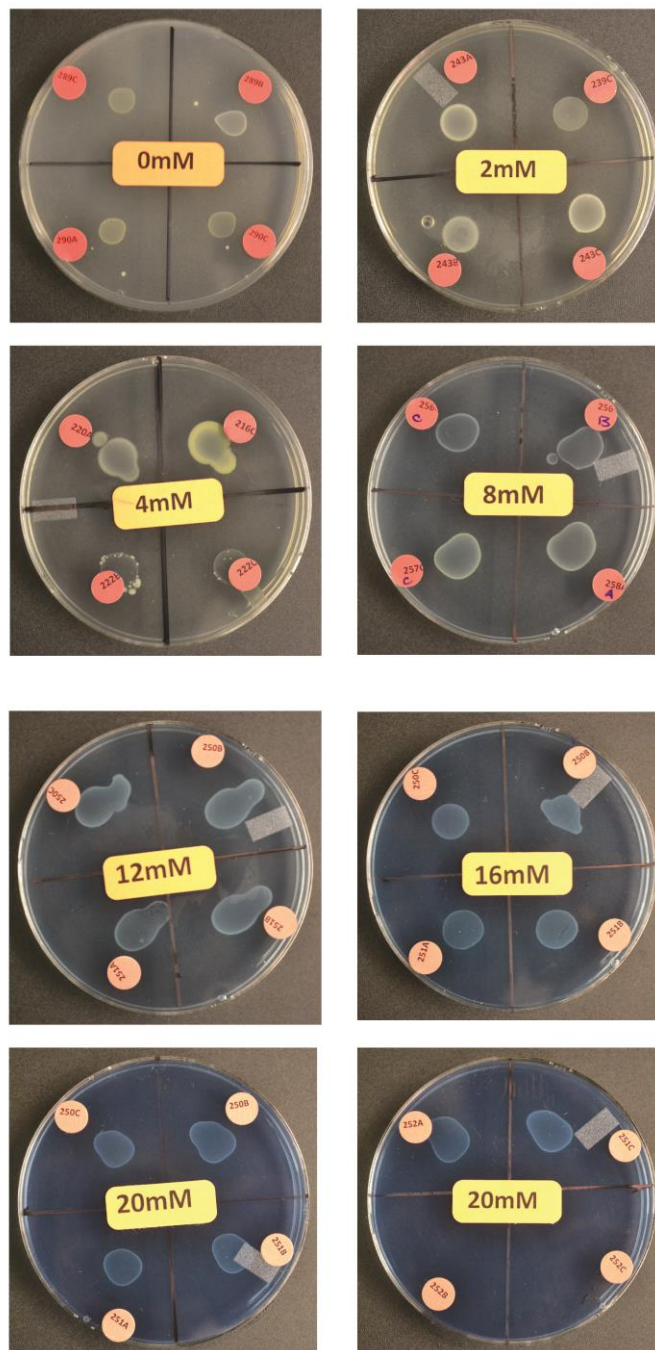


Figure A.2: Cumulative susceptibilities of *tcrB*-positive enterococcal isolates to copper

Appendix B - Schematic diagram showing intra and inter-species conjugation or transferability studies

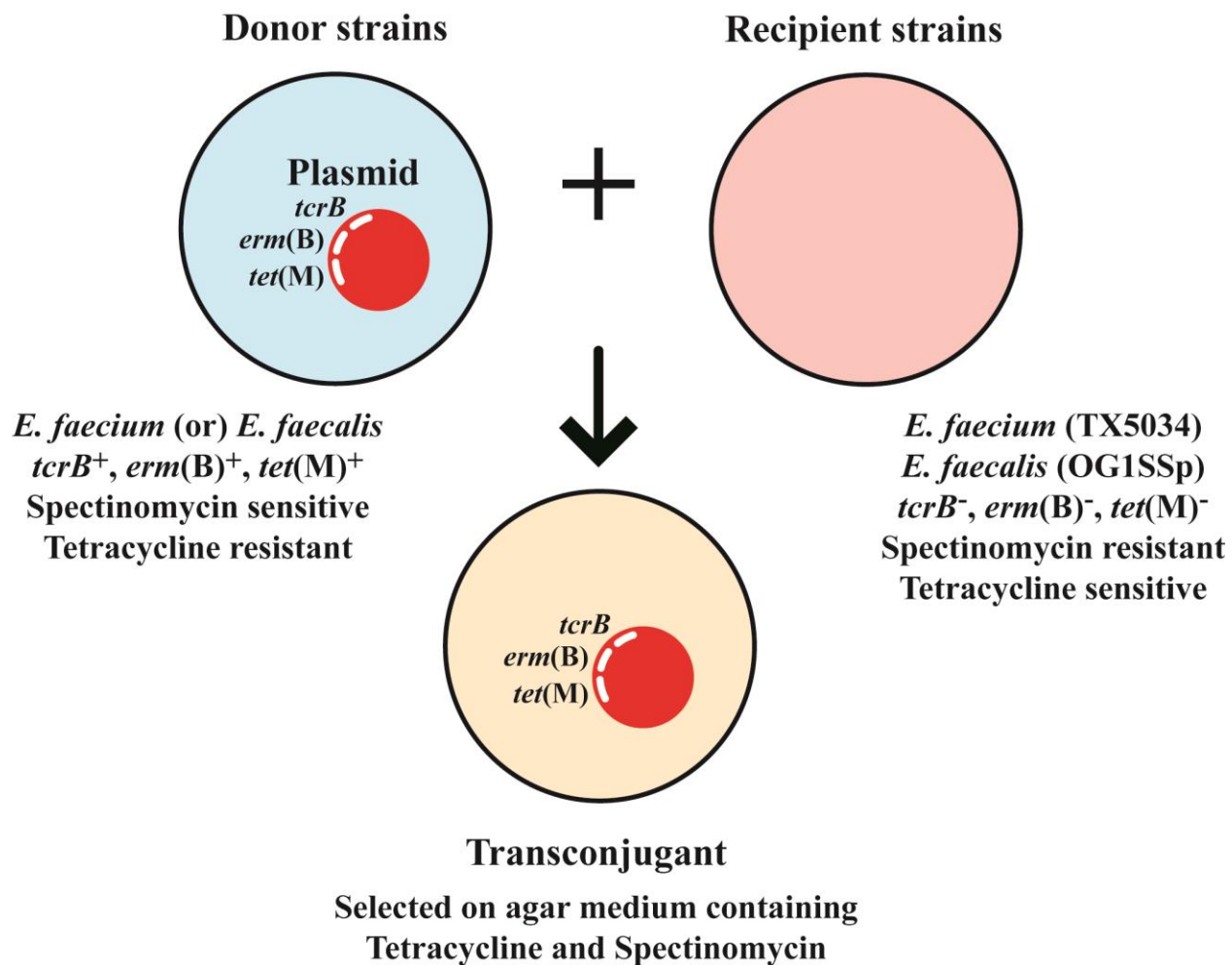


Figure B.1: Intra and inter-species transferability of *tcrB* gene

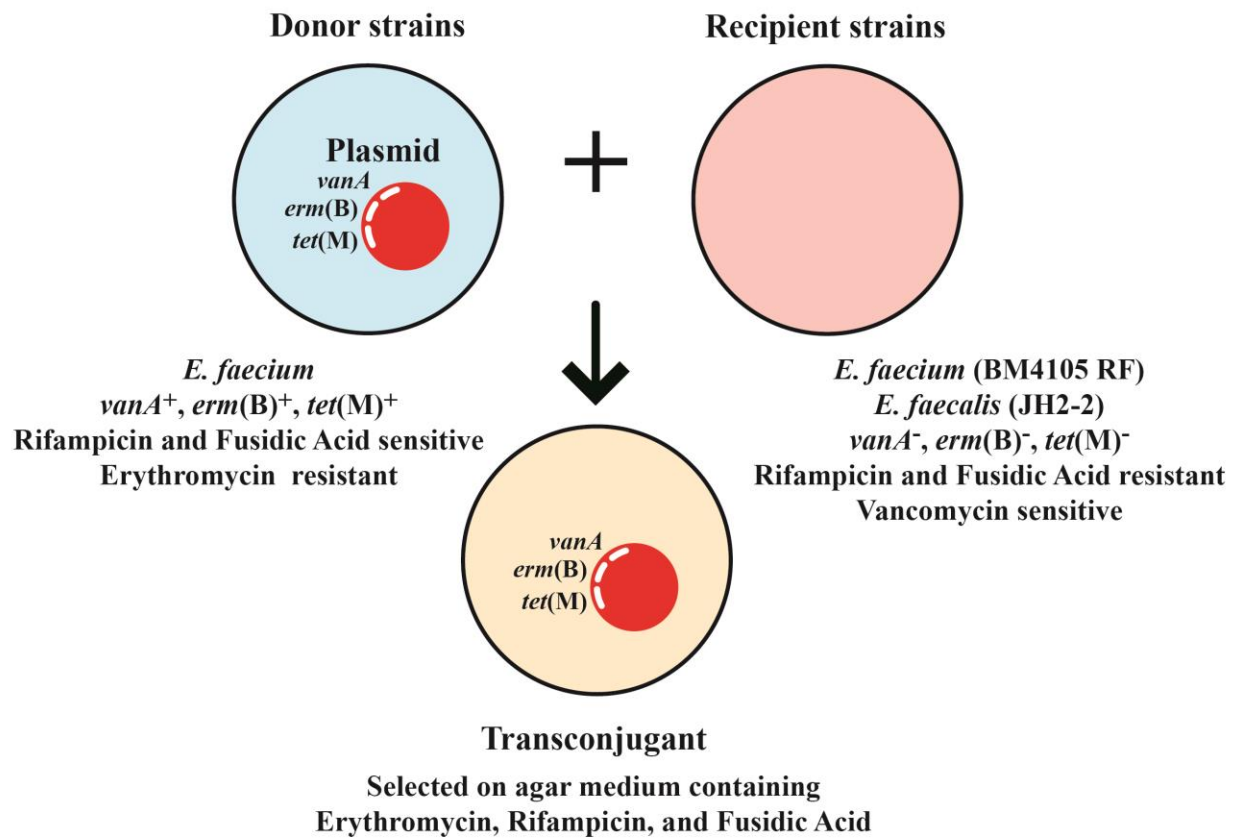


Figure B.2: Intra and inter-species transferability of *vanA* gene

Appendix C - Pictures from the swine trial















Appendix D - Pictures from the cattle trial







Appendix E - Miscellaneous pictures





