EFFECTS OF FEEDING ELEVATED CONCENTRATION OF COPPER ON PREVALENCE AND SELECTION OF FECAL ENTEROCOCCI POSITIVE FOR TRANSFERABLE COPPER RESISTANCE GENE IN PIGLETS

By

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Abstract

Copper, as copper sulfate, is often supplemented at elevated concentrations in swine diets, particularly in piglets, to promote growth. Growth promotional effects of copper are believed to be similar to that of antibiotics in that gut microbial flora is altered to reduce loss of nutrients and suppress pathogens. Bacteria exposed to copper may acquire resistance, and in *Enterococcus faecium* and *E. faecalis*, resistance is conferred by a plasmid-borne transferable copper resistance (*tcrB*) gene. The plasmid also carries macrolide [*erm*(B)] and glycopeptide (*vanA)* antibiotics resistance genes. The objectives of the research were to 1) determine the prevalence of *tcrB* gene in fecal enterococci of piglets in relation to normal (16.5 ppm) and elevated level (125 ppm) of copper supplementation, 2) determine the relationship of *tcrB* gene and susceptibilities to copper, erythromycin, and vancomycin, and 3) determine the transferability of *tcrB* gene in enterococci by conjugation. Weaned piglets, housed in pens, fed normal (16.5 ppm; control) or elevated level of copper (125 ppm) were used. Fecal samples were collected weekly for isolation of enterococci. Isolates were speciated by multiplex PCR and *sodA* gene sequence analysis. The prevalence of *tcrB*-positive enterococcal isolates was higher ($P < 0.05$) in the copper supplemented group than the control group. The prevalence of *tcrB* was affected by sampling days ($P < 0.05$) with a significant treatment and sampling time interaction ($P < 0.05$). The *tcrB* positive isolates were either *E. faecium* or *E. faecalis*, and majority of isolates was *E. faecium.* The mean MIC of copper for *tcrB*-positive isolates (21.1 mM) was higher ($P < 0.001$) compared to *tcrB*-negative isolates (6.1 mM). All isolates were resistant to erythromycin, tetracyclines and susceptible to vancomycin. The transferability of the *tcrB* gene from *tcrB*-positive strains to *tcrB*-negative strains was demonstrated by conjugation. The potential link between *tcrB* and

antibiotic resistance genes and the propensity of enterococci to transfer *tcrB* to other strains suggests the possibility that copper supplementation may exert selection pressure for antibiotic resistance. The positive association between copper supplementation and prevalence of *tcrB* gene has important implications for antimicrobial resistance and food safety, which warrants further investigation.

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CHAPTER 1 - Literature Review

Effect of supplementing elevated concentrations of copper in diets of piglets on the development of bacterial resistance to copper

INTRODUCTION

Use of Antimicrobial Agents in Food Animal Production

Antibiotic growth promotion in agricultural animal production has been practiced for about 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). The food animal production involves intensive optimization in every step of production process to maximize the efficiency of production (Aarestrup and Jenser, 2007). The early indications of its beneficial effect 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). Several classes of antimicrobials are used in food animal production for growth promotion both in United States and European Union (Table 1.1).

Table 1.1: Antimicrobials used for growth promotion in Europe and United States

Antimicrobial group	Antimicrobial growth promoter	United States	Europe	Related to antibiotic used in human treatment
Polypeptides	Bacitracin	In use (swine, poultry)	Banned (1999)	Bacitracin
Flavophospholipid	Flavomycin / Bambermycin	In use (broilers)	Banned (2006)	None
Glycopeptides	Avoparcin	Not used	Banned (2006)	Vancomycin, Teicoplanin
Ionophores	Monensin	Not used	Banned (2006)	None
	Salinomycin	Not used	Banned (2006)	None
Macrolides	Tylosin	In use (swine)	Banned (1999)	Erythromycin
	Spiramycin	Not used	Banned (1999)	Erythromycin
Oligosaccharides	Avilamycin	Not used	Banned	Evernimicin
Quinoxalines	Carbadox	In use (swine)	Until 1999	None
	Olaquindox	Not used	Until 1999	None
Streptogramins	Virginiamycin	In use (broilers)	Banned (1999)	Quinupristin/ Dalfopristin Pristinamycin
Sulfonamides	Sulfathiazole	In use (swine)	Not used	Sulfonamides
Tetracyclines	Tetracyclines	In use (swine)	Not used	Tetracyclines
Penicillin	Penicillin	In use (swine)	Not used	Penicillin
Pleuromuttilin	Tiamulin	In use (swine)	Prophylactic usage	None

Agriculture use of antibiotics accounts for at least half of antibiotics produced in the United States (Lipsitch et al., 2002). Antibiotics or antimicrobials are routinely used in livestock production to treat (therapeutic levels) and prevent (subtherapeutic levels) diseases. In addition, subtherapeutic concentrations of antimicrobials are commonly used as growth promoters in feed and drinking water (Chee-Sanford et al., 2009). Antimicrobial products work through a variety of bactericidal and bacteriostatic mechanisms that include inhibition of cell wall synthesis, inhibition of cell membrane synthesis, protein synthesis, folate synthesis, and DNA synthesis (Barton, 2000). Some of the antibiotics are primarily effective against specific groups of bacteria, such as gram-positive, gram-negative, aerobic, and anaerobic organisms.

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 as animals with clinical symptoms.
3. Prophylactics: Treatment of healthy animals to prevent disease in a period where they are stressed. This use of antimicrobial agents may indicate management problems, and is in most countries not legal or considered imprudent.
4. Growth promotion: Continuous inclusion of antimicrobial agents in the feed to improve growth.

There are hundreds of different antimicrobial agents, most of which belong to a few major family groups. Most of the members within a family arise from additions or substitutions of attachment to the drug's core structure.

The major family groups are:

1. Aminoglycosides
2. Beta-lactam subdivided into: penicillins, oxacillins, ampicillins, carbenicillins, ureidopenicillins, and cephalosporins of four generations, penems, monobactams, and betalactamase inhibitors.
3. Glycopeptides
4. Heavy metals (copper, zinc, mercury and arsenic)
5. Macrolides and Lincosamides
6. Nitrofurans
7. Nitroimidazoles
8. Orthosomycines (avilamycin)
9. Phenicols
10. Polyether antimicrobials (ionophores such as monensin, narasin, lasalocid and salinomycin)
11. Polypeptides (bacitracin, polymyxin)
12. Quinolones and Fluoroquinolones
13. Rifamycin
14. Steroid antimicrobials (fusidic acid)
15. Streptogramins (virginiamycin, quniopristin+dalfopristin)
16. Sulfonamides
17. Tetracyclines
18. Trimethoprim

The use of antimicrobials as growth promoters have been a regular practice in swine production since the early 1950's (Cromwell, 2001). Antimicrobial agents are necessary in swine production to treat diseases, increase weight gain, improve carcass quality, and for financial security (MacKinnon, 1993). The use of antimicrobial agents as growth promoters can be 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).

Definition of Antibiotics, Antimicrobials, and Antimicrobial resistance

Antibiotics: Refer to substances produced by microorganisms that have antibacterial effects (Prescott, 2000; Gustafson and Bowen, 1997; Gould, 2002).

Antimicrobials: Antimicrobials are substances of natural, synthetic or semisynthetic origin that kill or inhibit the growth of microorganisms by targeting cellular processes, and cause little or no damage to the host (Prescott, 2000; Walsh, 2003; Giguere, 2006; Guardabassi and Courvalin, 2006).

Antimicrobial resistance: Resistance is a relative term. It is defined in two ways, microbiological and clinical perspectives. According to the microbiological (in vitro resistance) definition, a bacterial strain is defined resistant if it grows in the presence of higher concentrations of the drugs compared with other phylogenetically related strains. But, clinically the strain is defined resistant when it survives antimicrobial therapy (Guardabassi and Courvalin, 2006).

Development of Antibiotic Resistance

A number of bacterial and fungal species possess the ability to produce antimicrobial compounds, generally to gain a competitive advantage in microorganism-rich environments (Amabile-Cuevas and Chicurel, 1992). It is thus likely that naturally occurring antibiotics have been in the environment even before the higher organisms evolved. Many of the antimicrobial products used in human and animal health are produced by organisms such as *Streptomyces*, *Bacillus*, *Penicillium*, *Cephalosporium*, and *Pleurotus* (Mathew et al., 2007).

Antibiotic resistance likely emerged also in nature prior to human use of drugs, as organisms producing antibiotic compounds required means to survive in the presence of their own products, and competing species also found ways to counteract effects of those antimicrobial compounds (Davies, 1997). Therefore, some of the resistance genes likely originated before the advent of modern medicine, and agricultural use of antibiotics. With the increase in use of antibiotics both in human medicine and veterinary medicine, selection pressure increased the advantage of maintaining resistance genes in diverse groups of bacteria, and bacterial evolutionary progress leading to mechanisms to retain, accumulate, and disperse resistance genes among bacterial populations (Aarestrup, 2006). However, use of antibiotics both in human medicine and food animal production may promote dissemination of resistance genes beyond that expected as a direct consequence of selective pressure on target organisms. The soil microbes provide a large reservoir of antibiotic resistance genes that can, under selection pressure of antibiotics, may disseminate into other microbial communities Numerous studies have demonstrated a link between antibiotic use in livestock and increased prevalence of antibiotic resistant organisms associated with farm animals and farm environment (McEwen and Fedorka-Cray, 2002; Witte, 2000). Of all the application of antibiotics, the use for growth promotion in food animal

agriculture has been the most controversial issue (Viola and DeVincent, 2006). It is well established that agricultural use of antibiotics results in increased prevalence of antibiotic-resistant bacteria in farm environments, thus contributing to the global pool of resistant organisms (Mathew et al., 2007). Foodborne pathogenic bacteria carrying resistance genes are the most likely route through which agricultural use of antibiotics could affect human health. However, evidence for direct animal to human transmission of resistant bacteria has also been reported (Box et al., 2005; Hunter et al., 1994).

Mechanisms for Bacterial Antimicrobial Resistance

Bacterial antimicrobial resistance can be divided into two broad categories, intrinsic and acquired. Intrinsic resistance reflects a bacterial genus or species lacking an appropriate target or the permeability needed for inhibition by an antimicrobial, whereas acquired resistance is observed once a particular strain has undergone chromosomal mutations or acquired genes encoding resistance (Schwarz and Chaslus-Dancla, 2001; Schwarz et al., 2006). Specifically, there are at least three general mechanisms by which bacteria resist antimicrobial activity:

Reduced intracellular accumulation is mediated by decreased intake or increased export of the compound (Schwarz and Chaslus-Dancla, 2001). Bacterial efflux mechanisms can be encoded by either chromosomal or plasmid genes, and belong to one of five classes of efflux pumps (Poole, 2005; Depardieu, et al., 2007). Plasmids, which are transferable between bacteria, often contain genes for specific efflux-mediated antimicrobial resistance, while multi-antimicrobial exporters normally are contained within the host genome. The effects of chromosomal efflux pumps typically occur after increased expression of the pumps (Walsh, 2003; Depardieu, et al., 2007).

a) Enzymatic inactivation can be coded for by genes in the host chromosome or in plasmids, gene cassettes, or transposons (Walsh, 2003; Schwarz et al., 2006). Antimicrobial inactivating enzymes have been found in both gram positive and gram negative bacterial species, including *Staphylococcus aureus* and *Escherichia coli* (Walsh, 2003; Schwarz et al., 2006). There are several broad mechanisms for enzymatic inactivation, including degradation and chemical modification of the antimicrobial (Schwarz, et al, 2006).

b) Target modification, which can occur chemically or through mutation or protection of the target site (Schwarz et al., 2006). An example of a target site modification is seen in macrolide, lincosamide, and streptogramin B co-resistance encoded for by *erm* genes from multiple bacterial genera (Schwarz et al., 2006). The *erm* genes encode rRNA methylases (mono- or di-), specific for a single adenine residue (position 2,058) conserved within 23S rRNA (Leclercq and Courvalin, 1991; Weisblum, 1995; Schwarz et al., 2006). In contrast to target modification, tetracycline resistance frequently is a result of ribosomal protection. Tetracycline protection involves proteins with homology to elongation factors, which are produced and interact with the ribosome, preventing tetracycline binding (Schwarz, et al., 2006). Both the *erm* genes and tetracycline protection proteins are encoded on genes that are present on transferable elements (Leclercq and Courvalin, 1991; Schwarz et al., 2006).

Transfer of Antimicrobial Resistance

Mobile resistance determinants likely originated in antibiotic-producing organisms before therapeutic or non-therapeutic use of antimicrobials (Boerlin and White, 2006). Mobile genetic elements can be passed through bacteria by vertical or horizontal transmission (Schwarz and Chaslus-Dancla, 2001). Populations of antimicrobial resistant bacteria are expanded by the acquisition of these resistance genes through horizontal transmission (Teuber et al., 1999). One specific type of mobile element, resistance-encoding plasmids, can be passed between bacterial types as seen by the presence of gram-positive resistance determinants expressed in gram-negative organisms (Courvalin, 1994). Doucet-Populaire and others (1992) have shown that plasmid DNA containing known kanamycin resistance could be transferred from *Enterococcus faecalis* to *Escherichia coli* isolates in the gastrointestinal tract of germ-free mice. Plasmids containing the *erm*(B) and *tet*(M) genes have the ability to transfer between *Lactobacillus plantarum* and *Enterococcus faecalis* within the gastrointestinal tracts of germ-free rats (Jacobsen et al., 2007). Movement of plasmids or other mobile elements is not limited to commensal bacterial species. Resistance genes located on mobile elements also can disseminate between commensal and pathogenic organisms (Boerlin and White, 2006). A precise determination of the rate at which horizontal transfer occurs within the complex gastrointestinal system of piglets has not been examined. Most recent studies have examined the involvement of lactic acid bacteria (LAB) in the spread of resistance genes in the environment and their potential role as reservoirs for resistance genes (Ammor et al., 2007). These resistance genes are more often carried on a plasmids and conjugative transposons (Toomey et al., 2009).

Public Health Consequences of Use of Antimicrobial Agents in Food Animals

Antimicrobials have had a more positive impact on human and animal health than any other medical discovery. However, antimicrobial agents are losing their effectiveness because of development, spread, and persistence of antimicrobial resistance. The use of antimicrobial agents in food animal production has caused major concern regarding the impact these uses have on human health. A major concern is the transfer of resistance genes via food chain from food producing animals to humans. The use of antimicrobials in animals (or humans) leads to an increased resistance in both pathogenic as well as the endogenous, normal flora. The resistant bacteria can get colonized in the host and potentially transfer the resistant genes to other bacteria within the gut microflora (van den Bogaard, 1993). There has been a controversy concerning the use of antibiotics as growth promoters for food producing animals. Use of any antibiotic is associated with the selection of resistance in pathogenic bacteria and it has been argued that the use of antibiotic growth promoters imposes selection pressure for bacteria that are resistant to antibiotics. The widespread use of antimicrobial agents in food animals is associated with increased antimicrobial resistance in foodborne pathogens, which subsequently may be transferred to humans. There is considerable controversy over the use of antibiotics because of the potential threat it poses to human health (Feber, 2003; Livermore, 2003; Arnold et al., 2004). It is accepted that subtherapeutic use of antimicrobials leads to increased antibiotic resistant fecal bacteria in the animals (Aarestrup et al., 2001; Hayes et al., 2004).

The transfer of these resistant bacteria or genetic determinants for resistance causes adverse health consequences in humans by increasing the number of food borne illnesses and increasing the potential for treatment failures (Anderson et al., 2003). The public health risks associated with the use of antimicrobial agents as growth promoters in animal husbandry has lead to

increased attention and documentation in some countries, including the European Union, and resulted in banning or in the process of phasing out the use (Anderson et al., 2003). The World Health Organization (WHO), following a series of consultations in 1997, 1999, and 2000, has recommended that, unless a risk based evaluation demonstrates their safety, the use of antimicrobial agents in food animals for growth promotion that belong to the classes of antimicrobial agents used in humans should be terminated (WHO, 1997; WHO, 1999; WHO, 2000; Anderson et al., 2003). Similar recommendations are made to discontinue the use of human antimicrobial agents in food producing animals as growth promoters, which have been implemented by several independent organizations in United States, including the Alliance of Prudent Use of Antibiotics (2002) and the Institute of Medicine of the National Academics (2003). In the United States, collaborative federal actions are undertaken to address antimicrobial resistance by Public Health Action Plan to Combat Antimicrobial Resistance, which was released in 2001, by interagency task force. To address this public health problem, overuse and misuse of antimicrobial agents in food animals and humans must be reduced. Several European countries have already demonstrated the feasibility of such measures and the effectiveness of these interventions to combat the antimicrobial resistance and reduce public health risk (DANMAP, 2005).

Antibiotic Resistance in Foodborne Pathogens

It is reported that 76 million people contract foodborne illnesses annually in United States (Mead et al., 1999) at an annual cost of nearly $ 6.9 billion (Allos et al., 2004). In most of the severe cases, treatment of foodborne illness may require the use of antibiotics, making antibiotic resistance in foodborne pathogens a considerable concern. The foodborne pathogens including *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., *Yersinia* spp., and *Enterococcus* spp., may be harbored by livestock and passed to humans via food chains. However, some of the other bacteria, such as *Enterococcus faecalis* and *E. faecium*, which are normal commensals of gastrointestinal tract, can pose zoonotic risks via environmental routes, and are responsible for nosocomial infections. Resistant strains of *Enterococcus* have become a major concern for the medical community as the number of infections has increased from past two decades (Huycke et al., 1998; Treitman et al., 2005).

Antibiotic Resistance Associated With Swine Production

According to NAHMS (USDA-NAHMS, 2005), 92% of the 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, and 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 based on the type of antibiotics.

Any usage of antimicrobials, even in the 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 promoter increased the need for other feed additives or alternatives to control disease outbreak, and to improve the growth rate in food producing animals. Generally, 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 more 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 changes at weaning. The dietary changes associated with the shift from sow milk to a diet composed of cereals supplemented with proteins sources, such as soyabean meal, skim-milk powder, and or fish meal. These diets are normally supplemented with minerals and vitamins according to current recommendations (NRC, 1998).

Alternatives to Antibiotic Use for Growth Promotion in Animals

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

1. Probiotics and competitive exculsion: 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, where bacterial cultures are allowed to establish in intestinal flora that will prevent colonization by pathogenic bacteria (Abe et al., 1995). Probiotics microorganisms, added to the feed, may protect piglets 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
    v. Affect the permeability of gut and increase uptake of nutrients.
2. Enzymes: Pigs have a variety of gastrointestinal enzymes to aid in digestion. However, newly weaned piglets may produce inadequate amount of enzymes. Therefore, the addition of enzymes to feed may be useful strategy 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 the resistance to disease. These substances include antibodies, cytokines, spray dried plasma, and other compounds. The growth promoting effects of antibodies or other immune active compounds is 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 digestion of proteins and 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). Studies reported that organic acids also improve the digestibility and absorption of proteins, minerals, and other nutrients in the diet (Overland et al., 2000).
5. Other feed supplements:
    a) Minerals: Copper and Zinc are added to piglet diets do significantly improved average daily weight gain, feed intake, and feed efficiency.
    b) Vitamins
    c) Conjugated linoleic acid
    d) Phospholipids
    e) Amino acids
    f) Carnitine
    g) Carbohydrates (Polysaccharides, Fiber)
    h) Herbs

Use of Heavy Metals in Swine Production

Metals form integral constituents of the earth's crust. All living organisms have evolved to use some metals, even though they are toxic. 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 incompletely filled d orbital. These d orbitals provide these heavy metal cations with the ability to form complex compounds which may or may not be redox-active. Thus heavy metals cations play an important role as "trace elements" in biological reactions (Nies, 1999). A large number of metals are used as trace amounts in adequate quantities in food animal production to maintain normal physiology and healthy status of animals. Two of these trace elements, copper and zinc are supplemented in animal feeds 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 seem to have an additional effect when supplemented in high doses (Hasman et al., 2006). In the United States, in addition to copper and zinc, livestock producers also use nonessential metal arsenic as a feed supplement to chicken, turkeys, and pigs (Hasman et al., 2006). The following table (Table 1.2) gives the dietary recommendations of trace minerals in swine.

Table 1.2: Dietary recommendations of trace minerals in swine (dose depends on age and performance)

Trace mineral	mg/kg
Copper	3-6
Zinc	50-100
Iron	40-100
Manganese	2-10
Selenium	0.10-0.30
Iodine	0.14

NRC (1998)

Zinc

Zinc is a component of various metalloenzymes, ranging from DNA and RNA transferases and synthetases, many digestive enzymes, and also the hormone, insulin. Hence, zinc plays a vital role in protein, carbohydrate, and lipid metabolism (NRC, 1998). The zinc requirement varies and depends on type of diet, age, sex, and breeding season (NRC, 1998). Zinc can be supplemented as zinc sulfate, carbonate, chloride, and zinc metal dust, as these are known to possess 100% bioavailability (Miller et al., 1991; NRC, 1998). Zinc, when supplemented with 3, 000 ppm for 14 days known to reduce post weaning scouring and increased weight gain (Poulsen, 1989; Kavanagh, 1992; Hahn and Baker, 1993; Carlson et al., 1995; Hill et al., 1996). It is found to be efficacious when fed with 250 ppm of copper sulfate and 3,000 ppm of zinc oxide, but with respect to growth promotion action the effects were not additive (Hill et al., 1996). Zinc toxicity in pigs was manifested by depression, arthritis, and hemorrhage in auxillary spaces, gastritis, and death.

Copper

The pig requires copper for the synthesis of hemoglobin and also for the synthesis and activation of several oxidative enzymes necessary for metabolism (Miller et al., 1979; NRC, 1998). Copper is also necessary for the normal immune function of the swine. Copper level of 5-6 ppm in the diet is sufficient 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 ppm as copper sulfate is known to stimulate the growth in pigs (Barber et al., 1955; Braude, 1967). This type of growth response in piglets may be independent of the addition of in-feed antibiotics (Stahly et al., 1980; Roof and Mahan et al., 1982; Edmonds et al., 1985). The mechanism through which copper promotes growth in piglets remains elusive. Earlier studies have attributed the growth promoting action of copper to its antimicrobial properties (Fuller et al., 1960), but the evidence supporting this action is lacking. Some studies have observed a correlation between the availability of copper and growth promoting action of copper (Bowland et al., 1961; Cromwell et al., 1989).

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.

Use of Copper as a Growth Promoter

Copper, as copper sulfate, in diets of weaned piglets has been known to exert growth promoting effects (Braude, 1967). Numerous 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 by studying the effects on the fecal micro flora (Fuller et al., 1960; Jensen, 1998; Kellogg et al., 1964). However, no data have shown a definite link of the effects of copper on the micro flora to the growth promotion. Despite this, it is generally accepted that the action of copper is attributed to its antimicrobial activity, which is in agreement with Hϕjberg et al. (2005) who showed that elevated levels of copper inhibited the coliforms. Several studies have been conducted to elucidate the mechanism of action of copper as growth promoter. Among these studies Li et al. (2006) and Shurson et al. (1990) found 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 tend to increase villous height of the jejunum (Zhao et al., 2007). The latter study also showed increase in growth rate and feed intake with increased amount of copper in the diet. The growth stimulating effect of feeding pharmacological concentrations of copper to swine is well documented (NRC, 2005). However, the nutritional requirements for copper, based on its biochemical functions are low in non ruminants (Underwood and Suttle, 1999). Generally, the response of weanling pigs to growth promoter is more pronounced in a conventional on farm nursery than in an experimental nursery (Coffey and Cromwell, 1995; Zhao et al., 2007). This may be suggestive of differences in sanitation and subclinical pathogen exposures between locations (Le Floch et al., 2006). The sanitation might modulate the effect of high concentrations of copper as well as zinc, which have

been associated with bactericidal effects similar to those of antimicrobial compounds (Hϕjberg et al., 2005; Dunning et al., 1998; Fuller et al., 1960). But, recent studies have reported that copper supplementation may result in microbial resistance (Hasman and Aarestrup, 2002).

Resistance to Heavy Metals

Bacteria have great capacity to absorb metals from solutions, on a biomass to dry weight metal basis (µg/mg dry wt.). It is always necessary to make a distinction between two types of heavy metals such as, heavy metals that are toxic *per se* and those which 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 sulphide groups. Exceptions to that being, the divalent cations of cobalt, copper, nickel, and zinc are medium sulphur lovers and these metals functions at low concentrations, but are toxic at high concentrations. It is necessary to achieve careful homeostasis of these metals to avoid either metal deprivation or metal toxicity.

There are two types of uptake system for heavy metals: (Nies, 1999)

1) Non specific and fast, used for a variety of substrates and it is constitutively expressed and driven by chemiosmotic gradient across the cytoplasmic membrane of bacteria.
2) Specific and slower with higher substrate specificity and uses ATP hydrolysis as the energy source and they are inducible. These specific systems are only produced by the cells in times of need.

When the concentration of a heavy metal increases in a cell, it will be transported into the cytoplasm by unspecific uptake systems, which are constitutively expressed. This is called the open-gate, since the gate cannot be closed once it is opened. This open-gate is the first reason

why heavy metal ions are toxic (Nies and Silver, 1995). This potential of heavy metal toxicity in connection with the open gate phenomenon has forced life in its early evolution to develop metal homeostasis factors and metal resistance determinants (Nies and Silver, 1995). The heavy metals are not degraded or modified like toxic organic compounds, thus leading to the development of heavy metal resistance system.

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 it from damaging metal-sensitive cellular targets (Gilotra and Srivatsava, 1997).
4) Extracellular sequestrations (Diels et al., 1995; Nies, 2000).
5) Transformation and detoxification (Rouch et al., 1995; Ji and Silver, 1995).

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 very acidic pH or in a complexed form. Because of its two oxidation states, it participates in biological redox reactions, thereby taking up and releasing electrons. However, copper homeostasis has to be tightly regulated, because it is toxic to the cells (Solioz and Stoyanov, 2003). The mechanism of copper toxicity in still not fully understood. Copper toxicity could occur via Fenton-type reaction leading to the production of hydroxyl radicals ($OH^{\cdot}$).

$$Cu^{+} + H_2O_2 = Cu^{2+} + OH^{-} + OH^{\cdot}$$

These hydroxyl radicals ($OH^{\cdot}$) are highly reactive, which in turn damage the 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 various homeostatic mechanisms.

Copper homeostasis in gram-negative bacteria (*Escherichia coli*)

Copper is essential for cells to function. However, it is toxic at higher concentrations. Therefore, intracellular copper concentrations need to be regulated within very narrow limits (Pena et al., 1999). Copper homeostasis in *E. coli* involves genes such as *cutC*, *cutF*, and *ndh*, but their exact roles have not been determined (Gupta et al., 1995; Rapisarda et al., 1999). Recent studies have shown the involvement of a two components signal transduction system in

copper homeostasis (Grass and Rensing, 2001). One is the copper-sensing locus, designated as *cus* locus. The sensor regulator pair containing *cusRS* genes activates the adjacent *cusCFBA* operon. The *cusCBA* genes of the operon are homologous to a family of proton-cation antiporter complexes involved in export of metal ions, xenobiotics, and drugs. The CusF is a putative periplasmic copper-binding protein (Munson et al., 2000). The other system involves CueR, which is known to regulate two genes, *copA* and *cueO* (Outten et al., 2000). CopA is a Cu (I)-translocating P-type ATPase, while CueO is a putative multi copper oxidase (Outten et al., 2000; Rensing et al., 2000).

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 four genes operon, *copYZAB* operon (Fig. 1.1). The extracellular copper reductase (CorA) converts Cu (II) to Cu (I), thereby it facilitates the supply of Cu I to CopA protein. The CopA (727 amino acids) protein takes up copper when copper is limiting and is encoded by *copA* gene. CopB protein (745 amino acids), coded by *copB* gene, extrudes copper when it reaches the toxic level. 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).

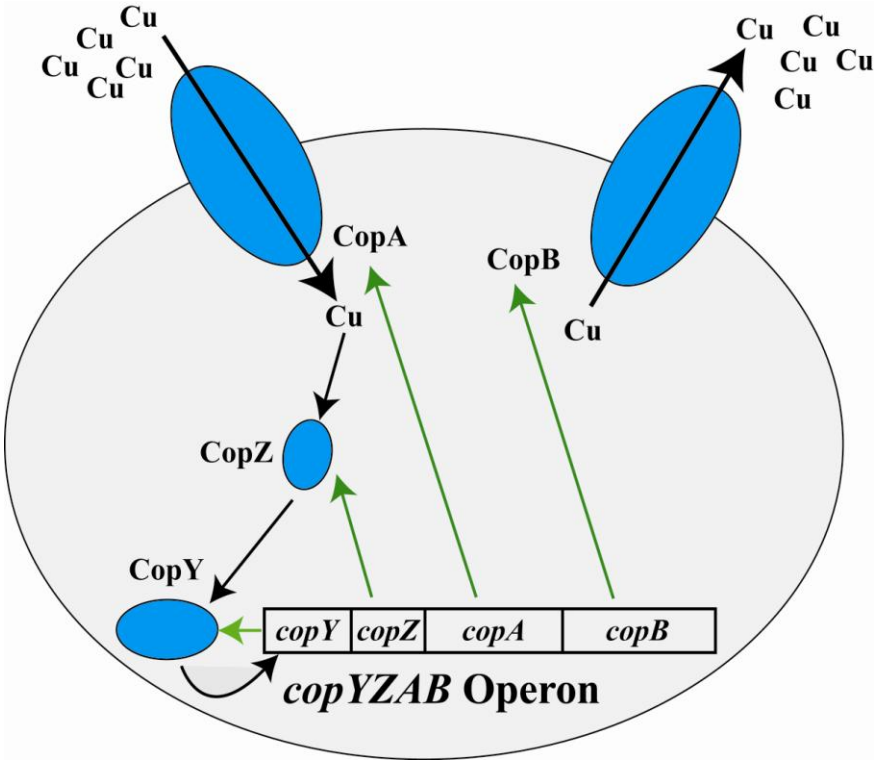

Figure 1.1: The *cop* operon and copper homeostasis in *Enterococcus hirae*

The heavy metal ATPases containing 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-negative bacteria

Escherichia coli can survive in copper rich environments, which would it normally overwhelm the chromosomally encoded copper homeostatic systems. But, some strains of *E. coli* harbor additional plasmid encoded genes that confer resistance to copper. Earlier studies in pigs which were supplemented with copper sulfate as a growth promoter showed the presence of conjugative plasmid pRJ1004, isolated from *E. coli* in the gut microflora, which confers copper resistance (Brown et al., 1995). Copper resistance in *E. coli* is mediated by plasmid borne *pco* gene cluster, which contains seven genes, *pcoABCDRSE* (Cooksey, 1994). Pco comprises of PcoA, which acts as a multi copper oxidase, PcoC and PcoE act as periplasmic chaperone, PcoB and PcoD, are the proteins of unknown function. Copper dependent expression is achieved by the two-component system PcoRS.

Copper resistance in *Pseudomonas syringae* is specified by the *cop* determinant, which has six genes, *copABCDRS*, which are arranged in a single operon and homologous to the pco gene cluster of *E. coli* (Brown et al., 1995; Cooksey, 1994; Silver and Phung, 1996). In both cases copper resistance is inducible (Mellano and Cooksey, 1988; Rouch and Brown, 1997; Rouch et al., 1995).

Copper resistance in gram-positive bacteria

Copper resistance in gram-positive bacteria, *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.

P-type ATPases consist of single, large catalytic monomer of 70-200 kDa. Heavy metal ATPases contain elements common to all P-type ATPases as well as several unique features (Lutsenko and Kaplan, 1995). 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 motif (CPx motif), hence the name CPx-type ATPases, contain conserved histidine-proline dipeptide (HP locus), which has 33 to 43 amino acids carboxy-terminal to the CPx motif, and possess unique number of topology of membrane spanning proteins. P-type ATPases based resistance to zinc was reported from *E. coli* and *P. putida* strain S4 (Beard et al., 1997).

Copper resistance in gram-positive bacterium, *Enterococcus hirae*, has led to the discovery of two putative copper transporting ATPases (*copA* and *copB*). But, in 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 copper resistance phenotype; a large plasmid (175 kb) was isolated from copper resistant *E. faecium* pig isolate (A17sv1). The plasmid also contained genes for glycopeptide and macrolide resistance. 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 *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 *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, but 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). The *tcr* gene operon was first characterized by Hasman (2005). The sequence studies of the flanking region 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*.

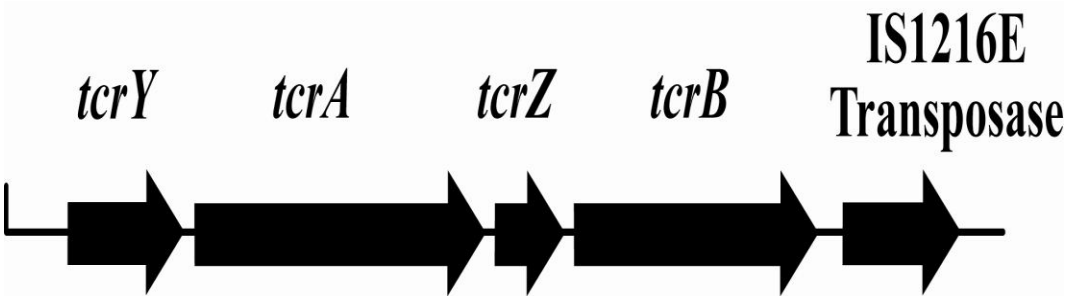

Figure 1.2: Organization of *tcrYAZB* operon including the flanking regions in *Enterococcus faecium* (Adapted from Hasman, 2005).

The first gene of the operon, *tcrY*, has 453 bp encoding 151 amino acids putative protein called TcrY. TcrY is homologous to CopY repressor from *E. hirae*, and consists of $CXCX_4CXC$ 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 putative copper-influx CPx-type ATPase called TcrA, made up of 2,433 bp and 811 amino acids, respectively. The TcrA has been known to have strong homology to CopA from *E. hirae*. TcrA is believed to possess a strong homology to CopA from *E. hirae*, and contained all features of CPx-type copper transporter (Solioz and Stoyanov, 2003). The *tcrZ* gene contains 204 bp in length, which encodes a putative chaperone protein called TcrZ with 68 amino acids, with a homology to other copper chaperones, including CopZ from *E. hirae*. The *tcrB* gene, last gene of the *tcrYAZB* operon encodes for copper efflux pump, TcrB protein homologous to CopB from *E. hirae*.

Table 1.3: Nucleotide and protein identities between the genetic elements of the *tcrYAZB* and the *copYZAB* operons of *E. hirae* (Adapted from Hasman, 2005).

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

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 wihin the cell, yet origin of the operon remains elusive, as the order of the genes is not the same as the *cop* genes of *E. hirae*. The nucleotide and protein identities between the *tcrYAZB* operon and the *copYZAB* operon of *E. hirae* are shown above (Table 1.3). 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 factor-independent transcriptional terminator (Hasman, 2005).

Enterococcus spp.

Enterococcus is an extremely versatile bacterial genus. Enterococci represent part of normal commensal 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 evolved and acquired resistance to many of the 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 the food of animal origin (Klein, 2003) and have been also associated with food-borne outbreaks due to their presence in foods and their capacity to carry and disseminate antibiotic resistance genes (Simjee et al., 2006). Recent studies 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.

The main focus of this review is to evaluate the effect of feeding copper on the resistance of *Enterococcus* spp., and its co-selection with other antibiotics from swine production systems.

Enterococcal infections

Enterococci occur in remarkable array of environments. They can be found in soil, water, food, and make up a significant portion of the normal gut flora of human 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* (Moellering, 2000). Most of the infectious diseases are caused by *Enterococcus faecalis*, which account for 80-90 % of the clinical strains. Enterococcal infections are more commonly seen in hospitalized individuals with underlying conditions comprised of wide spectrum of severity of illness and immune modulation. 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 are as follows:

a) Urinary tract infections: Most common form of infection caused by enterococci. Lower urinary tract infections, such as cystitis, prostatitis, and epidydimitis are common in older people (Graninger and Ragette, 1992). However, upper urinary tract infections are more common in young peoples, which can lead to severe bacteremia in later stages.
b) Bacteremia: Enterococci are observed in approximately 6-7% of the 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 infective 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.

CONCLUSION

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 be 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 ban on the nontherapeutic growth promoting use of antibiotics. 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, thus need for 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. Heavy metals, particularly copper and zinc are used in the diet of piglets as an alternative to antibiotics for growth promotion. Copper is a very important nutrient for young weaned piglets, and assist in control of diarrhea during the first week of weaning. A high dietary level of copper, as copper sulfate, is more often supplemented in the

diet of piglets for growth promotion purposes. It should be noticed that a recent studies from Denmark has indicated that high dietary copper may result in microbial resistance. 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 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, clonality, etc. 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.

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CHAPTER 2 - Occurrence of *tcrB*, a Transferable Copper Resistance Gene, in Fecal Enterococci of Swine

ABSTRACT

High concentration of copper, fed as copper sulfate, is 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. Our objectives were to determine the occurrence of *tcrB* in fecal enterococci from weaned piglets fed diets with normal supplemental (16.5 ppm) or elevated supplemental level (125 ppm) of copper and to determine the association of *tcrB* with copper, erythromycin, and vancomycin resistance. A total of 323 enterococcal isolates were examined and 15 (4.6%) isolates (14 *E. faecium* and 1 *E. faecalis*) were positive for *tcrB*. Fifteen *tcrB*-positive and 15 randomly chosen *tcrB*-negative isolates from piglets fed normal supplemental level of copper were tested for *erm*(B), *tet*(M), *vanA* and *vanB* genes and susceptibilities to copper, erythromycin, tetracyclines, and vancomycin. All *tcrB*-positive and -negative isolates contained *erm*(B) and *tet*(M), but not *vanA* and *vanB*. The mean MIC of copper for *tcrB*-positive (21.1 mM) was higher ($P < 0.001$) compared to *tcrB*-negative isolates (6.1 mM). All isolates were resistant to erythromycin, tetracyclines and susceptible to vancomycin. The transferability of the *tcrB* gene from *tcrB*-positive strains to *tcrB*-negative strains was demonstrated by conjugation. The potential link between *tcrB* and antibiotic resistance genes and the propensity of enterococci to transfer *tcrB* to other strains raises the possibility that copper supplementation may exert selection pressure for antibiotic resistant enterococci. This study is the first report on the occurrence of *tcrB* gene in enterococci isolated from swine in the U. S.

INTRODUCTION

Copper is an essential trace element for all living organisms, required for vital biological functions of cells (Krupanidhi et al., 2008). Copper, as copper sulfate, is supplemented in swine diets, at concentrations of 100-250 ppm, to reduce mortality and morbidity associated with bacterial enteric infections, particularly in piglets, and for growth promotion (NRC, 1998). One of the potential mechanisms of growth promotional effects of copper is believed to be similar to that of antibiotics in that gut microbial flora are altered to reduce fermentation loss of nutrients and suppress pathogens (Hϕjberg et al., 2005). Copper is highly reactive and toxic to cells because of their ability to generate intracellular superoxide or reactive oxygen radicals (Macomber and Imlay, 2009). Therefore, it is critical for a cell to regulate intracellular copper concentration. Bacteria utilize several mechanisms to regulate the intracellular copper concentration. Generally, copper homeostasis in a cell is regulated at three levels, influx, intracellular distribution and efflux (Reyes et al., 2008). The homeostatic mechanism is mediated mainly by the action of active efflux systems, which remove copper ion from the cell.

Copper homeostasis in gram positive bacteria, well documented in *Enterococcus hirae*, is mediated by a group of membrane spanning proteins, called CPx-ATPases, which are encoded by four genes in an operon, called *cop YZAB* (Solioz and Stoyanov, 2003). The *copA* and *copB* encode for ATPases, which are responsible for influx and efflux of copper, respectively. The *copZ* encodes a copper chaperone and *copY* acts as copper responsive repressor (Solioz and Stoyanov, 2003). Acquired copper resistance has been reported in gram negative bacteria, such as *Escherichia coli* and *Pseudomonas syringae* (Brown et al., 1995), and in certain gram positive bacteria, *Enterococcus hirae*, *Bacillus subtilis*, and *Lactococcus lactis*, (Solioz et al., 2010;

Hasman and Aarestrup, 2002; Leelawatcharamas et al., 1997). A copper resistance gene, designated as transferable copper resistance gene or *tcrB,* that confers copper resistance has been identified in *E. faecium, E. faecalis, E. gallinarum, E. casseliflavus*, and *E. mundtii* (Hasman and Aarestrup, 2002; Hasman et al., 2006b). The *tcrB* gene, harbored on a plasmid (Hasman et al., 2006b), is homologous to *copB* of *copYZAB* operon, and encodes a putative protein belonging to the CPx-type ATPase family (Hasman, 2005). Interestingly, the plasmid also carried genes, *erm*(B) and *vanA,* that encode resistance to macrolides and glycopeptides, respectively (Hasman and Aarestrup, 2002; Hasman et al., 2006 b), 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 by conjugation (in vitro).

MATERIALS AND METHODS

Animals, experimental design and sampling

The use of animals and the experimental procedure followed were approved by the Kansas State University Animal Care and Use Committee. Fecal enterococci for this study were obtained from weaned piglets fed diets supplemented with or without elevated supplemental level of copper supplied by copper sulfate. The study was designed and conducted to assess the performance benefits of supplementation of copper, zinc or antibiotics. One hundred and fifty weaned pigs (21 days old with an average body weight of 6.0 ± 1.0 kg) were randomly allocated to one of five dietary treatments. The five dietary treatments were: basal diet with 16.5 ppm of supplemental copper and 165 ppm of supplemental zinc (control), basal diet supplemented with 125 ppm of copper provided by copper sulfate (copper group), basal diet supplemented with 3,000 ppm of zinc provided by zinc oxide (zinc group), basal diet supplemented with 125 ppm copper and 3,000 ppm of zinc (copper and zinc group), and basal diet supplemented with neomycin sulfate and oxytetracycline (Neo/Oxy 10/10; Penfield Animal Health, Omaha, NE; antibiotic group) at 55 mg/kg of feed (1:1). In treatment groups that were supplemented with zinc (zinc and copper and zinc groups), the level of zinc supplementation was reduced from 3,000 to 2,000 ppm after 14 days (Smith et al., 1997). The levels of copper and zinc supplemented in the basal diet are typical supplementation rates provided in nursery diets to meet the nutrient needs of the piglets. The basal diet consisted of corn, soybean meal, and vitamins, amino acids, and trace mineral supplements, and piglets were housed in an environmentally controlled nursery facility. Each dietary treatment group had a total of 30 piglets assigned to 6 pens with 5 piglets per pen. Each pen contained a four-hole, dry, self feeder and a nipple water

to provide *ad libitum* access to feed and water. Pens had wire-mesh floor and that allowed for 0.3 m^2 per piglet. Piglets were fed the treatment diets for 5 weeks. During each week of the study, fecal samples were obtained from three randomly selected piglets per pen and the samples were transported on ice immediately to the laboratory.

Isolation and identification of enterococci

All culture media used in the study were from Difco (Becton and Dickson, Sparks, MD). Fecal samples were diluted (approx. 1 g in 10 ml) in phosphate buffered saline and 50 µl of the suspension was spread-plated onto M-*Enterococcus* agar. After 24 h incubation at 37°C, two colonies (pin-point red, pink or metallic pink) were picked, streaked on blood agar plates and incubated overnight at 37°C. For the genus confirmation, isolates were inoculated into 100 µl Enterococcosel broth in a 96-well microtiter plate (Becton and Dickinson, Franklin Lakes, NJ), incubated at 37° C for 4 h, and tested for esculin hydrolysis, followed by testing with the API 20 Strep kit (bioMérieux®, Durham, NC). Isolates confirmed as enterococci were stored in protect beads (Cryo-Vac®, Round Rock, TX) at -80ºC until further use.

PCR for detection of the *tcrB* gene

The *tcrB* gene in enterococcal isolates was detected by the procedure described by Hasman *et al.* (2006 b). For DNA extraction, each isolate from the protect bead was streaked on a blood agar plate and a single colony was suspended in nuclease free water with Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiled for 10 min. The primers (Table 2.1) for the PCR reaction were supplied by Integrated DNA Technologies (IDT, Coralville, IA). One of the two *tcrB*-positive *E. faecium* strains obtained from Denamrk (7430275-4 or 7430162-6) served as a positive control.

Speciation of *enterococci*

Species identification of *tcrB*-positive and an equal number of *tcrB*-negative enterococcal isolates, randomly chosen from the control group, were performed by a multiplex PCR that identifies *E. faecium, E. faecalis, E. gallinarum* and *E. casseliflavus* (Jackson et al., 2004 a). Additionally, superoxide dismutase (*sodA)* gene sequence analysis (Poyart et al., 2000) was used for confirmation of species. The DNA of the isolates was extracted as described above. Master mixes, primers (Table 1) and running conditions for the multiplex PCR were as described by Jackson et al. (2004 a). 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. The primers (Table 1) and PCR conditions for *sodA* sequence analysis were as described by Poyart et al. (2000). The primers used were supplied by Invitrogen Life Technologies (Invitrogen, Carlsbad, CA).

DNA Sequencing

PCR products (*tcrB* and *sodA*) were purified by QIAquick® Gel Extraction Kit (QIAgen Science, Valencia, CA). The eluted DNA samples were submitted for sequencing to Genomics Core, Institute for Integrative Genome Biology, University of California at Riverside. The sequences were analyzed by BLAST search in the NCBI GenBank database.

Detection of *erm*(B), *tet*(M), *vanA* and *vanB* genes

The primers (Table 2.1) and PCR conditions for detection of *erm*(B) and *tet*(M) genes were as per Jacob et al. (2008). *Enterococcus faecalis* MMH 594 and *E. coli* harboring plasmid pFD 310 (Aminov et al., 2001) served as positive controls for *erm*(B) and *tet*(M), respectively. The primers (Table 2.1) and PCR conditions used for detecting *vanA* and *vanB* genes were based on

Kariyama et al. (2000). The *E. faecium* (ATCC 51559) and *E. faecalis* V583 were used as positive controls for *vanA* and *vanB*, respectively.

Copper susceptibility determinations

Copper susceptibilities of enterococcal isolates were determined by agar dilution method (Hasman et al., 2006 b). The *tcrB*-positive isolates, including the two *E. faecium* strains from Denmark (7430162-6 and 7430275-4; provided by Henrik Hasman, National Food Institute, Technical University of Denmark) and fifteen *tcrB*-negative strains were included. Mueller Hinton agars plates containing 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 or 40 mM of copper sulfate (Fischer Scientific, Fair Lawn, NJ), adjusted to pH 7.0, were used for copper susceptibility determinations. The plates, in duplicates, were spot inoculated with 20 µl of bacterial growth that was adjusted to McFarland turbidity standard no. 0.5. Plates were incubated for 48 h at 37°C to determine growth or no growth. The susceptibility determination was repeated with different inocula preparations.

Antibiotic susceptibility determinations

Minimum inhibitory concentrations of antibiotics were determined by micro-broth dilution method (CLSI, 2002). Antibiotics tested were chlortetracycline, erythromycin, oxytetracycline, tetracycline and vancomycin (Sigma-Aldrich, St. Louis, MO). Stock solutions of antibiotics 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 concentration 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 microtiter plate and MIC determinations were repeated with different inocula preparations.

Transferability of the *tcrB* gene

A conjugation assay to demonstrate transferability of *tcrB* gene from *tcrB*-positive to *tcrB*-negative isolates was performed by filter mating procedure (Tendolkar *et al*., 2006). The donor strains (14 *tcrB*-positive *E. faecium* and 1 *E. faecalis*) were resistant to tetracycline, erythromycin, and streptomycin (MIC = >100 µg/ml) and susceptible to spectinomycin (mean MIC = 2.2 µg/ml). The *E. faecium* TX 5034 (provided by Dr. Barbara E. Murray, University of Texas Medical Center) strain resistant to spectinomycin, and erythromycin (MIC = >100 µg/ml) and susceptible to tetracycline (MIC = 0.78 µg/ml) and negative for *tet*(*M*), was used as the recipient for mating with *tcrB*-positive *E. faecium* isolates. The *E. faecalis* OG1SSp strain, resistant to streptomycin (MIC = >100 µg/ml) and spectinomycin (MIC >100 µg/ml) and susceptible to tetracycline (MIC = 0.39 µg/ml), was used as the recipient for mating with *tcrB*-positive *E. faecalis* isolate. Transconjugants were selected on BHI agar plates containing tetracycline (40 µg/ml) and spectinomcyin (500 µg/ml) for both *E. faecium* and *E. faecalis* isolates. Transconjugants were tested by PCR for *tcrB*, *erm*(B) and *tet*(M) genes. The MIC of copper for the two recipient strains and the 15 transconjugants were determined as described above. The transfer frequency for each isolate was calculated as the number of transconjugants per recipient CFU. The model of the conjugation assay is depicted in Fig. 2.4.

Pulsed field gel electrophoresis

The PFGE analysis of *tcrB*-positive isolates was done as per Murray et al. (1990 b) with minor modifications. Isolates were grown overnight in 5 ml of BHI broth at 37° C. The cells were pelleted by centrifugation and suspended in 0.85% NaCl. The plugs were prepared by mixing 200 µl of the cell suspension with 200 µl of 1.6% SeaKem gold agarose. The plugs were lysed by transferring them into a 10 ml lysis solution (6 mM Tris-HCl, pH-7.4, 100 mM EDTA, 1M NaCl, 0.5% sodium lauroyl sarcosine, 0.5% Brij, 0.2% deoxycholate, lysozyme [500 µg/ml] and RNaseA [20 µg/ml] for 4 h at 37° C with gentle shaking. The plugs were then transferred to ESP buffer (10 mM Tris-HCl, pH-7.4, 1 mM EDTA, 1% SDS and 50 µg/ml Proteinase K) and incubated overnight at 50° C with gentle shaking. The plugs were washed three times for 10 min each time with TE dilute buffer (10 mM Tris-HCl, pH-7.4, 0.1 mM EDTA) and stored at 4° C until used.

Restriction digestion of the plugs was performed by placing a small slice of agarose plug in 1.5 ml micro centrifuge tube with 2 µl of *Sma*I in 10 µl of 10X buffer and volume made to 100 µl with double distilled water for 4 h at 25° C. The digested plugs were transferred onto a gel (1% SeaKem Gold Agarose with 0.5X TBE) and the gel was placed in an electric field device, CHEF-DR II (BioRad®, Richmond, CA) and the pulse time for block 1 was 3.5 s initial time and 25 s final time at 200 V for 12 h; and for block 2 was 1 sec initial time and 5s final time at 200 V for 8h. Then the gel was stained with 0.0001% (or 1 µg/ml) ethidium bromide for 30 min followed by destaining in distilled water for 20 min each for 3 times. Gel images were captured with a Gel Doc 2000 system (BioRad®, Richmond, CA), and band patterns were analyzed and compared by using BioNumerics software (Applied Maths, Austin, TX). We used the band-based Dice similarity coefficient and the unweighted pair group mathematical average algorithm

method (UPGMA) for clustering with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% for band comparison. Isolates were grouped based on identical banding patterns (100% Dice similarity).

Statistical analysis

The prevalence of *tcrB* gene in fecal enterococcal isolates was evaluated using a generalized mixed model (PROC GLIMMIX, SAS, Version 9.1.3). The statistical model included the fixed effect of dietary treatment and sampling week as a random effect. Because all isolates were resistant (MIC > 100 µg/ml) to erythromycin, oxytetracycline, and tetracycline, these data were not analysed. The distribution of MIC values within copper or vancomycin and *tcrB*- negative or -positive isolates was evaluated for evidence of departure from normal distribution (PROC UNIVARIATE). Because the values were not normally distributed ($P < 0.05$), the values were transformed based on rank (PROC RANK) and analysis of variance (PROC GLIMMIX) was performed on the ranked values.

Nucleotide sequence accession number

The partial gene sequence of one *tcrB*-positive *E. faecium* (Strain KSU-263) was deposited in the NCBI GenBank database (Accession number EU869871).

RESULTS

Occurrence of the *tcrB* gene

A total of 323 enterococcal isolates were obtained from the feces of piglets collected at weekly intervals for 5 weeks (Table 2.2). All 323 enterococcal isolates were screened for the *tcrB* gene and 15 isolates (4.6 %) were identified as positive (amplicon size was 663 bp; Fig. 2.1). Sampling time (weekly) had no effect on the number of *tcrB*-positive enterococci isolated from the feces ($P > 0.05$). Similarly, the number of *tcrB*-positive enterococci isolated was not different among the five dietary treatment groups ($P > 0.05$; Table 2.2). Seven *tcrB*-positive isolates (7/109; 6.4%) were from piglets fed elevated supplemental level (125 ppm; copper or copper and zinc groups) and eight isolates (8/214; 3.7%) were from piglets fed normal supplemental level (16.5 ppm) of copper in their diets (control, zinc, and antibiotic groups).

Species identification

Based on API, the 15 *tcrB*-positive and the 15 randomly chosen *tcrB*-negative isolates were initially identified as *E. faecium*. The multiplex PCR revealed that one of the 15 *tcrB*-positive isolates, identified as *E. faecium* by API, was *E. faecalis*. The multiplex PCR confirmed the 15 *tcrB*-negative isolates as *E. faecium*. The *sodA* gene sequence analyses indicated that *tcrB*-positive or -negative *E. faecium* had 98-99% sequence homology with the *E. faecium* and the *tcrB*-positive *E. faecalis* isolate had 99% homology with *E. faecalis*.

Occurrence of *erm*(B), *tet*(M), *vanA* and *vanB*

All 15 *tcrB*-positive and 15 *tcrB*-negative isolates were positive for the *erm* (*B*) and *tet* (*M*) *genes* and negative for *vanA* and *vanB* genes.

Minimum inhibitory concentrations of copper

All *tcrB*-positive isolates obtained in our study (14 *E. faecium* and 1 *E. faecalis*) and the two Denmark strains of *E. faecium* grew on Mueller Hinton agar containing copper at 16 or 20 mM. In contrast, the 15 *tcrB*-negative isolates were able to grow at 4 or 6 mM of copper concentration. The mean MIC of copper for *tcrB*-negative and *tcrB*-positive isolates were 6.1 [SD (95% CI) = 2.205] and 21.1[SD (95% CI) = 4.677] mM, respectively, and the difference was significant at $P < 0.001$ (Fig. 2.2). The two *E. faecium* strains, 7430162-6 and 7430275-4 from Denmark had a MIC of 23 and 24 mM, respectively.

Minimum inhibitory concentrations of antibiotics

All isolates (14 *tcrB*-positive *E. faecium*, 1 *tcrB*-positive *E. faecalis*, 2 *tcrB*-positives *E. faecium* from Denmark and 15 *tcrB*-negative *E. faecium*) were resistant to tetracycline, chlortetracycline, erythromycin, and oxytetracycline with MIC greater than 100 µg/ml and susceptible to vancomycin. The MIC values for vancomycin were higher ($P < 0.001$) for *tcrB*-positive (0.39 µg/ml) than for *tcrB*-negative (0.098 µg/ml) isolates.

Transferability of the *tcrB* gene by conjugation

Fourteen *tcrB*-positive *E. faecium* isolates and 1 *tcrB*-positive *E. faecalis* were used to demonstrate transferability of *tcrB* gene by conjugation. The 14 transconjugant *E. faecium* and 1 *E. faecalis* strains were positive for *tcrB, erm*(B), and *tet*(M) genes (Table 2.3). As expected, the

transconjugants were resistant to tetracycline (MIC > 100 µg/ml) and were able to grow on BHI agar containing high copper concentrations (16 mM or higher). The mean MIC of copper for the 15 transconjugants was 17.6 mM. The mean transfer frequency for *tcrB*-positive *E. faecium* (14 isolates) was 1.01×10^{-5}. The *E. faecalis* isolate had a transfer frequency of 1.16×10^{-5}. The transfer frequency (number of transconjugants per recipient) for both intra-species and inter-species conjugal transfer of *tcrB* gene in *tcrB*-positive *E. faecium* and *E. faecalis* were shown in Table 2.4 and Table 2.5.

PFGE of *tcrB*-positive enterococcal isolates

The clonal relationship among the 15 *tcrB*-positive isolates was determined by PFGE typing (Fig. 2.3). A total of six PFGE patterns (< 95% Dice similarity) were observed among the 14 *tcrB* positive *E. faecium* isolates. Four of the 5 isolates from the zinc supplemented group had identical banding patterns (100% Dice similarity). Four of the 5 isolates from the copper and zinc supplemented group had different banding patterns (< 95% dice similarity). The two Denmark isolates were clonally different and had distinctly different banding patterns compared to our *tcrB*-positive *E. faecium* isolates (Fig. 2.3).

DISCUSSION

Antibiotics are supplemented in swine diets, particularly of weanling and starter pigs, to reduce morbidity and mortality, and increase growth rate (Zhao et al., 2007). Similarly, copper, at elevated levels, is used as a dietary supplement in pigs for growth promotion (Cromwell et al., 1989; NRC, 1998). In piglets, the growth response to copper supplementation is in addition to the response provided by antibiotics (Edmonds et al., 1985). However, in European countries like Denmark, copper is included increasingly in swine diets at elevated levels, as a replacement to in-feed antibiotics, which have been banned for use as growth promotants (Hasman et al., 2006 a). The mechanisms to explain the beneficial effects from copper supplementation are not fully known. It is suggested that copper exerts some systemic effect (Zhou et al., 1994) and more importantly beneficially alters gut microbial metabolism, which is because of the antimicrobial activity of copper (Gould et al., 2009; Hϕjberg et al., 2005). The inhibition of potential pathogens, such as coliforms, and increased availability of nutrients and energy because of reduced microbial activity in the gut are contributing factors in growth promotion (Hϕjberg et al., 2005).

In our study, only a small proportion of fecal enterococci (4.6%; 15/323), isolated from weaned piglets fed diets supplemented with normal or elevated level of copper, contained the *tcrB* gene. Majority of the *tcrB*-positive isolates (14/15) were *E. faecium* and only one was *E. faecalis*. In Denmark, the *tcrB* was also detected in *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* (Hasman et al., 2006b). The presence of *tcrB* gene was associated with approximately four-fold increase (6 vs. 21 mM) in resistance to copper. Hasman and Aarestrup (2002) have reported a seven-fold increase (4 vs. 28 mM) in copper resistance in *tcrB*-positive *E. faecium.* For *tcrB*-negative isolates, we determined MIC of copper of only 15 *E. faecium* isolates that

were randomly selected from 308 *tcrB*-negative enterococci. None of them had an MIC greater than 8 mM of copper. According to Hasman and Aarestrup (2002), all copper-resistant *E. faecium* strains isolated from feces, harbored the *tcrB* gene and none of the copper susceptible isolates contained *tcrB*. Therefore, in enterococci, presence of *tcrB* gene is associated with resistance to copper.

In our study, the *tcrB*-positive enterococci were isolated from all five treatment groups, regardless of copper level in the diet. Although we picked two esculin-positive colonies from each piglet sampled (three piglets per pen and six pens per treatment), we retained only one isolate after confirming the genus and species by esculin hydrolysis and the API and PCR analyses. This allowed us to screen a maximum of 18 isolates per sampling week per treatment group and the total isolates in each treatment group for the entire study ranged from 52 to 78. Interestingly, a higher proportion of enterococci positive for *tcrB* (7/109; 6.4%) were from groups supplemented with elevated level (125 ppm) of copper (copper or copper and zinc group) compared to 3.7% (8/214) of isolates from groups fed normal supplemental level (16.5 ppm) of copper in their diets (control, zinc, or antibiotic). Of the seven *tcrB*- positive isolates in groups fed elevated level of copper, six were *E. faecium* and they represented only four PFGE types based on *Sma*I restriction digestion. The clonal identities of the three isolates suggest possible pen to pen transmission. Of the eight *tcrB*-positive isolates from treatment groups fed normal level of copper, only one isolate (from the antibiotic group) was clonally identical to an isolate (PFGE type 3) from the copper-supplemented group. The four *tcrB*-positive isolates from the zinc treatment group were from the same pen and had identical banding patterns (PFGE type 6), suggesting possible pig to pig transmission. Another explanation for the occurrence of the same clones may be the common source (same barn or litter mates) of piglets used in the study.

Because of the limited number of isolates examined in the study, we could not ascertain whether elevated copper level in the diet exerted pressure to select *tcrB*-positive entercocci. In testing different populations of *E. faecium* of animal or human origin in Denmark, Hasman and Aarestrup (2002) reported the highest level of copper resistance (76%) in isolates from pigs that had received 165 ppm of copper sulfate during the post weaning growth phase. In an *in vivo* animal challenge study (Hasman *et al*., 2006 b), feeding 175 ppm of copper resulted in the selection of *tcr*-mediated copper resistance in *E. faecium* compared to piglets fed low levels of copper (6 ppm). The influence of zinc supplementation on copper resistance is not known, although there is evidence that in *Psedomonas aeruginosa,* copper exposure induces resistance to not only copper but also zinc (Callie et al., 2007).

An interesting aspect of copper resistance in enterococci is the genetic link of *tcrB* to macrolide and glycopeptide resistance (Hasman and Aarestrup, 2002). A 175-kb plasmid from a pig isolate of *E. faecium* that contained genes for vancomycin resistance (*vanA*) and macrolide resistance [*erm*(B)] along with the *tcrB* gene has been identified (Hasman and Aarestrup, 2002). In our study, all 15 *tcrB*-positive isolates contained the *erm*(B) gene and were phenotypically resistant to erythromycin (MIC = >100 µg/ml). However, the *tcrB*-positive isolates did not contain either the *vanA or vanB* gene and were susceptible to vancomycin (MIC = < 0.7 µg/ml). Neither presence of *erm*(B) nor absence of *van* genes in enterococci is surprising. The *erm*(B) is the most common gene associated with enterococcal isolates resistant to macrolides (Jensen et al., 1999). Although erythromycin is not commonly used in swine, tylosin, another macrolide, is used in the feed to treat enteric bacterial infections and for growth promotion (Jackson et al., 2004 b). We tested our isolates for both *vanA* and *vanB.* The *vanB* gene shares 76% of its amino acid sequence with *vanA,* and *vanA* is the most predominant resistant determinant in human

enterococcal isolates in Europe, whereas *vanB* is most common in the United States (Cetinkaya et al., 2000). Occurrence of glycopeptide resistance in enterococci of pigs and chickens in Europe is associated with the use of avoparcin, a glycopeptide, for growth promotion (Wegener et al., 1999; Aarestrup et al., 2001). However, the use of avoaprcin in Europe was banned in 1997. Also, a genetic link between *erm*(B) and *vanA* has been documented in population of *E. faecium* isolates from pigs and chickens in Europe and Japan (Aarestrup, 2000; Yoshimura et al., 1998). The absence of glycopeptide resistance in enterococcal isolates of piglets in our study, which is in agreement with Poole et al. (2005), was possibly because avoparcin was never approved for use as a growth promotant in the United States (McDonald et al., 1997).

Enterococcal isolates in our study were also positive for *tet*(M) and phenotypically resistant to tetracyclines. There is evidence of co-selection of *tet*(M) with *erm*(B) in enterococci (Cauwerts et al., 2007). The *tet*(M) gene is most often carried by transposons of the Tn*916*-Tn*1545* family, which are promiscuous conjugative transposons in both gram negative and gram positive bacteria (Chopra and Roberts, 2001). The carriage of *tet*(M) is associated with *erm*(B) gene in Tn*1545*-like transposons (Clewell, 1995).

Antimicrobial resistant enterococci are important opportunistic and nosocomial pathogens in humans (Moellering, 1992; Arias and Murray, 2008; Murray, 2000). Because of frequent occurrence of resistant enterococci in animals, it has been suggested the enterococci may serve as a reservoir of resistance genes capable of transferring to humans (Kojima et al., 2010). The medical importance of enterococci is related to the propensity of these organisms to participate in the horizontal transfer of the antibiotics and virulence genes. We were able to demonstrate the transferability of the *tcrB* gene from all strains of *tcrB*-positive enterococci isolated in our study.

Our results suggest that *tcrB*, *erm*(B), and *tet*(M) genes may be present on conjugative plasmids, but this remains to be determined.

An increased use of copper at elevated levels in swine feeds may exert selection pressure for copper resistance, mediated by the *tcrB*. Because of the genetic link between the *tcrB* gene and antibiotic resistance genes, it could be argued that copper supplementation may co-select for resistance to antibiotics, such as tetracyclines and macrolides. One of the reasons for persistence of macrolide and glycopeptides resistance in enterococci in the years following the ban of in-feed antibiotics in Europe is thought to the use of copper as a growth promotant (Boerlin et al., 2001; Hasman and Aarestrup, 2005).

Our study is the first report on the occurrence of the *tcrB* gene in enterococci of swine in the U. S. The significance of this finding is the potential association between copper resistance and resistance to other antibiotics and the propensity of enterococci to transfer *tcrB* and antibiotic resistance genes to other strains within the same species and to other enterococcal species. Further studies are needed to determine whether supplementation with elevated levels of copper selects for copper resistance, and in turn co-selects for antibiotic resistance.

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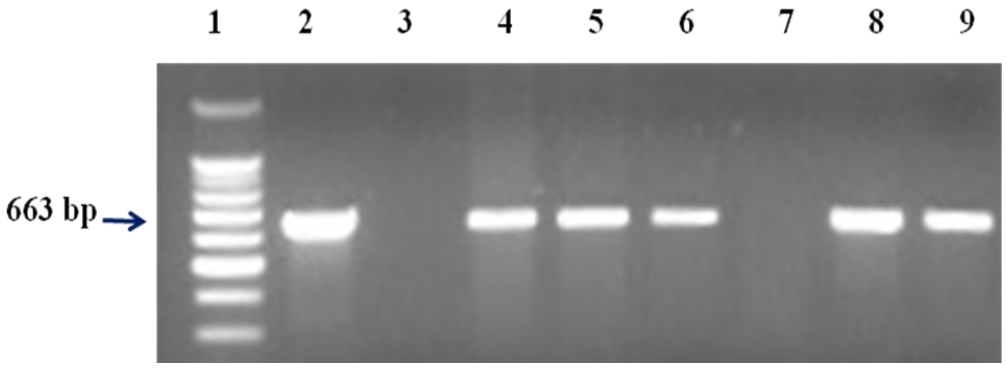

Figure 2.1: PCR identification of the *tcrB* gene of enterococcal isolates from piglets fed diets supplemented with copper, zinc, copper and zinc, or neomycin and oxytetracycline. (Lane 1: 100 bp molecular marker, Lane 2: Positive control (Denmark strain), Lane 3: Negative control and Lanes 4-9: Isolates from this study).

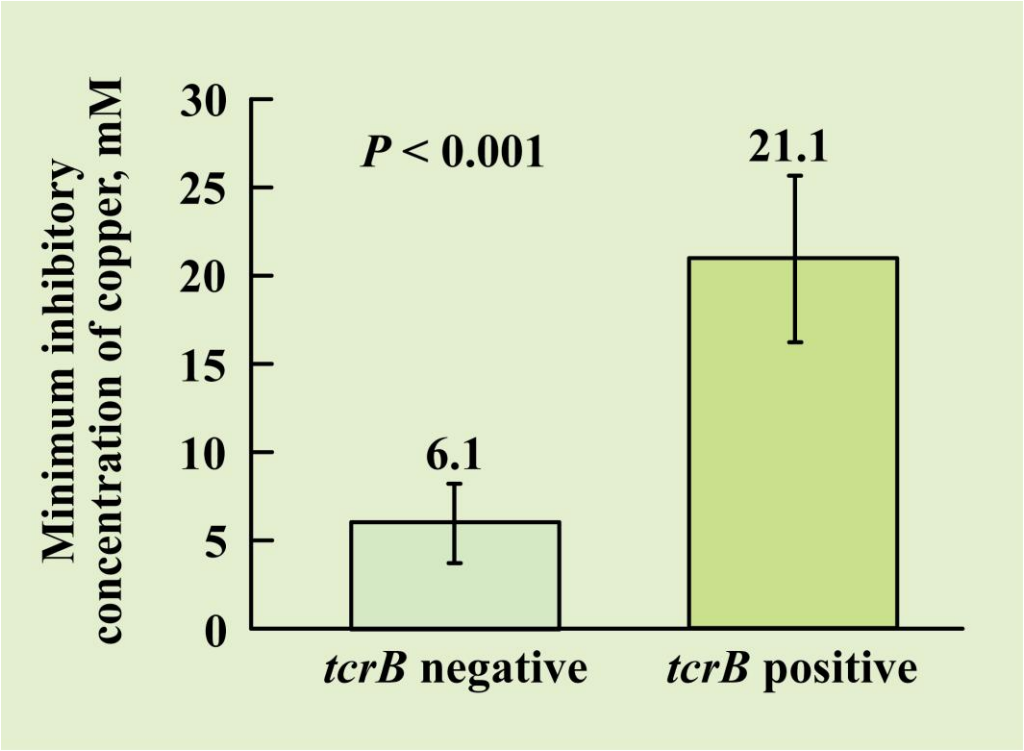

Figure 2.2: Minimum inhibitory concentrations (95% Confidence Interval) of copper for *tcrB*-positive and *tcrB*-negative enterococcal isolates from piglets fed diets supplemented with copper, zinc, copper and zinc, or neomycin and oxytetracycline.

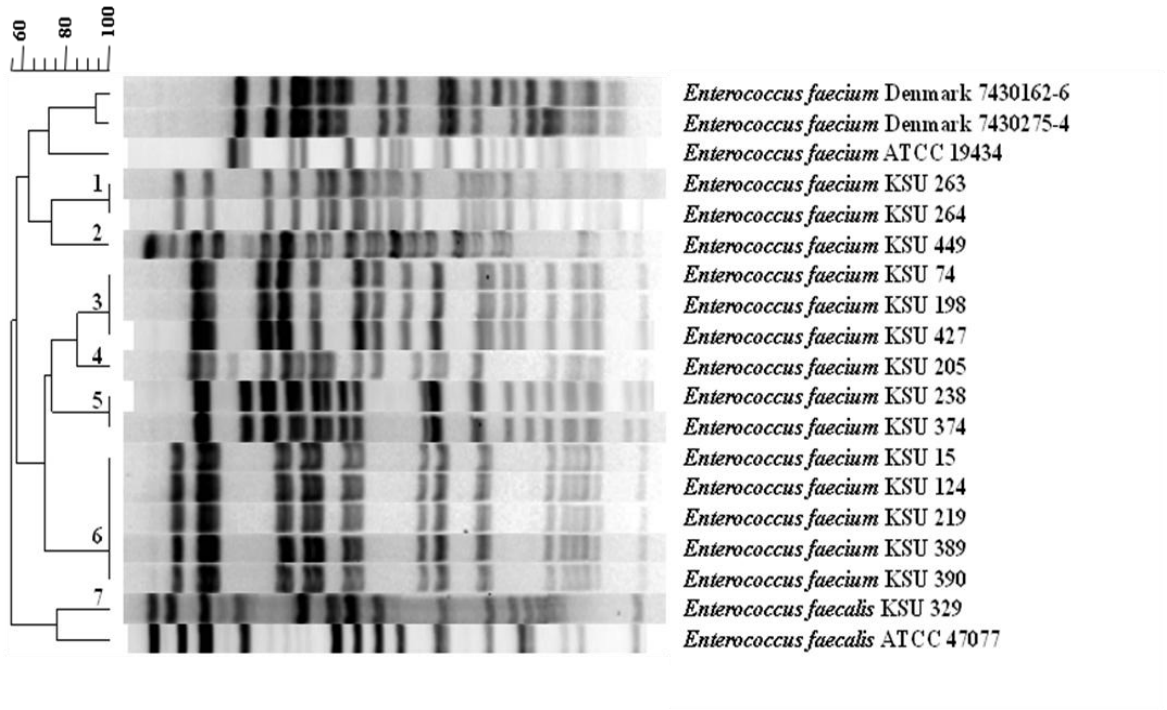

Figure 2.3: Pulsed-field gel electrophoresis patterns of *Sma*I-digested genomic DNA of enterococcal isolates from piglets fed diets supplemented with copper, copper and zinc, or neomycin and oxytetracycline.

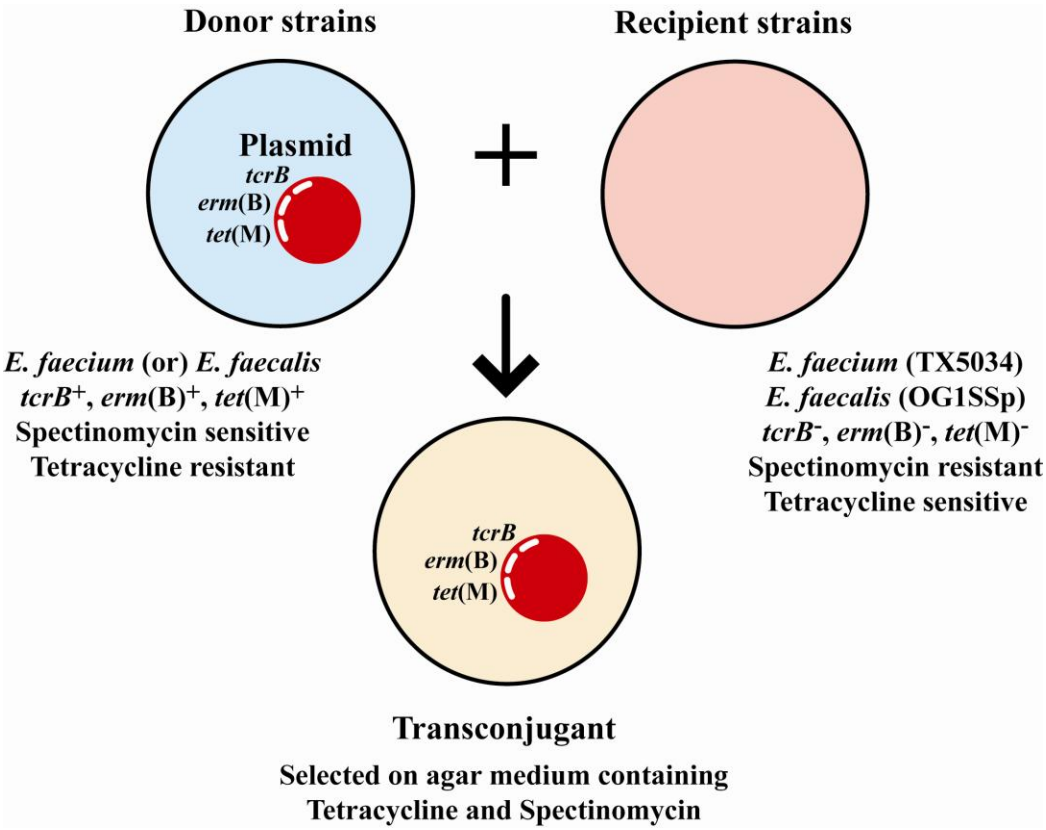

Figure 2.4: Model for the conjugative transfer of plasmid mediated antibiotic resistance determinants

Table 2.1: Primers used in the present study

Gene	Sequence (5 ′-3 ′)	Product size (bp)	Reference
tcrB	F: CATCACGGTAGCTTTAAGGAGATTTTC R: ATAGAGGACTCCGCCACCATTG	663	Hasman *et al*., 2006
tet(M)	F: CTGTTGAACCGAGTAAACCT R: GCACTAATCACTTCCATTT	156	Jacob *et a*l., 2008
erm(B)	F: GAATCCTTCTTCAACAATCA R: ACTGAACATTCGTGTCACTT	175	Jacob *et al*., 2008
sodA	F: CCITAYICITAYGAYGCIYTIGARCC R: ARRTARTAIGCRTGYTCCCAIACR	438	Poyart *et al*., 2000
vanA	F: CATGAATAGAATAAAAGTTGCAATA R: CCCCTTTAACGCTAATACGATCAA	1,030	Kariyama *et a*l., 2000
vanB	F: GTGACAAACCGGAGGCGAGGA R: CCGCCATCCTCCTGCAAAAAA	433	Kariyama *et al*., 2000
Enterococcus gallinarum (*vanC1*)	F: GGTATCAAGGAAACCTC R: CTTCCGCCATCATAGCT	822	Kariyama *et al*., 2000
Enterococcuscassel iflavus (van C2/C3)	F: CGGGGAAGATGGCAGTAT R: CGCAGGGACGGTGATTTT	484	Kariyama *et al*., 2000
Enterococcus faecalis	F: CAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	Dutka-Malen *et al*., 1995
Enterococcus faecium	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	Dutka-Malen *et al*., 1995
16s rRNA gene	F: GGATTAGATACCCTGGTAGTCC R:TCGTTGCGGGACTTAACCCAAC	320	Kariyama *et al*., 2000

Table 2.2: Occurrence of the *tcrB* gene in fecal enterococcal isolates from piglets fed diets supplemented with copper, zinc, copper and zinc, or neomycin and oxytetracycline.

Treatment	Sampling weeks (No. of isolates positive for *tcrB* gene/total isolates tested)					TOTAL
	1	2	3	4	5	
Control	0/9	0/14	0/12	0/17	1/15	1/67
Copper	0/11	0/17	2/13	0/11	0/5	2/57
Zinc	1/16	1/17	1/8	0/14	2/14	5/69
Copper + zinc	1/11	0/14	2/8	1/14	1/5	5/52
Neomycin + oxytetracycline	0/16	0/15	1/18	0/16	1/13	2/78
TOTAL	2/63	1/77	6/59	1/72	5/52	15/323

Table 2.3: Transferability of the *tcrB* gene of *Enterococcus faecium* or *E. faecalis* to *tcrB*-negative strains of *E. faecium* or *E. faecalis*.

Species and strains	*tcrB*	*erm*(B)	*tet*(M)	Copper MIC, mM	Species and strains	*tcrB*	*erm*(B)	*tet*(M)	Copper MIC, mM
					Recipient *E. faecium* (TX 5034)	-	+	-	2
Donor *E. faecium*					Transconjugant *E. faecium*				
KSU 15	+	+	+	23	KSU- TC-15	+	+	+	16
KSU 74	+	+	+	21	KSU-TC-74	+	+	+	18
KSU 124	+	+	+	19	KSU-TC-124	+	+	+	16
KSU 198	+	+	+	19	KSU-TC-198	+	+	+	20
KSU 205	+	+	+	20	KSU-TC-205	+	+	+	20
KSU 219	+	+	+	24	KSU-TC-219	+	+	+	16
KSU 238	+	+	+	22	KSU-TC-238	+	+	+	20
KSU 263	+	+	+	20	KSU-TC-263	+	+	+	18
KSU 264	+	+	+	24	KSU-TC-264	+	+	+	16
KSU 374	+	+	+	23	KSU-TC-374	+	+	+	16
KSU 389	+	+	+	22	KSU-TC-389	+	+	+	16
KSU 390	+	+	+	22	KSU-TC-390	+	+	+	16
KSU 427	+	+	+	24	KSU-TC- 427	+	+	+	20
KSU 449	+	+	+	17	KSU-TC- 449	+	+	+	16
Donor *E. faecalis* KSU 329	+	+	+	17	Recipient *E. faecalis* (OG1SSp)	-	-	-	4
					Transconjugant *E. faecalis* KSU-TC-329	+	+	+	16

Table 2.4: Intra-species conjugal transfer of *tcrB* gene in *tcrB*-positive *Enterococcus faecium* and *Enterococcus faecalis*

Donor	Recipient	Transfer frequency (no. of transconjugants per recipient)
E. faecium KSU 15	*E. faecium* TX5034	8.73×10^{-6}
E. faecium KSU 74	*E. faecium* TX5034	8.85×10^{-6}
E. faecium KSU 124	*E. faecium* TX5034	1.19×10^{-5}
E. faecium KSU 198	*E. faecium* TX5034	6.76×10^{-6}
E. faecium KSU 205	*E. faecium* TX5034	1.00×10^{-5}
E. faecium KSU 219	*E. faecium* TX5034	9.78×10^{-6}
E. faecium KSU 238	*E. faecium* TX5034	8.02×10^{-6}
E. faecium KSU 263	*E. faecium* TX5034	6.21×10^{-6}
E. faecium KSU 264	*E. faecium* TX5034	1.03×10^{-5}
E. faecalis KSU 329	*E. faecalis* OG1SSp	1.16×10^{-5}
E. faecium KSU 374	*E. faecium* TX5034	1.24×10^{-5}
E. faecium KSU 389	*E. faecium* TX5034	1.13×10^{-5}
E. faecium KSU 390	*E. faecium* TX5034	1.29×10^{-5}
E. faecium KSU 427	*E. faecium* TX5034	1.36×10^{-5}
E. faecium KSU 449	*E. faecium* TX5034	1.21×10^{-5}

Table 2.5: Inter-species conjugal transfer of *tcrB* gene in *tcrB*-positive *Enterococcus faecium* and *Enterococcus faecalis*

Donor	Recipient	Transfer frequency (no. of transconjugants per recipient)
E. faecium KSU 15	*E. faecalis* OG1SSp	8.60×10^{-6}
E. faecium KSU 74	*E. faecalis* OG1SSp	5.75×10^{-6}
E. faecium KSU 124	*E. faecalis* OG1SSp	1.12×10^{-5}
E. faecium KSU 198	*E. faecalis* OG1SSp	7.74×10^{-6}
E. faecium KSU 205	*E. faecalis* OG1SSp	1.25×10^{-5}
E. faecium KSU 219	*E. faecalis* OG1SSp	1.07×10^{-5}
E. faecium KSU 238	*E. faecalis* OG1SSp	1.51×10^{-5}
E. faecium KSU 263	*E. faecalis* OG1SSp	1.15×10^{-5}
E. faecium KSU 264	*E. faecalis* OG1SSp	8.43×10^{-6}
E. faecalis KSU 329	*E. faecium* TX5034	6.85×10^{-6}
E. faecium KSU 374	*E. faecalis* OG1SSp	6.91×10^{-6}
E. faecium KSU 389	*E. faecalis* OG1SSp	7.85×10^{-6}
E. faecium KSU 390	*E. faecalis* OG1SSp	7.43×10^{-6}
E. faecium KSU 427	*E. faecalis* OG1SSp	4.21×10^{-6}
E. faecium KSU 449	*E. faecalis* OG1SSp	8.94×10^{-6}

CHAPTER 3 - Selection of *tcrB* gene mediated copper resistant fecal enterococci in piglets fed diets supplemented with copper

ABSTRACT

Copper, as copper sulfate, is often supplemented at elevated concentrations in swine diets to promote growth. Bacteria exposed to copper acquire resistance and among *Enterococcus faecium* and *E. faecalis* in Denmark, a plasmid-borne, transferable copper resistance (*tcrB*) gene has been reported. The plasmid also carried genes for macrolide [*erm*(B)] and glycopeptide (*vanA*) resistance. In a preliminary study, we confirmed the presence of the *tcrB* gene in fecal enterococci of piglets fed diets with normal or elevated concentration of copper. We conducted a full-scale study to determine the relationship between copper supplementation and the prevalence of *tcrB*-positive enterococci in feces of piglets. Sixty weaned piglets, housed in 10 pens with 6 piglets per pen, fed normal (16.5 ppm; control) or elevated concentration of copper (125 ppm; copper) were used. Fecal samples were collected randomly from three piglets per pen on days 0, 14, 28, and 42, and three enterococcal isolates were obtained from each sample. The speciation of enterococci was done by multiplex PCR and *sodA* gene sequence analysis. A total of 45 enterococcal isolates were obtained per treatment group at each sampling day and tested for *tcrB* gene by PCR (360 isolates in total). Overall, prevalence of *tcrB*-positive enterococci was 2.8% (5/180) in the control group and 21.1% (38/180) in those piglets fed elevated concentration of copper ($P < 0.05$). The *tcrB* positive isolates belonged to either *E. faecium* or *E. faecalis*, and majority of isolates (35/43) was *E. faecium.* All *tcrB*-positive isolates contained *erm*(B) and *tet*(M) genes but none harbored the *vanA* gene reported in the Danish study. The mean MICs of copper for *tcrB*-negative and *tcrB*-positive enterococci were 6.2 and 22.2 mM,

respectively ($P < 0.001$). Based on PFGE typing, a total of 17 PFGE types were obtained among the 35 *tcrB*-positive *E. faecium* isolates and 4 PFGE types were obtained among the 8 *tcrB*-positive *E. faecalis* isolates. The majority of the *tcrB*-positive isolates obtained on different sampling days belonged to different PFGE types. Only one isolate of *E. faecium* in the control was clonally identical to an isolate from the copper group suggesting that the occurrence of *tcrB*-positive enterococci in the control group was not because of transmission from the copper group. Conjugation assay demonstrated cotransfer of *tcrB* along with *erm*(B) and *tet*(M) genes within and between *E. faecium* and *E. faecalis*. The higher prevalence of *tcrB*-positive enterococci in feces of piglets fed elevated copper compared to normal copper concentration suggests that copper supplementation in swine diets selects for *tcrB* mediated resistant population of enterococci. The genetic link between *tcrB* and antibiotic resistance genes explains the potential importance of elevated copper supplementation in propagation and selection for antibiotic resistance.

INTRODUCTION

Copper is an essential trace element required for vital biological functions in both prokaryotic and eukaryotic cells (Krupanidhi et al., 2008). High concentration of copper (100-250 ppm), as copper sulfate, is routinely added to the diets of piglets to promote growth (NRC, 1998). The growth response to copper in piglets is independent of and in addition to the response of other commonly used antibiotics in the feed (Edmonds et al., 1985). Although the exact mechanism of action is unknown, the growth-promoting action of dietary copper is generally attributed to its antimicrobial activity on the gut microbiota (Gould et al., 2009), including suppression of enteric bacterial pathogens (Hϕjberg et al., 2005).

Copper in excess amount is toxic to cells because of the production of intracellular reactive oxygen radicals, which inactivate cell components, such as nucleic acids, lipids, and proteins (Macomber and Imlay, 2009). Therefore, cells tightly regulate intracellular copper concentration to avoid copper toxicity (Ridge et al., 2008). The copper homeostasis mechanism is well studied in certain gram-positive bacteria, such as *Enterococcus hirae*, *Lactococcus lactis*, and *Bacillus subtilis* (Solioz et al., 2010). The normal intracellular copper concentration is maintained by *copYZAB* operon, where *copA* and *copB* encode for copper transport ATPases, which are responsible for influx and efflux of copper, respectively. The *copY* acts as a copper responsive repressor and *copZ*, encodes for a copper chaperone (Solioz & Stoyanov, 2003).

Some bacteria acquire resistance to copper, which may be either chromosome mediated (Franke et al., 2003) or plasmid mediated (Lim et al., 1993). A plasmid- borne copper resistance gene, designated as transferable copper resistance (*tcrB*) gene, homologous to *copB* of the *copYZAB* operon, was reported in *E. faecium* and *E. faecalis* from piglets, calves, poultry and

humans in Denmark (Aarestrup et al., 2002). The strains harboring the *tcrB* gene resisted up to 24 mM of copper *in vitro*, in contrast to strains that lacked the gene resisted only 8 mM of copper (Hasman, 2005 ; Hasman et al., 2006a). The plasmid also carried genes, *erm*(B) and *vanA*, that encode resistance to macrolides and glycopeptides, respectively (Hasman & Aarestrup, 2002; Hasman et al., 2006a), suggesting a potential genetic link between copper resistance and antibiotic resistance. We have confirmed the occurrence of *tcrB* gene in fecal enterococci of piglets fed diets with normal or elevated level of copper (Amachawadi et al., 2010). Because of the limited number of isolates tested in our previous study, we were unable to ascertain whether copper supplementation in the diet exerted pressure to select for *tcrB*-positive enterococci. Therefore, the present study was undertaken to determine the relationship between supplementation of copper at elevated level and prevalence of fecal *tcrB*-mediated copper resistant enterococci in piglets.

MATERIALS AND METHODS

Animals, experimental design and sampling

The use of animals and the experimental procedure followed were approved by the Kansas State University Animal Care and Use Committee. Sixty newly weaned piglets (21 days old) with an average body weight of 7.0 Kg (± 1 Kg) were randomly allocated to two dietary treatments. The two dietary treatments were: basal diet with 16.5 ppm of copper (control group) or basal diet supplemented with 125 ppm of copper as copper sulfate (copper group). The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements, and piglets were housed in an environmentally controlled nursery facility. Each treatment group had a total of 30 piglets assigned to 5 pens with 6 piglets per pen. Each pen was provided with a self feeder, containing four holes and a nipple water so that animals can have access to *ad libitum* feed and water. Each pen had a wire-mesh floor that allowed for 0.3 m^2 per piglet. Piglets were fed treatment diets for 42 days. Fecal samples were collected randomly from 3 pigs in each pen on days 0, 14, 28, and 42, placed in individual bags, and transported to the laboratory.

Isolation of enterococci

Unless otherwise mentioned all culture media used were from Difco (Becton and Dickson, Sparks, MD). Fecal samples were processed by diluting 1 g of feces in 10 ml of phosphate buffered saline and spread-plating 50 µl of the suspension onto M-*Enterococcus* agar. The plates were incubated for 24 h at 37°C. Five colonies (pin-point red, pink or metallic pink) were picked and streaked onto blood agar plates and incubated overnight at 37°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 incubateing at 37° C for 4 h. Three

esculin hydrolysis positive isolates per fecal sample (9 per pen and 45 per treatment and sampling time) were selected and stored in protect beads (Cryo-Vac®, Round Rock, TX) at -80ºC until further use.

PCR for the detection of *tcrB* gene

The *tcrB* gene was detected according to the procedure described by Hasman et al. (2006a). Each isolate from the protect bead was streaked on to blood agar plate and incubated overnight at 37°C. 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. A *tcrB*-positive *E. faecium* strain (7430275-4 or 7430272-6; kindly provided by Dr. Henrik Hasman, National Food Institute, Technical University of Denmark) served as a positive control.

Speciation of *tcrB*-positive enterococci

Species identification of the enterococcal isolates that were *tcrB*-positive and an equal number of *tcrB*-negative isolates, randomly chosen from the control group, were performed by a multiplex PCR that identifies *E. faecium*, *E. faecalis E. gallinarum* and *E. casseliflavus* (Jackson et al., 2004a). Additionally, superoxide dismutase (*sodA)* gene sequence analysis (Poyart et al., 2000) was used for species confirmation. The DNA template was prepared as before. 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 (Table 1) and running conditions for the multiplex PCR and *sodA* gene PCR were as described by Jackson et al. (2004a) and Poyart et al. (2000), respectively. The *sodA* gene products were purified by Wizard® SV Gel and PCR clean-up system (Promega, Madison, WI). The purified PCR products were sequenced at Genomics Core, Institute for Integrative Genome Biology,

University of California, Riverside. The sequences were analyzed by BLAST search in the NCBI GenBank database.

Detection of *erm*(B) and *vanA* genes

The primers (Table 3.1) and PCR conditions for detection of *erm*(B) and *vanA* genes were as per Jacob et al. (2008) and Kariyama et al. (2000), respectively. *Enterococcus faecalis* MMH 594 (Dr. Lynn Hancock, Dept. of Biology, Kansas State University) and *E. faecium* (ATCC 51559) served as positive controls for the *erm*(B) and *vanA* genes, respectively.

Copper susceptibility determinations

Copper susceptibility determinations of *tcrB*- positive and an equal number of *tcrB*-negative enterococcal isolates were done by agar dilution method (Hasman & Aarestrup, 2002; Hasman et al., 2006a). The two *tcrB*-positive *E. faecium* strains from Denmark (7430162-6 and 7430275-4) were included as positive controls. Mueller Hinton (MH) agar plates prepared with concentrations of copper, added as copper sulfate (Fischer Scientific, Fair Lawn, NJ), at 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 mM and with pH adjusted to 7.0 were used. The plates were spot inoculated with 20 µl of bacterial culture that was adjusted to McFarland turbidity standard no. 0.5 (Remel®, Lenexa, KS) and incubated at 37°C for 48 h to determine growth or no growth. The susceptibility determinations were repeated on another day with different inocula preparations.

Antibiotic susceptibility determinations

Micro-broth dilution method was used to determine the minimum inhibitory concentrations of erythromycin and vancomycin (Sigma-Aldrich, St. Louis, MO) as per CLSI guidelines (CLSI, 2002). Antibiotic stock solutions containing final concentration of 1,000 µg/ml, based on

potency, were prepared in sterile distilled water. The bacterial inocula were prepared by inoculating a single colony in 10 ml MH broth, incubating for 6 h, and diluting 100 fold with sterile MH broth. 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 antimicrobial susceptibility test was performed in 96-well micro titer plates (Becton and Dickinson) and inoculated plates were incubated at 37° C for 24 h, and results were recorded as growth or no growth. The MIC determinations were repeated one more time with different inocula preparations.

Pulsed field gel electrophoresis

The PFGE analysis of *tcrB*-positive isolates was done as per Murray et al. (1990b) with minor modifications. A single colony of the isolate was inoculated into 5 ml of Brain-Heart infusion broth and incubated at 37° C. The bacterial cells were suspended in 0.85% NaCl and pelleted by centrifugation. The plugs were prepared by mixing 200 µl of the cell suspension with 200 µl of 1.6% SeaKem gold agarose. The plugs were lysed by transferring them into a 10 ml lysis solution (6 mM Tris-Hcl, pH-7.4, 100 mM EDTA, 1M NaCl, 0.5% sodium lauroyl sarcosine, 0.5% Brij, 0.2% deoxycholate, lysozyme [500 µg/ml] and RNaseA [20 µg/ml] for 4 h at 37° C with gentle shaking. The plugs were then transferred to ESP buffer (10 mM Tris-Hcl, pH-7.4, 1 mM EDTA, 1% SDS and 50 µg/ml Proteinase K) and incubated overnight at 50° C with gentle shaking. The plugs were washed three times for 10 min each time with TE dilute (10 mM Tris-Hcl, pH-7.4, 0.1 mM EDTA) and stored at 4° C till to be used.

Restriction digestion of the plugs was performed by placing a small slice of agarose plug in 1.5 ml micro centrifuge tube with 2 µl of *Sma*I in 10 µl of 10X buffer and volume made to 100 µl with double distilled water for 4 h at 25° C. The digested plugs were transferred on to a gel (1% SeaKem Gold Agarose with 0.5X TBE) and the gel was placed in a electric field device,

CHEF-DR II, clamped homogenous electric field (BioRad®, Richmond, CA) and the pulse time for block 1 was, 200v, initial time 3.5 s, final time 25 s, 12 h; and for block 2, initial time 1 sec, final time 5s, 8h. Then the gel was stained with 0.0001% (or 1 µg/ml) ethidium bromide for 30 min followed by destaining in distilled water for 20 min each for 3 times. Gel images were captured using a Gel Doc 2000 system (BioRad®), and band patterns were analyzed and compared by using BioNumerics software (Applied Maths, Austin, TX). For clustering with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% for band comparison, we used the band-based Dice similarity coefficient and the unweighted pair group mathematical average algorithm method (UPGMA). Isolates were grouped based on identical banding patterns (100% Dice similarity).

Inter-species transferability of *tcrB* gene

A conjugation assay was performed using the filter mating procedure (Tendolkar et al., 2006) to demonstrate the transferability of *tcrB* gene from *tcrB*-positive *E. faecium* isolates to *tcrB*-negative *E. faecalis* isolates and vice-versa. The interspecies conjugation was done with five isolates each of *tcrB*-positive *E. faecium* and *E. faecalis*. The *tcrB*-positive donor strains (5 *E. faecium* and 5 *E. faecalis*) were resistant to tetracycline (MIC > 100 µg/ml) and streptomycin (MIC >100 µg/ml) and susceptible to spectinomycin (MIC = 12.5 µg/ml). The *E. faecium* (TX 5034; provided by Dr. Barbara E. Murray, University of Texas Medical School) resistant to spectinomycin (MIC > 100 µg/ml), *tet*(*M*) negative, and susceptible to tetracycline (MIC = 0.78 µg/ml), and *E. faecalis* (OG1SSp provided by Dr. Ludek Zurek. Kansas State University) resistant to streptomycin (MIC > 100 µg/ml) and spectinomycin (MIC > 100 µg/ml), *tet*(M) negative and sensitive to tetracycline (MIC = 0.39 µg/ml) were used as recipient strains. The donors and recipients were grown on BHI agar plates containing tetracycline (40 µg/ml) and

spectinomycin (500 µg/ml), respectively. The resultant transconjugants were selected on BHI agar plates containing both tetracycline and spectinomycin. The transconjugants were tested for *tcrB* 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.

Statistical analysis

The *tcrB* gene prevalence in treatment groups was analyzed using a generalized mixed model (PROC GLIMMIX, SAS, Version 9.2). The statistical model included the fixed effect of dietary treatment (control or copper) and pen as the random effect. The analysis was performed to determine the effects of sampling days and copper supplementation. Copper and vancomycin MIC values were analyzed for evidence of normal distribution (PROC UNIVARIATE) within and between *tcrB*-positive and -negative isolates. The MIC values were transformed based on rank (PROC RANK) and analysis of variance (PROC GLIMMIX) was performed on the ranked values since the MIC values were not normally distributed ($P < 0.05$). Data were not analyzed for erythromycin because all *tcrB*-positive and negative isolates were resistant.

RESULTS

Prevalence of the *tcrB, erm*(B), and *vanA* genes

A total of 360 enterococcal isolates consisting of 45 isolates (3 isolates per fecal sample, 3 piglets sampled per pen and 5 pens per treatment) per treatment group (control or copper group) and sampling day (days 0, 14, 28, and 42) were obtained. All 360 enterococcal isolates were screened for the *tcrB* gene and 43 (11.9 %) isolates were positive (amplicon size of 663 bp). Out of 43 *tcrB*-positive isolates, five (5/180; 2.8%) were from piglets fed basal diet with normal amount of copper (16.5 ppm) and 38 isolates (38/180; 21.1%) were from piglets supplemented with elevated amount of copper (Table 3.2). The overall prevalence of *tcrB*-positive enterococcal isolates was higher ($P < 0.05$) in the copper supplemented group than the control group. The prevalence of *tcrB* was affected by sampling days ($P < 0.05$) and also had a significant treatment and sampling time interaction ($P < 0.05$). All *tcrB*-positive and *tcrB*-negative isolates were positive for *erm*(B) gene and negative for *vanA* gene.

The 43 *tcrB*-positive and 44 *tcrB*-negative isolates randomly chosen from the control group were used for the species identification. Based on multiplex PCR and *sodA* gene sequence analyses, 35 *tcrB*-positive isolates were *E. faecium* and 8 isolates were *E. faecalis*. Among 44 *tcrB*-negative isolates, 25 isolates were *E. faecium* and 19 isolates were *E. faecalis*.

Minimum inhibitory concentrations of copper, erythromycin, and vancomycin

All *tcrB*-positive isolates (35 *E. faecium* and 8 *E. faecalis*) grew on MH agar containing copper at 16 or 20 mM. However, the *tcrB*-negative isolates were able to grow only at 2 to 8 mM of copper concentration. The mean MIC of copper for *tcrB*-negative and *tcrB*-positive isolates were 6.2 and 22.2 mM, respectively, and the difference was significant at $P < 0.001$ (Fig. 3.1). The two reference strains obtained from Denmark [7430162-6 and 7430275-4] had an MIC of 23 and 24 mM, respectively.

All *tcrB*-positive and *tcrB*-negative isolates were resistant to erythromycin and tetracycline (MIC >100 μg/ml) and susceptible to vancomycin. The MIC values for vancomycin were higher for the *tcrB*-positive isolates (0.42 μg/ml) than the *tcrB*-negative isolates (0.22 μg/ml) and the difference was significant at $P < 0.001$ (Table 3.3).

PFGE for *tcrB*-positive enterococcal isolates

The clonal relationship among the 43 *tcrB*-positive isolates was determined (Fig. 3.2 & 3.3) by PFGE typing. Based on species specific distribution of PFGE patterns within and between copper and control group, a total of 17 PFGE types were obtained (< 95 % dice similarity) among the 35 *tcrB*-positive *E. faecium* isolates and the 8 *tcrB*-positive *E. faecalis* isolates had 4 PFGE types. Among the five *tcrB*-positive isolates from the control group, three *E. faecalis* isolates belonged to two different PFGE types and the two *E. faecium* isolates had different banding patterns. Among the 38 *tcrB*-positive isolates obtained from copper group, 33 *E. faecium* isolates belonged to 15 different PFGE types and five *E. faecalis* isolates shared two PFGE patterns. Six *E. faecium* isolates (type 13) with identical banding pattern (100% Dice similarity) were obtained from four pens in the copper group on sampling day 14. Similarly,

four *E. faecium* isolates (type 4) with identical banding pattern (100% Dice similarity) were obtained from two pens in the copper group on sampling day 14. On day 28, three *E. faecalis* isolates obtained from the same pen had identical banding pattern. Among the two *tcrB*-positive *E. faecium* isolates in the control group, one isolate had identical PFGE pattern with that of two *tcrB*-positive isolates (obtained from the same pen) in the copper group. All *tcrB*-positive isolates obtained on three different sampling days (14, 28, and 42) belonged to different PFGE types except for two *E. faecium* isolates obtained from the same pen on day 28 matched with another isolate (from a different pen) obtained on day 42. Few of the *tcrB*-positive isolates obtained within each pen over three different sampling days (14, 28, and 42) belonged to different PFGE types. In one pen of the copper group that had five *tcrB*-positive *E. faecium* isolates (on sampling day 42) four had identical PFGE banding patterns. Among the isolates obtained from day 14, one PFGE type was distributed among three different pens, which was same with other two different PFGE types shared between pens. On day 28, we observed only one PFGE that was common between two pens. In one pen (pen 32) that had 12 *tcrB* positive isolates obtained on three different sampling days, there were six different PFGE types. In pens 7 and 13 that had seven *tcrB*-positive isolates each, there were five and six different PFGE types, respectively. The pen 21 with nine *tcrB*-positive isolates had five different PFGE types. The pen 10 with two *tcrB*-positive isolates had two PFGE types.

Interspecies transferability of *tcrB* gene

Five each of *tcrB*-positive *E. faecium* and *E. faecalis* isolates were used to demonstrate the interspecies transferability of *tcrB* gene by conjugation. The ten resultant transconjugants were positive for *tcrB, erm*(B), and *tet*(M) genes. As expected, the transconjugants were able to grow on BHI agar containing high copper concentrations (16 or 20 mM). The mean copper MIC of

the ten transconjugants was 18.4 mM. The mean transfer frequency for *tcrB*-positive *E. faecium* and *E. faecalis* isolates were 9.3×10^{-6} and 8.2×10^{-6}, respectively (Table 3.4).

DISCUSSION

The present study examined the effect of feeding elevated amount of copper on the prevalence of *tcrB*-mediated, copper-resistant enterococcal isolates in the feces of piglets. Only a small proportion of fecal enterococcal isolates (11.9%; 43/360) obtained from piglets fed diets supplemented with normal or elevated level of copper harbored the *tcrB* gene. This suggests that acquired resistance to copper was not widespread in enterococci. In contrast to our study, Hasman and Aarestrup (2002) have reported a prevalence of 76% (45/59) of copper resistant enterococcal isolates from pigs at the time of slaughter from Denmark. The higher prevalence of *tcrB* gene in the Danish piglets may be related to higher level or longer duration of copper supplementation (Hasman et al., 2006 b). In our study, the *tcrB* gene was detected only in two species, *E. faecium* and *E. faecalis,* and majority of the isolates (35/43; 81%) were *E. faecium*. Our results are in agreement with Denmark studies, where the prevalence of *tcrB* gene was higher among the *E. faecium* isolates from pigs (Hasman, 2005; Hasman and Aarestrup, 2005). In Denmark, the *tcrB* was also detected in *E. gallinarum*, *E. casseliflavus,* and *E. mundtii* (Hasman and Aarestrup, 2002; Hasman *et al*., 2006b). Even among the randomly chosen *tcrB*-negative isolates (44 isolates) that were subjected for species identification, we did not find species other than *E. faecium* and *E. faecalis.* Studies conducted in swine have generally reported *E. cecorum, E. durans, E. faecalis, E. faecium, E. hirae,* and *E. malodoratus* as predominant species (Devriese et al., 1994; Thal et al., 1995). The differences in the species distribution can be attributed to factors, such as age, diet, use of in-feed antibiotics, identification methods employed, and the geographical location (Jackson et al., 2004a). However, for our study samples were collected during the nursery stage, the predominant types of species may be

representative of the overall population of swine at that particular stage of production (Jackson et al., 2004a).

The higher prevalence of *tcrB* in enterococci from piglets fed elevated level of copper compared to the control (21.1% vs. 2.8%) suggests exertion of pressure to select for copper resistance. The presence of *tcrB*-positive isolates in the control group fed normal level of copper in the diet suggests the occurrence of naturally resistant isolates. The occurrence was not because of possible pen to pen transmission of *tcrB*-positive from the copper group because only one isolate (among five) of *E. faecium* was clonally identical to an isolate from the copper group. We obtained three enterococcal isolates per sample (nine per pen), which allowed us to screen 45 isolates per treatment group and sampling day. Although the prevalence of *tcrB*-positive isolates was detected only after initiation of copper supplementation, we did not find a linear increase in the prevalence with continued supplementation of elevated level of copper. In fact, the highest prevalence was on day 14 of copper supplementation. Also, there was no clone (PFGE type) of *tcrB* positive enterococci that became dominant or persistent with continued supplementation of elevated level of copper. Almost all isolates obtained on different sampling days (14 days apart) belonged to different PFGE types. Based on an *in vivo* pig challenge study, Hasman et al. (2006 a) reported that feeding elevated level of copper resulted in the selection of *tcrB*- mediated copper resistant *E. faecium*. The *in vivo* animal challenge study consisted of piglets fed low-copper (6 ppm) or high-copper diet (175 ppm) that were orally inoculated with *tcrB*-positive (copper resistant) and *tcrB*-negative (copper sensitive) *E. faecium* strains.

The presence of *tcrB* gene was associated with an almost four-fold increase in resistance to copper (6 mM vs. 24 mM) compared to *tcrB*-negative isolates. Hasman and Aarestrup (2002) have reported a seven-fold increase in resistance to copper in enterococcal isolates harboring

tcrB gene. Copper is an essential micronutrient for both prokaryotic and eukaryotic cells for vital biological functions, because of its involvement as a cofactor in a range of enzymes (Magnani and Solioz, 2005). In piglets, copper is added to diets at concentrations above those physiologically required by the animal because of growth promoting effects (NRC, 1998; Hasman et al., 2006 b). The exact mechanism of action of copper as growth promoter has not been elucidated, but suggested mechanisms include altered gut microbial populations or increased availability of nutrients and energy because of the reduced microbial activity in the gut, attributed to the antimicrobial effects of copper (Hϕjberg et al., 2005; Gould et al., 2009). In European countries, copper is supplemented in swine diets at elevated levels, as a replacement to in-feed antibiotics, which have been banned for use as growth promotants (Aarestrup et al., 2001; Hasman et al., 2006b).

Copper shuttles between Cu^{++} and Cu^{+} at biologically relevant oxidation potential and interacts with molecular oxygen, which produces toxic oxygen radicals (Solioz et al., 2010). Because of its toxicity, cells have evolved homeostatic mechanisms to regulate normal intracellular concentration of copper. In bacteria, these homeostatic mechanisms are very well documented and are mediated by a group of membrane spanning proteins, called CPx-type ATPases, encoded by genes located on an operon called *copYZAB*, which in concert work as efflux systems (Solioz and Stoyanov, 2003). Generally gram negative bacteria, exposed to elevated levels of copper, possess both plasmid and chromosome mediated resistant mechanisms (Brown et al., 1995; Silver, 1996; Hasman et al., 2006 b). In gram positive bacteria, plasmid encoded copper resistance has been shown in *Lactococcus lactis* and *Enterococcus* genus (Solioz et al., 2010). In enterococci, a plasmid borne transferable copper resistance gene (*tcrB*) that encodes for CPx-type ATPase and confers resistance to copper was first documented in swine

isolates in Denmark (Hasman and Aarestrup, 2002). The *tcrB* gene was identified as part of an operon, *tcrYAZB*, which is organized similar to *copYZAB* (Hasman, 2005). The *tcrB* gene was detected in *E. faecium* and *E. faecalis* isolates of piglets, poultry, calves, and humans (Hasman and Aarestrup, 2002). The strains harboring *tcrB* gene had a copper MIC of 24 mM compared to 2 to 8 mM for strains which were negative for *tcrB* gene (Hasman and Aarestrup, 2002).

In our study, the PFGE examination of *tcrB*-positive isolates revealed that there was no dominant or persistent PFGE type in the copper supplemented group.. Among the two *tcrB*-positive *E. faecium* isolates in the control group, one isolate had identical PFGE pattern with that of two *tcrB*-positive isolates from the same pen in the copper group. This suggests transmission of *tcrB*-positive isolate across pens; although potential horizontal transfer cannot be excluded.

An interesting aspect of copper resistance in enterococci of piglets is the potential genetic linkage of *tcrB* gene to macrolide and glycopeptide resistance (Hasman and Aarestrup, 2002). Hasman et al. (2006 b) have shown that elevated levels of copper fed to piglets co selected for erythromycin and vancomycin resistance in enterococcal isolates obtained in Denmark and Spain. A plasmid of 175 kb containing *tcrB* gene along with *erm*(B) and *vanA* genes has been identified from an *E. faecium* isolate of piglets (Hasman et al., 2002). In our study, both *tcrB*-positive and *tcrB*-negative isolates contained *erm*(B) gene and were phenotypically resistant to erythromycin (MIC = > 100 μg/ml). . However, our *tcrB*-positive isolates did not contain the *vanA* gene and were susceptible to vancomycin (MIC = 0.42 μg/ml). It was not surprising to observe the presence of *erm*(B) and absence of *vanA* genes in our enterococcal isolates from piglets. The *erm*(B) gene is the most common determinant associated with enterococci resistance to macrolides (Jackson et al., 2004 b) and tylosin, an erythromycin derivative, is used in the feed of piglets to treat enteric infections and also for growth promotion (Jackson et al.,

2004b). The presence of *vanA* in enterococcal isolates of piglets and chickens in European countries was possibly because of the use of avoparcin, a glycopeptide, for growth promotion. However, the use of avoaprcin in Europe was banned in 1997. The absence of glycopeptide resistance in enterococcal isolates in our study is in agreement with Poole et al. (2005) and is possibly because avoparcin or other related glycopeptide derivates have never been used in the swine industry in the United States (McDonald et al., 1997).

The copper susceptibility determinations of *tcrB*-positive isolates showed that all strains were able to grow in media with copper concentrations of 16 to 24 mM compared to the *tcrB*-negative isolates which tolerated only 4 to 8 mM. This relationship between *tcrB* and copper resistance is in agreement with studies from Denmark. Antibiotic resistant enterococci are opportunistic and nosocomial pathogens in humans (Murray, 2000). Because of the widespread occurrence of resistant enterococci in animals, it has been suggested that the enterococci may serve as reservoir of potential resistant genes capable of transferring to humans (Kojima et al., 2010). The importance of enterococci is related to their involvement in the conjugal transfer of resistance genes, mostly associated with the plasmids or transposons. In our previous study (Amachawadi et al., 2010), we demonstrated the transferability of *tcrB* gene between strains within the same species of enterococci. In the present study, we demonstrated the interspecies transferability of *tcrB* gene between *E. faecium* and *E. faecalis*. The transconjugants possessing *erm*(B), *tet*(M), and *tcrB* genes were suggestive of their presence on a conjugative plasmid, but this remains to be confirmed. The resistance determinants, *tet*(M), and *erm*(B), are frequently carried on conjugative transposon, Tn*916* and Tn*916*/Tn*1545* (Clewell, 1995). These conjugative transposons have broad host range and are capable of being transfered by horizontal gene transfer to a variety of gram positive and gram negative bacteria in the gut bacterial community

(Bertram et al., 1991). A critical issue is the co-selection of metal and antibiotic resistance on the same genetic determinant, often on a plasmid or a transposon (Nandi et al., 2004). These plasmids or transposons are of concern because they harbor antibiotic resistance genes and have the potential to spread between species (Nandi et al., 2004). The reports on the relationship between the copper and antibiotic resistance are limited, and a definitive link (Hasman et al., 2006 b) is lacking. The existence of a metal associated co selective mechanism could be a major issue relative to public health and need further studies (Hasman et al., 2006 b). In conclusion, our study showed a relationship between the elevated copper supplementation and prevalence of copper resistant enterococci in piglets.

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Table 3.1: Primers used in the present study

Gene	Sequence (5′-3′)	Product size (bp)	Reference
tcrB	F:CATCACGGTAGCTTTAAGGAGATTTTC R: ATAGAGGACTCCGCCACCATTG	663	Hasman et al., 2006
erm(B)	F: GAATCCTTCTTCAACAATCA R: ACTGAACATTCGTGTCACTT	175	Jacob et al., 2008
vanA	F: CATGAATAGAATAAAAGTTGCAATA R: CCCCTTTAACGCTAATACGATCAA	1,030	Kariyama et al., 2000
Enterococcus faecium	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	Dutka-Malen et al., 1995
Enterococcus faecalis	F: CAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	Dutka-Malen et al., 1995
sodA	F: CCITAYICITAYGAYGCIYTIGARCC R: ARRTARTAIGCRTGYTCCCAIACR	438	Poyart et al., 2000

Table 3.2: Occurrence of *tcrB* gene positive fecal enterococci in piglets fed diets supplemented with or without copper.

	No. of isolates positive for *tcrB* gene/total isolates tested				
	Sampling days				Total (%)
Treatment	0	14	28	42	
Control	0/45	3/45	1/45	1/45	5/180 (2.8)
Copper	0/45	19/45	8/45	11/45	38/18(21.1)

Table 3.3: Susceptibility of *tcrB*-positive or negative enterococcal isolates to antibiotics

Antibiotics	*tcrB* negative isolates (n =44)	*tcrB* positive isolates (n=43)
Erythromycin, µg/ml	> 100	> 100
Tetracycline, µg/ml	> 100	> 100
Vancomycin, µg/ml	0.2	0.4*

* $P < 0.001$

Table 3.4: Transfer frequency of *tcrB* gene in *Enterococcus faecium* and *Enterococcus faecalis*

Donor	Recipient	Transfer frequency (no. of transconjugants per recipient)
E. faecium KSU 143	*E. faecalis* OG1SSp	1.18×10^{-5}
E. faecium KSU 150	*E. faecalis* OG1SSp	7.88×10^{-6}
E. faecium KSU 153	*E. faecalis* OG1SSp	6.97×10^{-6}
E. faecium KSU 160	*E. faecalis* OG1SSp	9.48×10^{-6}
E. faecium KSU 167	*E. faecalis* OG1SSp	1.02×10^{-5}
E. faecalis KSU 101	*E. faecium* TX5034	1.05×10^{-5}
E. faecalis KSU 102	*E. faecium* TX5034	8.15×10^{-6}
E. faecalis KSU 201	*E. faecium* TX5034	7.43×10^{-6}
E. faecalis KSU 234	*E. faecium* TX5034	7.67×10^{-6}
E. faecalis KSU 269	*E. faecium* TX5034	6.96×10^{-6}

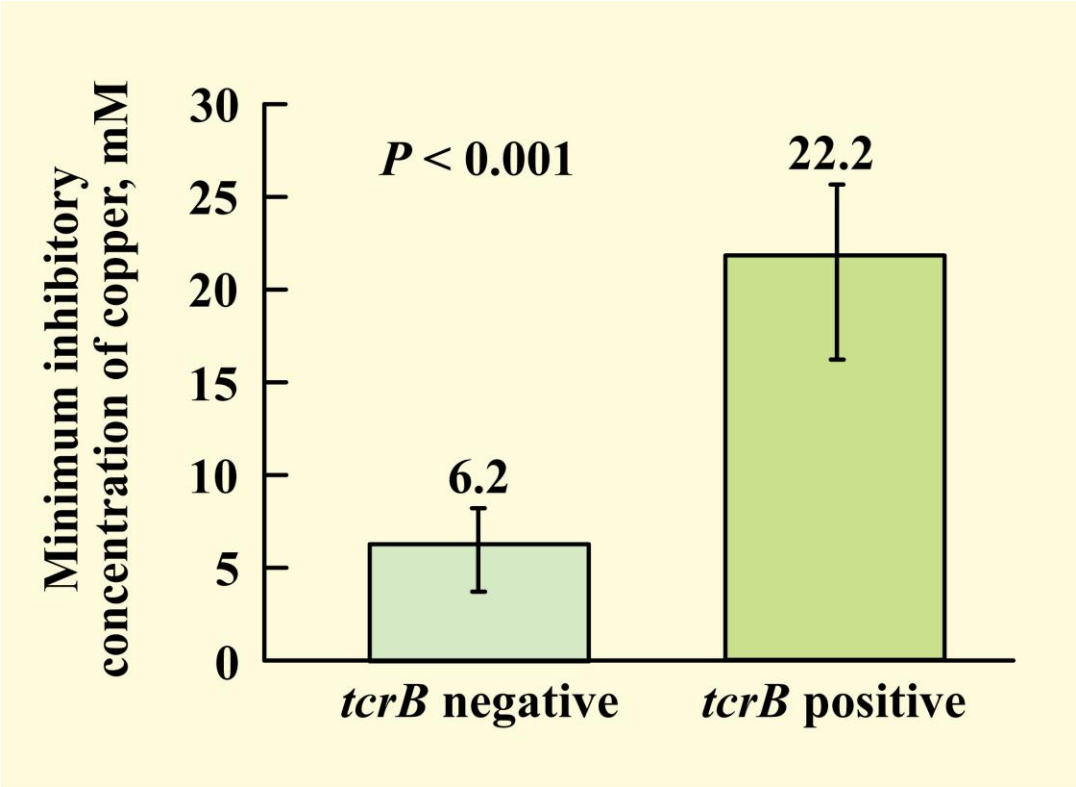

Figure 3.1: Minimum inhibitory concentrations (95% Confidence Interval) of copper for *tcrB*-positive and *tcrB*-negative enterococcal isolates from piglets fed diets supplemented with or without copper.

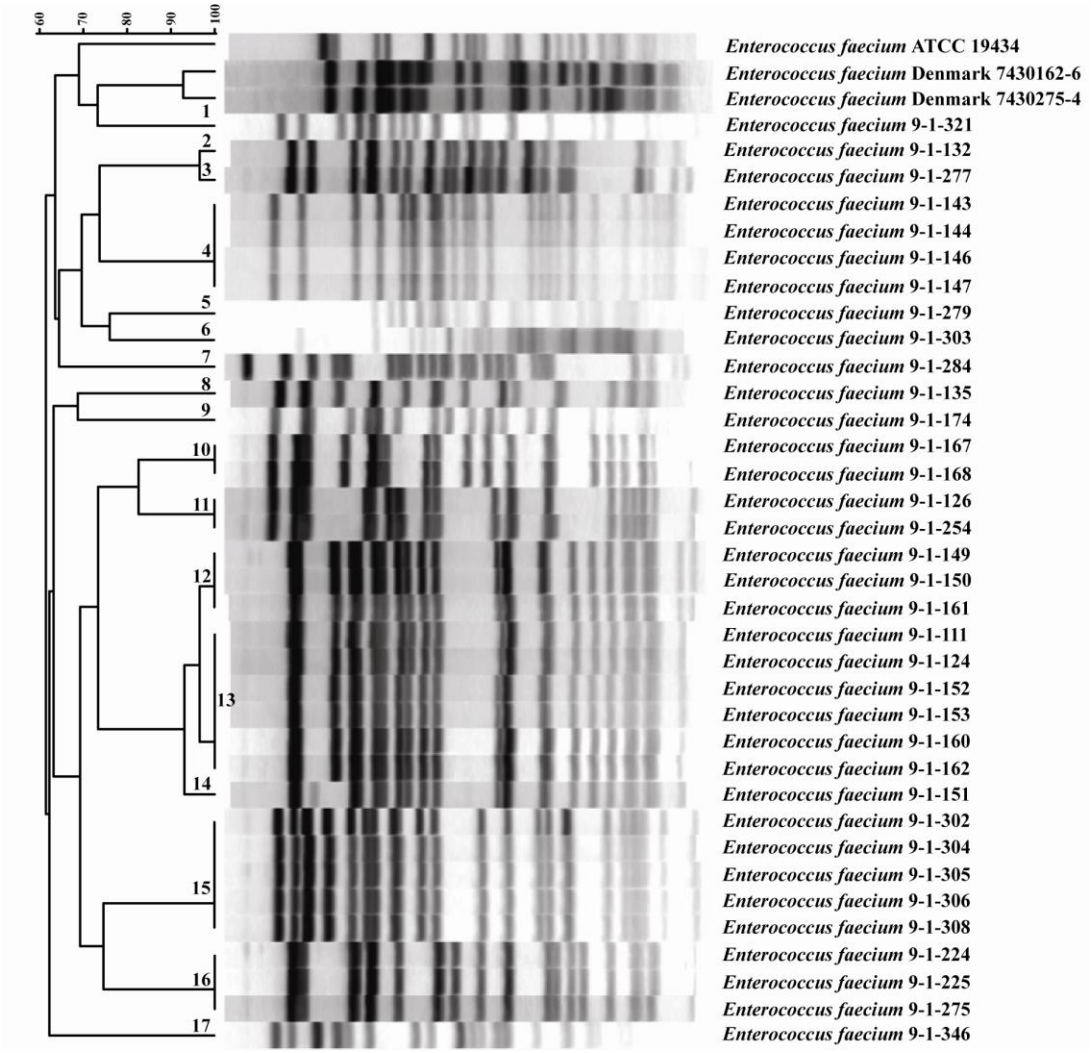

Figure 3.2: Pulsed-field gel electrophoresis patterns of *Sma*I-digested genomic DNA of *tcrB*-positive *Enterococcus faecium* isolates from piglets fed diets supplemented with or without copper.

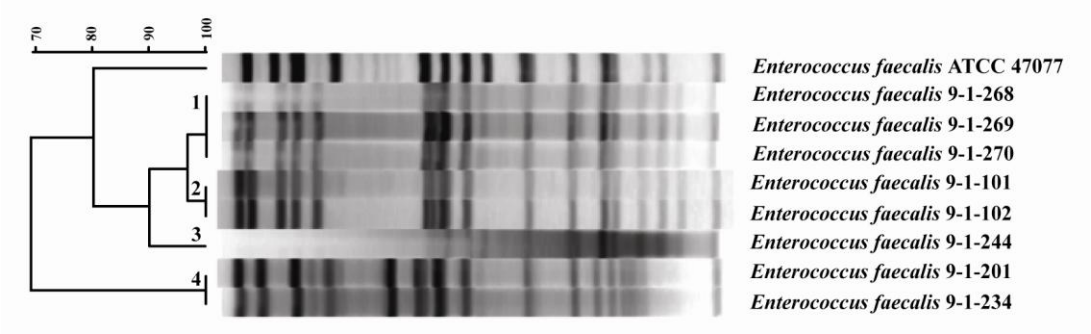

Figure 3.3: Pulsed-field gel electrophoresis patterns of *Sma*I-digested genomic DNA of *tcrB*-positive *Enterococcus faecalis* isolates from piglets fed diets supplemented with or without copper.