Copper supplementation and antimicrobial resistance in swine and *Salmonella enterica* in liver abscesses of cattle

by

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Abstract

Copper is an essential micronutrient that is supplemented in swine diets as a growth promoter. Previous studies suggest a link between copper supplementation and co-selection of antimicrobial resistance (AMR) in *Enterococcus*, but the data are inconsistent. Therefore, the objective of this study was to assess the impact of copper supplementation, alone or with chlortetracycline (CTC), on prevalence and concentration of copper resistance gene, *tcrB*, prevalence of tetracycline [*tet*(M)] and macrolide resistance [*erm*(B)] genes, and AMR in fecal enterococci of weaned piglets. A total of 320 weaned piglets at 21 days of age were allocated into 64 individual pens distributed equally among two barns. Pen-pair was the experimental unit (n=32). Four treatments were used: a basal diet as the control, a basal diet with 200 ppm of copper as copper sulfate, a basal diet with chlortetracycline (CTC) at 400 g/ton of feed, and a basal diet with 200 ppm of copper and CTC at 400 g/ton of feed. The study period was 35 days with days -7 to -1 as an acclimation period and days 0 to 28 as the treatment period. Direct fecal samples were collected on days 0, 14, and 28. Prevalence of *tcrB*-positive enterococci was not affected by copper and or CTC supplementation ($P > 0.05$). Prevalence of *tcrB*-positive enterococci was higher on day 14 than other sampling days ($P = 0.002$). Prevalence of *tet*(M)-positive enterococci was not affected by treatment group or sampling day ($P > 0.05$). Prevalence of *erm*(B)-positive enterococci had a significant treatment and sampling day interaction ($P = 0.0213$). The total copy number of the *tcrB* gene was quantified as a percent of total bacterial cells in the feces. The median copper MIC of *tcrB*-negative and -positive isolates was 3 mM and 20 mM, respectively ($P < 0.0001$). For day 0 (n=64) and day 28 (n=63), all *Enterococcus* isolates were susceptible to gentamicin, kanamycin, streptomycin, daptomyicin, and tigecycline, with majority of isolates resistant to chloramphenicol, erythromycin, lincomycin, linezolid,

tetracycline, tylosin tartrate, and synercid. For day 0 (n=64) and day 28 (n=63), respectively, a total of 61 (95.3%) and 47 (74.6%) isolates were resistant to erythromycin, 51 (79.7%) and 41 (65.1%) to tylosin, and 60 (93.8% and 95.2%) to tetracycline. The results of this study show that supplementing copper, with or without a selection pressure of chlortetracycline, did not increase copper-resistant enterococci and did not co-select resistance to any other antibiotics.

Liver abscesses in feedlot cattle have a significant economic impact because of reduction in cattle performance, and carcass yield and liver condemnation at harvest. *Fusobacterium necrophorum* is the primary causative agent of the liver abscesses. Recently, *Salmonella enterica* has been isolated from liver abscesses of cattle. Our objectives of this study were to determine prevalence of *Salmonella,* compare conventional (serological) and commercially available Check &Trace serotyping methods, and to describe the antimicrobial susceptibility patterns of *Salmonella* isolated from liver abscesses of feedlot cattle. In the 2014 study, the number of liver abscesses positive for *Salmonella* were higher ($P < 0.05$) in cattle fed no tylosin in the diet (66/200; 33%) compared to tylosin-fed cattle (31/183; 16.9%). In the 2015 study, *Salmonella* prevalence tended to be higher in liver abscesses categorized as severe (29/106; 27.4%) compared to mild liver abscesses (38/174; 21.8%), but the difference was not significant. Out of the 164 *Salmonella* isolated, 152 (92.7%) were used for serotyping and 164 strains were used for antimicrobial susceptibility testing. Serotyping was done by serological method, which is considered as the gold standard, and the commercial Check & Trace method, which is a molecular method based on differences in their DNA sequence. A total of 11 serotypes were identified with Lubbock (66/152; 43.4%) being the predominant serotype, followed by Agona (24/152; 15.8%), Anatum (20/152; 13.2%), and Montevideo (18/152; 11.8%). The commercial identified only a few serotypes correctly suggesting that the method requires further validation.

Antimicrobial susceptibility testing was done by microbroth dilution method according to Clinical Laboratory Standards Institute guidelines. A majority of the *Salmonella* strains were pansusceptible to the antimicrobials included in the panel. Overall, 10 strains (10/164; 6.1%) were resistant to one or more antibiotics and belonged to serotypes Agona, Anatum, Cerro, Lubbock, Mbandaka, and Reading. The top three of nine resistant antibiotics were chloramphenicol (5/10; 50%), streptomycin (5/10; 50%), and tetracycline (6/10; 60%). Whether *Salmonella* contributes to liver abscess formation or just happen to survive in an abscess initiated by the primary etiologic agent, *Fusobacterium necrophorum,* remains to be determined.

Table of Contents

List of Figures

List of Tables

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Dedication

To God, Sully, Doc, and my family.

Chapter 1 - Copper Supplementation in Swine and Cattle and its Suspected Link to Antimicrobial Resistance: A Review of Literature

Copper as an Element

Copper, a heavy metal and an essential trace element exists in two forms or oxidation states, Cu^{+1} as cuprous copper and Cu^{+2} as cupric copper. In the presence of oxygen or other electron-accepting particles, the cuprous form is oxidized to cupric copper, which is the most common form in biological systems. The reverse, non-enzymatic reduction reaction, mediated by cupric copper's ability to accept electrons, also occurs. Copper's ability to navigate one-electron reactions is a key reason why copper plays a crucial role in reduction-oxidation reactions of living systems (Galhardi et al., 2004).

Copper serves as a co-factor to several metabolic enzymes, like superoxide dismutase, which aids in the protection against free radicals (Tainer et al., 1983). There are various proteins involved in the absorption, transport, and excretion of copper across cellular membranes. Copper is thought to be absorbed in the stomach and duodenum of mammals, followed by transportation to the liver via albumin, transcuprein, and amino acids (Linder, 1991) with intestinal absorption of dietary copper facilitated by L-amino acids (Sandstead, 1982). Cellular transport of copper is highly conserved with no key differences between animals, bacteria, fungi, and plants (Harris, 2000). In fact, yeast is a primary model for copper metabolism (Freitas et al., 2003). The use of chaperone proteins in intracellular transit keeps copper bound until received by the target protein. In many cases, the trans-Golgi network, which houses two cation-transporting P-type ATPases (Wilson and Menkes ATPases) responsible for regulating copper, is the first stop (Carroll et al., 2004; Arredondo and Nunez, 2005). Chaperones also carry copper to other locations/targets in the cell such as superoxide dismutase in the cytoplasm, or to the mitochondria, where copper is

required to aid cytochrome-c-oxidase in ATP synthesis (Linder and Hazegh-Azam, 1996). It has been shown that cells of the intestines (Caco-2 cells), liver (HepG2 cells), and kidney's (Hek293 cells) regulate the uptake of copper (Arredondo et al., 2000, 2004). If copper exceeds normal levels, homeostatic cellular conditions are achieved by P-type ATPases providing cuproproteins with copper, allowing for the excretion of copper from the trans-Golgi network to either the plasma membrane or an intracellular vesicular compartment (Arredondo and Nunez, 2005). For example, in hepatocytes, the Wilson-ATPase mediates the disposal of excess copper in the bile. Processes like this serve as efficient means to regulate total copper in the body, which is crucial to the livelihood of cells.

Although copper plays an important role in living systems, it can be cytotoxic in both eukaryotic and prokaryotic cells if regulatory controls of the element fail. Copper has two cytotoxic properties. The first property is linked to the element's ability to participate in intracellular redox reactions where cuprous copper reacts with dihydrogen peroxide to form cupric copper and a highly damaging hydroxyl radical. The second is copper's ability to bind to non-specific amino acids, which can interfere with protein synthesis (Koch et al., 1997). For example, in respiration, dioxygen is reduced to superoxide, a harmful anion that causes oxidative stress. This stress is alleviated by actions of the enzyme copper-zinc superoxide dismutase, therefore, it lessens or prevents oxidative stress. Another complication is Menkes syndrome where the intestinal absorption of copper is highly reduced due to an X-chromosome-linked deficiency (Mercer et al., 1993; Koch et al., 1997). This causes inadequate copper levels in the brain and liver resulting in decreased activity of enzymes who require copper. One of many enzymes includes lysyl oxidase which plays a role in initiating covalent cross-linkage of elastin

and collagen and has been suggested as a cause of myocardial ischemia (Balamurugan and Schaffner, 2006; Sibon et al., 2005).

Copper as a Mineral Supplement

Copper is found in a variety of foods and supplements in many forms including sulfate salts and oxides (Johnson et al., 1998). When copper is used as a mineral supplement, careful consideration toward dietary zinc (Solomons, 1983; Johnson and Murphy, 1988), iron (Solomons, 1983; Johnson and Murphy, 1988; Haschke et al., 1986), and manganese (Gubler et al., 1954; Johnson et al., 1992) should be considered as they may alter copper status. For example, it is suggested that zinc could support the synthesis of copper-binding ligands thereby decreasing copper availability for absorption (Fischer et al., 1981). It is also suggested that depletion of dietary copper could affect the release of iron from the duodenal mucosa and hepatic parenchymal cells to plasma because iron release from tissue to plasma requires that ferrous ions be oxidized by the plasma copper protein, ceruloplasmin (Lee et al., 1968). Additionally, dietary supplementation of manganese could result in excess manganese in the liver, which may be the cause of impaired biliary copper excretion and excess copper in the liver (Mercadante et al., 2016). As listed in the Swine Nutrition Guide provided by the Kansas State University Agricultural Experiment Station and Extension Service, dietary supplementation in swine varies based on the objective of a given diet (general, breeding, starter herd, and grower-finisher).

Sulfate forms of these minerals are generally higher in bioavailability than the oxide forms (Baker et al., 1991; Wedekind and Baker, 1990; Lowe et al., 1994), although it has been found that cupric oxide is used extensively in human foods and supplements (Baker et al., 1991) but is seemingly unavailable in other species like poultry (Jackson and Stevenson, 1981) and animals that are commonly used as research models for humans, like swine (Cromwell et al.,

1989). However, manufacturing processes could alter the bioavailability of cupric oxide, creating a more soluble product for absorption and biological use (Cromwell et al., 1989).

There are many food sources that contain copper including organ meats, nuts, seeds, chocolate, and shellfish (Murphy et al., 1975; Pennington and Calloway, 1974). Additional copper sources include potatoes and legumes (Ma et al., 2000) along with vegetables and dairy products (Wapnir, 1998). While foodstuffs are one way to attain copper, common food processing practices can reduce mineral content. Removing the bran and germ from grains reduces dietary copper content by 45%(Clydesdale, 1988). Heat treatment acts as a catalyst in the formation of sugar-amino acid condensation products resulting in a reduction of free amino acids and metal-nitrogen bond formation (Andrieux, 1984), as mentioned by Wapnir, 1998. Copper bioavailability may decrease due to aging (Massie et al., 1990; Harman, 1965; Brewer, 2010), supplementation of chemically-similar minerals, altered fiber content of the diet, and health conditions like Menkes disease which was discovered in 1972 (Danks et al., 1972) and leads to copper deficiency that causes anemia and bone abnormalities (Fosmire, 1990; Uauy et al., 1998) among many other issues that put a subject at high risk for cardiovascular disease (Klevay, 1983; Reiser et al., 1987). Copper bioavailability may increase due to consumption of proteins, soluble carbohydrates, and promotion of low-molecular-weight chelates (Wapnir, 1998).

Copper in Swine and Cattle Diets

Traditionally, copper is fed in animal diets as a requirement for cellular metabolism (Harris, 2000; Okonkwo et al., 1979; Schroeder et al., 1965), and to promote growth (Hill et al., 2000), lactation (Ballantine et al., 2002), reproduction performance (Kellogg et al., 2003), and to reduce morbidity (Jones and Suttle, 1981; Arthur and Boyne, 1985) and mortality (Reiser et al.,

1983). Copper, as salt in animal feed, is largely bioavailable as either copper acetate, carbonate, chloride, or sulfate and is used in both oxidative forms (Wapnir, 1998).

In cattle, cupric copper has a higher bioavailability than cuprous copper (Chapman et al., 1963) and the sulfate salts are generally more bioavailable (Kegley and Spears, 1994) than oxides, although some studies suggest that cupric oxide can be a usable source of copper for cattle (Suttle, 1981, 1994). Copper investigations in cattle include assessing the effects of copper deficiency on iron metabolism where plasma copper concentrations were positively correlated with plasma iron concentrations (Hansen et al., 2010). The comparison of footbaths containing either copper sulfate or copper sulfate with an acidifier for the prevention of digital-dermal lesions in dairy cattle was observed and results showed that copper sulfate, alone and in combination with an acidifier effectively reduced and prevented digital-dermatitis lesion occurrence (Reichenbach et al., 2017). Evaluations on copper status and metabolism across cattle breeds demonstrated that Angus steers had greater copper absorption and retention as compared to Simmental steers and greater copper plasma concentrations than Charolais steers suggesting that Simmental and Charolais steers may have higher copper requirements than Angus cattle (Ward et al., 1995). Overall, the scope of study and use of copper in cattle is very broad.

In contrast to cattle, cupric oxide is completely unavailable to swine (Cromwell et al., 1989) but other forms are bioavailable. In swine, dietary copper has been used to better understand the relationship of calcium and iron to copper where copper had a significant effect on growth at higher calcium levels and reduced the incidence and severity of parakeratosis in weaned pigs fed varying levels of calcium at varying combinations of zinc and iron supplementation (Hoefer et al., 1960). Additionally, performance criteria, elemental liver store rate, and efficiency of gain in finishing pigs were evaluated and were improved by the addition

of 250 parts per million of copper to high calcium feed (Prince et al., 1984). Furthermore, copper resistant enterococci have been monitored in pigs fed diets supplemented with copper where a higher prevalence of copper resistant Enterococcus were found in diets supplemented with copper as compared to a control diet (Amachawadi et al., 2011). Overall, the scope of study and use of copper in swine is very broad, as it is in cattle.

Copper as a Growth Promoter

Copper is most commonly used as a dietary supplement to meet nutritional requirements and to promote growth in livestock. Generally, copper is supplemented in the diet, but other studies have demonstrated copper's growth promotional effects by intravenous injection in swine (Zhou et al., 1994). For this review, I will focus on the studies that have demonstrated growth promotional effects via dietary supplementation of copper either alone or in combination with other products.

Many studies have focused on the comparison of copper based products alone, compared to other supplements in livestock diets, particularly swine and cattle (Armstrong et al., 2004; Perez et al., 2011; Dias et al., 2013; Feldpausch et al., 2016; Lu and Lindemann, 2017). One swine study demonstrated that basal diets supplemented at either 100 or 200 ppm of copper from copper chloride or copper sulfate resulted in improved average daily gain (ADG) and improved feed to gain ratios for diets with 200ppm of copper from copper sulfate and both 100 and 200 ppm copper from copper chloride diets, respectively (Cromwell et al., 1998). Another study demonstrated differences in dietary copper sulfate and copper proteinate when fed to copper depleted steers as average daily gain was greater in steers fed copper proteinate as compared to copper sulfate (Wittenberg et al., 1990).

Other studies have focused on enhancing absorption and bioavailability of copper by combining the mineral with other products like amino acids (Coffey et al., 1994; Apgar et al., 1995; Apgar and Kornegay, 1996; Hatfield et al., 2001; Carpenter et al., 2017) particularly, lysine. One study evaluated two copper-lysine complexes; One had 50% copper in complex form, and the second had 100% copper in complex form. The study determined that adding copper as copper sulfate, copper-lysine 50%-complex, or copper-lysine 100%-complex all improved pig performance with no difference between 100 or 200 ppm doses of copper on ADG, daily feed, or feed to gain ratio. However, growth rate and feed intake were greater for the copper-lysine complexes compared to copper sulfate alone (Coffey et al., 1994).

Lastly, some studies have focused on the combination of copper with antibiotics, particularly in swine (Stahly et al., 1980; Ribeiro et al., 1981; Edmonds et al., 1985; Amachawadi et al., 2015). Although antibiotic based products can no longer be used for growth promotion in livestock in the United States (FDA, 2017), it has been shown that copper supplementation in combination with sub-therapeutic antibiotics can result in performance gains larger than that of supplementing copper alone. For example, one study demonstrated improved average daily feed intake and gain when pigs were supplemented with 200 ppm copper sulfate in combination with carbadox as compared to diets supplemented with either copper sulfate or copper chloride, alone (Cromwell et al., 1998).

Copper and Copper Resistance Mechanisms

In agriculture, copper's toxic effect on microorganisms has been taken advantage of and has been used to control disease. Just as bacteria develop resistance to antibiotics, they will and have develop resistance to threatening environmental pressures like copper. The ever-changing microbial world has led scientists to investigate microbial resistance to copper ions.

Polysaccharide capsules isolated from *Klebsiella aerogenes* efficiently bound copper and were more tolerable to the substance than non-capsulated strains (Bitton and Freihofer 1977). Efflux resistance mechanisms were suggested when swine origin *Escherichia coli* with plasmid associated copper resistance accumulated less copper than cells lacking the plasmid (Tetaz and Luke 1983; Rouch et al., 1985). *Mycobacterium scrofulaceum* with plasmid associated copper resistance illustrated intracellular precipitates after consuming copper (Erardi et al., 1987). *Pseudomonas syringae* avoided the otherwise toxic effect of excess cellular copper by using Cop-protein mediated copper binding outside of the cytoplasm (Cooksey, 1990; Cha and Cooksey 1991). Others have studied copper resistance by determining the prevalence of *tcrB*, homologous to *copB* in *Enterococcus* of swine fed diets supplemented with varying levels of copper (Amachawadi et al., 2010, 2011, 2015).

There are many ways that both Gram-negative and -positive bacteria utilize copper and combat the cytotoxic effects of the element. Copper utilization is less understood than cellular expulsion methods, with active efflux of copper from the cell being the most commonly studied.

For Gram-negative's, like *Escherichia coli*, transportation of copper from the cytoplasm to the periplasm is mediated through the copper-transporting ATPase that resides in the cytoplasmic membrane (Rensing and Grass, 2003). Once the copper particle is in the periplasm, the chromosomally encoded or similar plasmid encoded copper efflux system, CusCFBA, alongside the copper reduction-oxidation control, CueO, work together to expel the toxic element (Rouch et al., 1985). Other cellular resources like copper-binding proteins aid in the defense against copper (Kim et al., 2010). While copper utilization is not well understood, some believe metalation of cuproenzymes takes place in the periplasmic space without the need for copper uptake systems for transport about the cytoplasmic membrane (Grass et al., 2011).

For Gram-positive's, like *Enterococcus,* the periplasmic space and outer membrane are absent, and extrusion systems differ than those of Gram-negative bacteria. A single circuit regulates only CopA-type exporter expression for the expulsion of copper from the cell (Solioz and Stoyanov, 2003; Solioz et al., 2010). Other cellular resources like copper-reductases aid in the defense against copper (Magnani and Solioz, 2007). Further research on copper-dependent novel proteins discovered in Gram-positive cocci (Magnani et al., 2008) may unveil methods of copper utilization. However, overall regulation of copper in Gram-positive bacteria, like Enterococcus, is orchestrated by the copYZAB operon with copA, B, Y, and Z encoding for an influx ATPase, export ATPase, copper-responsive repressor, and copper chaperone, respectively (Solioz and Stoyanov, 2003).

Copper as a Suspected Link to Antimicrobial Resistance

Acquired copper resistance is attributed to a plasmid-borne transferrable copper resistance gene, *tcrB*, homologous to *copB*, which has been reported in fecal enterococci of swine and cattle (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2010, 2011). The *tcrB*-carrying plasmid has also been shown to carry macrolide [*erm*(B)] and glycopeptide (*van*A) resistance genes in fecal enterococci of swine and cattle in Europe (Hasman and Aarestrup, 2002; Hasman et al., 2006), and *erm*(B) and tetracycline [*tet*(M)] resistance genes in fecal enterococci of swine in the United States (Amachawadi et al., 2010, 2011, 2015).

A dose related *tcrB* prevalence in enterococci to copper supplementation in weaned piglets has been reported in the United States (Amachawadi et al., 2011). *TcrB*-positive enterococci have been observed in Denmark (Hasman and Aarestrup 2002; Hasman et al. 2006). In the Danish studies, high prevalence of *tcrB* in *Enterococcus* isolates from swine collected at slaughter are thought to be associated with the age of the animal (development of resistant

microflora) or the duration and concentration of copper exposure (birth to slaughter and 175 to 200ppm). *TcrB*-positive *Enterococcus* have been associated with four- and seven-fold increase in resistance to copper in both the United States and Denmark (Amachawadi et al., 2010; Hasman and Aarestrup, 2002). It is known that feeding dietary copper to piglets can co-select for erythromycin and vancomycin resistance in Denmark and Spain (Hasman et al., 2006), and both macrolide and tetracycline resistance have been found in *tcrB*-positive isolates in the United States (Amachawadi et al., 2010; 2011).

The genetic link, shown by presence of *tcrB* and AMR genes on the same plasmid (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2010, 2011, 2015) suggests potential association of copper supplementation with antibiotic resistance. A public health concern exists and is heightened because of the propensity of nosocomial pathogens like Gram-positive *Enterococcus* to become multidrug resistant. Additionally, *Enterococcus* can horizontally transfer resistance genes to other bacteria in the gut, including *Salmonella* and *Campylobacter* spp. (Winokur et al., 2001; Carattoli, 2009) all of which contribute to major illness in both humans and animals.

More research is needed to determine the propensity of copper resistance to co-select for antimicrobial resistance. Therefore, the objective of this study was to evaluate the impact of copper, alone or in combination with chlortetracycline on AMR of fecal enterococci of weaned piglets.

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Chapter 2 - Impact of copper supplementation, alone or in combination with chlortetracycline, on antimicrobial resistant of fecal enterococci of weaned piglets

Abstract

Copper is an essential micronutrient that is supplemented in swine diets as a growth promoter. Previous studies suggest a link between copper supplementation and co-selection of antimicrobial resistance (AMR) in *Enterococcus*, but the data is inconsistent. Therefore, the objective of this study was to assess the impact of copper supplementation, alone or with chlortetracycline (CTC), on prevalence and concentration of copper resistance gene, *tcrB*, prevalence of tetracycline [*tet*(M)] and macrolide resistance [*erm*(B)] genes, and AMR in fecal enterococci of weaned piglets. A total of 320 weaned piglets at 21 days of age were allocated into 64 individual pens distributed equally among two barns. Pen-pair was the experimental unit (n=32). Four treatments were used: a basal diet as the control, a basal diet with 200 ppm of copper as copper sulfate, a basal diet with chlortetracycline (CTC) at 400 g/ton of feed, and a basal diet with 200 ppm of copper and CTC at 400 g/ton of feed. The study period was 35 days with days -7 to -1 as an acclimation period and days 0 to 28 as the treatment period. Direct fecal samples were collected on days 0, 14, and 28. Prevalence of *tcrB*-positive enterococci was not affected by copper and or CTC supplementation ($P > 0.05$). Prevalence of *tcrB*-positive enterococci was higher on day 14 than other sampling days ($P = 0.002$). Prevalence of *tet*(M)-positive enterococci was not affected by treatment group or sampling day ($P > 0.05$). Prevalence of *erm*(B)-positive enterococci had a significant treatment and sampling day interaction ($P = 0.0213$). The total copy number of the *tcrB* gene was quantified as a percent of total bacterial

cells in the feces. The median copper MIC of *tcrB*-negative and -positive isolates was 3 mM and 20 mM, respectively ($P < 0.0001$). For day 0 (n=64) and day 28 (n=63), all *Enterococcus* isolates were susceptible to gentamicin, kanamycin, streptomycin, daptomyicin, and tigecycline, with majority of isolates resistant to chloramphenicol, erythromycin, lincomycin, linezolid, tetracycline, tylosin tartrate, and synercid. For day 0 (n=64) and day 28 (n=63), respectively, a total of 61 (95.3%) and 47 (74.6%) isolates were resistant to erythromycin, 51 (79.7%) and 41 (65.1%) to tylosin, and 60 (93.8% and 95.2%) to tetracycline. The results of this study show that supplementing copper, with or without a selection pressure of chlortetracycline, did not increase copper-resistant enterococci and did not co-select resistance to any other antibiotics.

Key words: antimicrobial resistance, copper supplementation, *Enterococcus*, swine

Introduction

Copper is an essential chemical element required for basic cellular metabolism in eukaryotic and prokaryotic cells. In swine, copper is included, generally as copper sulfate, in trace amounts in the diets (5-20 ppm) as part of a multi-mineral supplement to meet nutritional requirements. At elevated concentrations in the diet, generally at 100 to 250 ppm, copper has growth promotional benefits, which are attributed to alteration of gut microflora, retention of nutrients, reduction in gut fermentation losses, and reduced morbidity and mortality, particularly in piglets (Hojberg et al., 2005; NCR, 2012). High concentrations of copper have antimicrobial activities because of production of intracellular superoxides, which inhibit basic cellular processes that utilize lipids, nucleic acids, and proteins (Macomber and Imlay, 2009). Acquired copper

resistance attributed to a plasmid-borne transferrable copper resistance gene, *tcrB*, homologous to *copB,* has been reported in fecal enterococci of swine and cattle (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2010, 2011). The *tcrB*-carrying plasmid has also been shown to carry *erm*(B) and glycopeptide (*vanA*) resistance genes in fecal enterococci of swine and cattle in Europe (Hasman and Aarestrup, 2002; Hasman et al., 2006), and *erm*(B) and *tet*(M) resistance genes in fecal enterococci of swine in the US (Amachawadi et al., 2010, 2011, 2015). This genetic link between copper and antimicrobial resistance (AMR) genes suggests potential association of copper supplementation with AMR. The concern is heightened because of the propensity of *Enterococcus* to become multidrug resistant and its ability to horizontally transfer resistance genes to other bacteria in the gut, including *Salmonella* and *Campylobacter* spp., (Winokur et al., 2001; Carattoli, 2009).

In previous studies, data on the effects of copper supplementation on acquisition of resistance to copper and co-selection of copper and AMR have been inconsistent. Although a dose related *tcrB* prevalence in enterococci to copper supplementation in weaned piglets has been reported (Amachawadi et al., 2011), in other studies (Amachawadi et al., 2010; 2015), copper supplementation at elevated concentration did not always result in increased prevalence of *tcrB*-positive in fecal enterococci. Because of this somewhat inconsistent and even conflicting data, there is a need to reevaluate the impact of copper on selection of copper resistance and co-selection of AMR in gut bacteria of swine. Therefore, the objective of this study was to evaluate the impact of copper, alone or in combination with an antibiotic selection pressure, chlortetracycline (CTC), on *tcrB* prevalence and antimicrobial resistance of fecal enterococci of weaned piglets. To assess the impact of copper and CTC on *tcrB*, a real-time, quantitative PCR

(qPCR) assay was developed, validated, and utilized to quantify the abundance of *tcrB* in fecal community DNA.

Materials and Methods

Animals, experimental design, and sampling schedule

The Kansas State University Animal Care and Use Committee approved the use of animals and the experimental procedures for this research. The study was conducted at Kansas State University's Segregated Early Weaning Swine facility. A total of 320 weaned, DNA Genetics 200 X 400 barrow piglets were used. Piglets at 21 days of age with an average initial BW of 7.4 kg were allocated into 64 pens (5 piglets per pen) distributed in two barns. Specifically, each barn housed 32 pens that were oriented in four rows with 8 pens in each row. Per barn, row one adjoined row two and row three adjoined row four creating two 16-pen blocks per barn which were separated by a mid-way. Additionally, there were walk-ways flanking the outside of each 16-pen block in each barn. Per 16-pen block, there were 8 pen-pairs that allowed piglets to make contact through vertical bars between rows, and restricted within-row piglet contact using wood boards. The availability of contact between rows (same treatment) resulted in the experimental unit being expressed by pair of pens. Therefore, each barn housed 32 individual pens, represented by 16-pen pairs, equaling a total of 16 experimental units per barn, totaling 32 experimental units in the study. Pen-pairs were randomly assigned to one of four treatments in a two-by-two complete factorial design with 4 pen-pairs (16 pens) per treatment in each barn. Treatment allocation followed a block design, ensuring that adjacent pen-pairs alternated among treatment groups and treatment allocation ensured each pen-pair treatment was in contact with equal number of other pen-treatments.

The four treatment groups were: a control group fed a basal diet (NRC, 2012) with a normal level of copper (17.5 ppm) and no antibiotic supplementation, a copper-supplemented group fed the basal diet with 200 ppm of copper as copper sulfate, an antibiotic-supplemented group fed the basal diet with CTC at 400 g/ton of feed (22 mg/kg BW), and a copper and CTC group fed the basal diet with 200 ppm of copper and CTC at 400 g/ton of feed. The basal diet was a corn-soybean meal based diet suitable for this age of pigs and meeting the nutrient needs suggested by NRC (2012). *Ad libitum* access to feed and water was provided in each pen with a four-hole, dry self-feeder and a nipple water. The study period was 35 days with days -7 to -1 as an acclimation period and days 0 to 28 as the treatment period. Treatments containing CTC were administered under two veterinary feed directives with the first fed from days 0 to 13 and the second fed from days 15 to 28 allowing for a one-day no CTC on day 14, thereby complying with the Food and Drug Administration (FDA) guidelines for CTC administration.

Rectal fecal samples were collected randomly from three of the five piglets in each pen on days 0, 14, and 28.. Fecal samples from each pen were pooled and transported, in a cooler with ice, to Kansas State University's Pre-harvest Food Safety Laboratory for analysis. Laboratory and farm personnel were blinded to the treatment groups.

Quantification, isolation and speciation of enterococci

Difco (Becton and Dickson, Sparks, MD) was the source of all culture media used in this study, unless otherwise mentioned. One gram of pooled fecal sample was suspended in 9 ml of phosphate-buffered saline (1:10), thoroughly vortexed, then 100 µl of the fecal suspension was spiral plated using the Eddy Jet V. 1.23 spiral plater (IUL, S. A, Barcelona, Spain) onto M-*Enterococcus* agar (ME), ME agar with 8 mM of copper as copper sulfate (Fisher Scientific, Pittsburgh, PA; MECu), and ME agar with 16 µg/ml of CTC (Sigma-Aldrich, St. Louis, MO)

(MECTC) to determine the concentrations of total enterococci and enterococci resistant to copper or CTC, respectively. Plates were incubated for 24-48 h at a minimum of 37°C and colonies were counted using a counting grid, which relates colonies on the spiral plate to the volume deposited in the area, per the manufacturer's instructions and guidelines outlined in the FDA Bacteriological Analytical Manual (Maturin and Peeler. 2001). Two putative enterococcal colonies (pin-point, red, pink, or metallic pink) were picked from the ME spiral plates, plated onto blood agar, and incubated for 24 h at 37°C. Isolates were then subjected to preliminary genus confirmation by performing an esculin hydrolysis test. A colony from the blood agar plate was suspended in 100 µl of enterococcsel broth in a 96-well microtiter plate (FALCON 96-well U-bottom plate Corning Inc., NY), and incubated for 4 h at 37°C. Two esculin hydrolysis positive isolates per fecal sample were suspended in tryptic soy broth with 15% glycerol and stored at -80°C until used.

Bacterial DNA was prepared by suspending colonies in nuclease-free water with 5% Chelex 100 Resin from Bio-Rad Laboratories (Hercules, CA) followed by 10 min of incubation at 95°C using an Eppendorf nexus GSX1 flexlid Mastercycler (Hamburg, Germany). Speciation of enterococci was performed by a duplex PCR assay that identifies *E. faecalis* and *E. faecium* (Jackson et al., 2004). The PCR running conditions, primers, and master mixes for the PCR are described by Kariyama et al. (2000). Positive controls for the PCR assay were ATCC strains 29212 and 19434 of *E. faecalis* and *E. faecium*, respectively (American Type Culture Collection, Manassas, VA). Isolates that were not *E. faecium* or *E. faecalis* were subjected to superoxide dismutase (*sodA*) gene sequence analysis, described by Poyart et al. (2000) for speciation. Gene Clean Turbo Kit (Montreal, CA) was used to purify the *sodA* gene products. The purified gene products were sequenced using Genewiz Sanger Sequencing (South Plainfield, NJ). Sequences

were analyzed using the National Center for Biotechnology Information (NCBI) GenBank database Biological Local Alignment Search Tool (BLAST) search.

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

Bacterial DNA was extracted as before and used for the detection of *tcrB* (Hasman et al., 2006), *erm*(B) and *tet*(M) genes (Jacob et al., 2008). *E. faecium* (7430272-6) served as the positive control for *tcrB*. Field strains of *E. faecium* (AGR 15) and *E. faecalis* (AGR 329) served as positive controls *for erm*(B) and *tet*(M) PCR, respectively.

Development and validation of a real-time PCR (qPCR) to determine the relative abundance of tcrB against total bacterial load in fecal DNA

The NCBI BLAST database was used to analyze the *tcrB* gene target. All sequences available at the time were downloaded and aligned using CLC main workbench 7.6 (Qiagen, Valencia, CA). Primers and probes were designed from the conserved regions of the alignment result. Heterodimer analysis was evaluated *in silico* using the OligoAnalyzer tool (Integrated DNA Technologies, Inc.). To test interactions, *tcrB* and 16S rRNA (Yang et al. 2002) targets were amplified individually and concurrently from serially diluted DNA extracted from a strain of *tcrB*-carrying *E. faecium* (A17Sv1) and from a swine fecal sample positive for *tcrB*. Primer and probe sequences were as follows: *tcrB*-F-Primer: 5'-AGTAGCATTGGATTCAGCCGA -3'; *tcrB*-R-Primer: 5'-CATCTTACGTGTGGTCTTCTGAG -3'; *tcrB*-Probe: 5'- /5MAXN/ TCTTGACACAGTCAGACCCTGG/ 3BHQ_2/ -3'; Primer and probe sequences of the 16S rRNA were used according to Yang et al. 2002. However, to optimize multiplex reaction chemistry, Cy5 channel and BHQ-3 quencher were selected. 16S-F: 5'-

TGGAGCATGTGGTTTAATTCGA -3'; 16S-R: 5'-TGCGGGACTTAACCCAACA -3'; 16S-Probe 5'-/5Cy5/ CACGACCTGACGACARCCATGCA/ 3BHQ_2/ -3'.

The 20 µl qPCR reaction mixture was comprised of 5 µl of DNA template, 1 µl of 10 µM *tcrB* primer mix (Invitrogen, Carlsbad, CA), 0.5 µl of 10 µM *tcrB* probe (IDT, San Jose, CA), 1 µl of 10 µM 16S primer mix (Invitrogen, Carlsbad, CA), 0.5 µl of 10 µM 16S probe (IDT, San Jose, CA), 10 µl of 2X Bio-Rad powermix (Bio-Rad, Hercules, CA), and 2 µl nuclease free water per reaction. The qPCR running conditions consisted of an initial denaturation for 10 min at 95°C, followed by 45 cycles for 15 s at 95°C and 45 s at 60°C using a Bio-Rad CFX96 machine (Hercules, CA). The raw data were analyzed using the Bio-Rad CFX Manager software. Initially, the assay was tested with *tcrB*-positive (*E. faecium* A17Sv1) and *tcrB*-negative (*E. faecium* ATCC 29212) strains of *Enterococcus*. DNA was extracted with QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Germantown, MA).

Analytical sensitivity of the assay was determined using a *tcrB*-positive strain (A17Sv1). The *tcrB*-positive strain was grown on blood agar and a single colony was inoculated into 10 ml Luria-Bertani (LB) broth and incubated overnight. One-hundred microliters of the culture was inoculated into 10 ml LB broth and grown to an absorbance of 0.45 at 600 nm (approximately 3.5 to 6 h at 37°C and 10^8 CFU/ml). Serial ten-fold dilutions of the culture were prepared. To determine viable cell counts, 100 µL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread-plated onto blood agar in triplicate and incubated at 37°C overnight. DNA was extracted from the 10^8 CFU/ml culture using the DNeasy Blood and Tissue Kit (Germantown, MA) and was serially diluted. DNA was tested in quintuplicate with the qPCR assay. Standard curves were generated from the resulting data and PCR efficiency and correlation coefficient (R^2) values were calculated from the standard curves.

Fecal samples from all sample days were processed using the QIAamp® DNA Mini Stool Kit (Germantown, MD 20874) and were stored for future use. The DNA from days 0 and 28 were used in the qPCR assay to determine the relative abundance of *tcrB* in total fecal DNA.

Copper susceptibility determinations

Copper susceptibilities of enterococcal isolates were determined by agar dilution method (Hasman, et al., 2006; Amachawadi et al., 2010). All *tcrB*-positive isolates (n=39), including two Denmark strains (7430162-6 and 7430275-4) and an equal number of *tcrB*-negative strains (n=39) were included. Mueller-Hinton agar plates containing 0, 2, 4, 8, 12, 16, 20, 24, 24, 28, 32, 36, and 40 mM of copper sulfate (Fischer scientific), adjusted to pH 7.0, were used. All isolates were grown to 0.5 McFarland standard and spot inoculated (20 µl) on each plate, in duplicate, and incubated for 48 h at 37°C to determine growth or no growth.

Antibiotic susceptibility determinations

Minimum inhibitory concentrations were determined by micro-broth dilution method, as per CLSI guidelines (2013), for enterococcal isolates from days 0 (n=64) and 28 (n=63). The *E. faecalis* strain, 29212 (ATCC) was used as a reference quality control. Stock solutions of all antibiotics, sourced from either Sigma-Aldrich (St. Louis, MO) or TOKU-E (Bellingham, Washington), were prepared with sterile distilled water to a final concentration of 1,000 µg/ml based on their individual potencies. Antibiotics tested were chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin and vancomycin similar to the National Antimicrobial Resistance Monitoring System (NARMS;

CMV3AGPF) Sensitire panel. All antibiotics except tigecycline were tested at concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/ml. Tigecycline was tested at 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0125, and 0.003125 µg/ml. A single colony of each isolate was inoculated into 10 ml Muller-Hinton (MH) broth and grown to 0.5 McFarland standard, followed by a 1:100 dilution of the culture. The antimicrobial susceptibility tests were performed in 96-well micro titer plates (Becton and Dickinson, Franklin Lakes, NJ). Plates were incubated at 37° C for 20-24 h and results were recorded as growth or no growth.

Statistical analysis

Statistical analysis was conducted with STATA MP (v. 14.2). The combination of 2 pens segregated by solid pen dividers served as the experimental unit. Prevalence outcomes were statistically described before multivariable analysis. Poisson-model (log-linear regression) with log rank assumption of normality for bacterial counts and gene copies was used to analyze quantification data. Main effects of copper, chlortetracycline and their combination were analyzed first on prevalence, then, interaction terms. Descriptive, bivariate, and multivariate analyses were used in a multi-level mixed model framework for each outcome. From the final model, marginal predicted probabilities were obtained for the proportion of enterococci positive for *tcrB*, *erm*(B), and *tet*(M) genes along with their 95% confidence intervals. Due to the clustering of pens within treatment, the clustering of animals in pens, and within-animal dependency (due to re-sampling of animals), multilevel and longitudinal data was considered. Both, antibiotic and copper susceptibility data were log2-transformed before analyzing by survival analysis. Results were considered statistically significant at a *P*-value of < 0.05.

Results

Concentrations of total, copper-, and tetracycline-resistant Enterococcus

The average concentration of fecal enterococci on ME agar for the control, copper, CTC, and copper with CTC treatment groups across all days were $9.5x10^5$, $9.3x10^5$, $6.1x10^5$, and $1.0x10^6$, respectively (Table 1). There was neither sampling day nor treatment effect on fecal concentration of enterococci. However, there was a significant sampling day by treatment interaction (P =0.008). There was a log reduction in the concentration of enterococci on ME agar in day 28 compared to day 0 in the copper with CTC group. Neither the ME-copper nor the ME-CTC plates had any growth.

Species identification of Enterococcus isolates

Four *Enterococcus* isolates were obtained from of all experimental units (pen-pairs) on sample days 0, 14, and 28 (n=384) for which two isolates were speciated (n=192). One isolate from the CTC group on sample day 14 did not grow, therefore, was not characterized (n=191) (Table 2). The predominant species of *Enterococcus* in all treatment groups were *E. faecalis* (97/191; 50.8%), *E. faecium* (33/191; 17.3%), and *E. hirae* (39/191; 20.4%). The other species identified were *E. casseliflavus* (14/191; 7.3%), *E. villorum* (5/191; 2.6%), *E. gallinarum* (2/191; 1.0%) and *E. mundtii* (1/191; 0.5%). The species distribution was not affected by treatment, except for *E. faecalis,* which was lower in the Cu group (18/48; 37.5%) compared to the Copper with CTC group (32/48; 66.7%) (Table 2.2).

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

A total of 384 *Enterococcus* isolates represented by four isolates per experimental unit (EU) at each of the three sample days were tested for three genes (Table 6). Of the 384 enterocossal isolates, 39 (10.2%) of the isolates were positive for the *tcrB* gene (Figure 1). The overall prevalence of *tcrB*-positive enterococcal isolates was not significantly affected by Cu or CTC ($P > 0.05$). However, the prevalence of *tcrB*-positive enterococcal isolates was higher in samples from day 14 than other sampling days ($P = 0.002$). Only 12 of the 39 *tcrB*-positive isolates were spectated. The species distribution included *E. casseliflavus* (n=5), *E. faecalis* (n=3), *E. faecium* (n=3), and *E. gallinarium* (n=1). For *tcrB*-positive isolates (n=39), 32 (82.1%) and 37 (94.9%) were also positive for *erm*(B) and *tet*(M), respectively. For *tcrB*-negative isolates (n=345), 270 (78.3%) and 166 (48.1%) were also positive for *erm*(B) and *tet*(M), respectively.

Of the 384 enterocossal isolates, 203 (52.9%) were positive for the *tet*(M) gene. Out of these *tet*(M)-positive isolates, 50 (50/203; 24.6%) were from piglets in the control group, 46 (46/203; 22.7%) were from piglets fed the Cu group, 52 (52/203; 25.6%) were from piglets fed the CTC supplemented diet, and 55 (55/203; 27.1%) were from piglets fed the copper and CTC supplemented diet (Figure 2). The overall prevalence of *tet*(M)-positive enterococcal isolates was not significantly affected by treatment group or sample day ($P > 0.05$). A total of 191 isolates were speciated, which included 176 *tet*(M)-positive isolates, and of those 94 were *E. faecalis*, 29 were *E. faecium*, 37 were *E. hirae*, 8 were *E. casseliflavus*, 2 were *E. gallinarium*, 1 was *E. mundtii*, and 5 were *E. vilorum*.

Of the 384 enterococcal isolates, 302 (78.6%) isolates were positive for the *erm*(B) gene. Out of these *erm*(B)-positive isolates, 72 (72/302; 23.8%) were from piglets in the control group, 67 (67/302; 22.2%) were from piglets in the copper group, 80 (80/302; 26.5%) were from piglets in the CTC group, and 83 (83/302; 27.5%) were from piglets fed the CuCTC group (Figure 3).

Prevalence of *erm*(B) gene had a significant treatment and sampling day interaction ($P = 0.02$). A total of 191 isolates were speciated, which included 150 *erm*(B)-positives, and of those 88 were *E. faecalis*, 27 were *E. faecium*, 21 were *E. hirae*, 9 were *E. casseliflavus*, 1 was *E. gallinarium*, 1 was *E. mundtii* and 3 were *E. vilorum*.

Real-time quantitative PCR (qPCR) to determine the relative abundance of tcrB in fecal DNA

Analytical sensitivity: Both *tcrB*-positive (*E. faecium*, A17Sv1) and *tcrB*-negative (ATCC 29212) strains of *Enterococcus* were tested initially. Results indicated that the gene target was amplified in *tcrB*-positive *Enterococcus* and not in *tcrB*-negative *Enterococcus*. The detection limit of the qPCR assay for serially diluted *tcrB*-positive pure culture was 4×10^2 per ml (Table 7). The analytical sensitivity of the assay was tested using A17Sv1 DNA in quintuplicate to determine the Ct difference between 16S and *tcrB* to calculate the mean copy-number difference of the two genes which was used to normalize the calculation of the percentage of bacteria in a sample that carries the *tcrB* gene. Our results indicated a PCR efficiency of 94.0% and 107.1% for the *tcrB* and 16S targets, respectfully. The R^2 values were 0.997 and 0.983 for the *tcrB* and 16S target, respectfully. The slope was -3.474. The average Ct's of each serial dilution used to generate the standard curve, and calculate the 16S copy-number difference in fecal samples for five technical replicates are listed in Table 8.

PCR Optimization: Primer and probe interaction analysis, amplifications of *tcrB* alone, 16S alone, and the combination of *tcrB* and 16S for 1x, 10x, 100x, and 1000x dilutions indicated no interactions when our *tcrB* and 16S reactions were duplexed.

Real-time qPCR to determine the relative abundance of *tcrB* in fecal DNA: Overall, the average percent of *tcrB* in total fecal DNA relative to the total bacterial load per EU was 0.24%,

0.19%, 0.09%, and 0.34% for the control, copper, CTC, and copper with CTC groups, respectfully. The abundance of *tcrB* gene in fecal samples was not affected by copper and or CTC supplementation. The distribution of *tcrB* prevalence can be seen in table (Table 9).

Copper susceptibility determinations

Copper susceptibilities of 78 isolates consisting of 39 *tcrB*-positive and 39 *tcrB*-negative isolates from days 0, 14, and 28 were determined by an agar gel dilution method on MH agar plates containing 0, 2, 4, 8, 12, 16, 20, 24, 24, 28, 32, 36, and 40 mM of copper sulfate adjusted to pH 7.0 (Table 10).

This analysis was done strictly on an isolate level, not an EU level where multiple isolates from the same experimental unit could be used in comparison to other isolates. This data is reported to illustrate the difference of copper susceptibility among *tcrB*-positive versus *tcrB*-negative *Enterococcus* only. The median copper MIC of *tcrB* negative isolates was 3 mM when compared to *tcrB* positive isolates with 20 mM ($P<0.0001$) (Table 10) (Figure 4).

Antimicrobial susceptibility determinations

Minimum inhibitory concentrations were determined by micro-broth dilution method per CLSI (2013) guidelines for 16 antimicrobials defined by the National Antimicrobial Resistance Monitoring System Gram-positive panel plates (CMV3AGPF, *Enterococcus* spp.) for isolates obtained from days 0 (n=64) and 28 (n=63). All isolates, from both sample days, were susceptible to gentamicin, kanamycin, and streptomycin. Per the Clinical and Laboratory Standards Institute only susceptible breakpoints have been established for daptomycin (≤4 μg/ml), and tigecycline (≤0.25 μg/ml). Among the macrolide antibiotics, there were a total of 61

(95.3%) and 47 (74.6%) isolates resistant to erythromycin, and a total of 51 (79.7%) and 41 (65.1%) isolates resistant to tylosin, with a similar distribution across all treatment groups, from days 0 and 28, respectively. There were 60 (93.8% and 95.2%) tetracycline resistant isolates, distributed similarly across all treatment groups, from days 0 and 28. In the glycopeptide antibiotic class, there were 1 (1.6%) and 4 (6.3%) vancomycin resistant isolates from sample days 0 and 28, respectively, with the sample day 0 isolate in the copper with CTC treatment group, and the sample day 28 isolates in the copper (n=1), CTC (n=2), and copper with CTC (n=1) groups. For lincomycin, there were 62 (96.9%) and 63 (100.0%) resistant isolates similarly distributed across all sample groups for days 0 and 28, respectively. For linezolid, there were 36 (56.3%) and 39 (61.9%) resistant isolates similarly distributed across all treatment groups from days 0 and 28, respectively. For chloramphenicol, there was 51 (79.7%) and 37 (58.7%) resistant isolates similarly distributed across all treatment groups from days 0 and 28, respectively. For penicillin, there were 3 (4.7%) resistant isolates from day 0. For nitrofurantoin, there were 2 (3.1%) and 18 (28.6%) resistant isolates from days 0 and 28, respectively. For ciprofloxacin, there were 9 (14.1%) and 19 (30.2%) resistant isolates from days 0 and 28, respectively. For synercid, there were 63 (98.4%) and 37 (58.7%) resistant isolates from days 0 and 28, respectively (Table 11).

Of the 5 *tcrB*-positive isolates within days 0 and 28, 3 were resistant to chloramphenicol, 2 to ciprofloxacin, 3 to erythromycin, 5 to lincomycin, 3 to linezolid, 3 to synercid, 5 to tetracycline, 3 to tylosin tartrate, and 1 to vancomycin. A comparison of *tcrB*-positive versus *tcrB*-negative antibiotic susceptibilities can be found in Table 12.

Discussion

In swine, copper is included, generally as copper sulfate, in trace amounts (5-20 ppm) as part of a multi-mineral supplement in diets. At elevated levels of 100 to 250 ppm, copper has growth promotional benefits, which are attributed to alteration of gut microflora, retention of nutrients, reduction in fermentation losses, and reduced morbidity and mortality, particularly in piglets (Hojberg et al., 2005; NCR, 2012). Because antibiotics are no longer used for growth promotion, implementation and development of antibiotic alternatives are crucial to maintain food-animal production and health. One alternative is to supplement copper in the diet, which has been implemented, but due to the potential link with AMR, raises concerns similar to those that drove the ban for sub-therapeutic or growth promotional antibiotics in food-animal diets. A similar ban on sub-therapeutic antibiotic use occurred in Europe between the years of 1997 and 1999, beginning with the ban of avoparcin, followed by the ban of bacitracin, spiramycin, tylosin and virginiamycin, which led to the general conclusion that not only did the new regulation increase veterinary use of antibiotics for therapeutic purposes but also had seemingly no effect on the reduction of AMR in humans or animals (Casewell et al., 2003). To truly understand the effects of employing regulations, and using either traditional or alternative antibiotic therapies, it is crucial to understand the complexity of pathogen resistance including the acquisition of resistance genes, and what selective pressures are beneficial or harmful for specific circumstances. In this study, we evaluated the effect of feeding normal and elevated levels of copper, alone or in combination with CTC, on the prevalence, and quantification of *tcrB*, the prevalence of *tet*(M) and *erm*(B), and the antimicrobial and copper susceptibility determinations of *Enterococcus* isolates from the feces of weaned piglets.

Species identification was performed on 191 isolates of which 12 were *tcrB*-positive (6.3%). In this study, the *tcrB*, gene was detected in four *Enterococcus* species: *E. faecalis*, *E. faecium E. casseliflavus* and *E. gallinarium.* In previous studies, *tcrB* gene was detected in only two of four screened species, *E. faecalis*, and *E. faecium* (Amachawadi et al., 2011). Species distributions may equate differently based upon number of samples collected, number of isolates selected for speciation, and number of screened species in each study. The species we detected in this study were similar to Danish (Hasman and Aarestrup, 2002; Hasman et al., 2006) and Australian (Mazaheri and Barton, 2010) studies. The overall species distribution in this study was unlike other study reports as most species reported in the United States other than *E. faecalis* and *E. faecium*, are *E. durans*, and *E. malodoratus* (Devriese et al., 1994; Thal et al., 1995). The variation of *Enterococcus* species across studies could be due to many factors such as age at sample time which could represent a given animal that has had either more or less time to develop a diverse microflora. Additionally, selecting just a few isolates of many colonies from a fecal sample could, by chance, be a limiting factor in unveiling the true variance of *Enterococcus* species in each sample.

There was only a small proportion of *tcrB* positive isolates (39/384; 10.2%) in this study. The low prevalence of *tcrB* in enterococci observed in this study was comparable to a previous study (Amachawadi et al., 2011) and suggests that this resistance gene is not being transferred at a high rate in enterococci, specifically. Regardless, enterococci remain significant to monitor as they are increasingly important pathogens that can cause illness like bacteremia and infective endocarditis (Arias and Murray, 2008). Other studies in Denmark that used high levels of copper, found high prevalence of *tcrB* in *Enterococcus* isolates from swine collected at slaughter which could be associated with the age of the animal (development of resistant microflora), or the

duration and concentration of copper exposure (birth to slaughter and 175 to 200 ppm) (Hasman and Aarestrup, 2002; Hasman et al., 2006). The presence of *tcrB* in the control group (8/39; 20.51%) suggests that there are naturally resistant isolates present in the piglets. The prevalence of *tcrB*-positive isolates did not have a significant treatment effect despite being numerically higher in the copper with CTC treatment group compared to other treatment groups. Most *tcrB*-positive *Enterococcus* isolates were from sample day 14 (56.4%) compared to days 0 (15.4%) and 28 (28.2%). The reason for the decreased prevalence of *tcrB* at sample day 28 when compared to sample day zero is unknown, but one possibility could be due to alteration of gut microbial population and activity (Gould et al., 2009; Hojberg et al., 2005) *tcrB* is on a plasmid that bacteria can lose or gain through conjugal transfer. The sub-population of *Enterococcus* from a given fecal sample represents a small portion of bacteria that may not represent the average *Enterococcus* population in the fecal sample.

In this study, in addition to the prevalence of *tcrB* in *Enterococcus* specifically, we also quantified *tcrB* in total fecal DNA. Using the NCBI primer BLAST tool, we identified seven cocci bacteria, in addition to *Enterococcus*, that contained the *tcrB* sequence as 100% matches. These cocci included three species of Trichococcus (*pasteurii*, *collinsii*, and *ilyis*), *Aerococcus viridans*, *Acetobacterium dehalogenans*, and two species of *Streptococcus* (*pseudoporcinus* and *agalactiae*). These cocci may be associated with *tcrB* amplification in our qPCR assay.

Enterococci that possess the *tcrB* gene, homologous to *copB*, are more capable of exporting copper from the cell, thereby avoiding copper's cytotoxic effect, than bacteria that lack the *tcrB* gene. This is likely the reason we saw a near-seven-fold increase in resistance to copper in *tcrB*-positive enterococci as compared to *tcrB*-negative enterococci (20mM vs 3mM) which is supported by former studies (Amachawadi et al., 2010; Hasman and Aarestrup, 2002).

It is known that macrolide resistance gene, *erm*(B), and vancomycin resistance gene, *vanA*, reside on the same plasmid as *tcrB* (Hasman and Aarestrup, 2002) and that feeding dietary copper to piglets can co-select for erythromycin and vancomycin resistance in Denmark and Spain (Hasman et al., 2006). In our study, both *tcrB*-negative and *tcrB*-positive isolates were positive for the *erm*(B) gene, and were resistant to erythromycin, disabling us from evaluating the co-selection of macrolide resistance in conjunction with the selection of *tcrB*. Additionally, both *tcrB*-negative and *tcrB*-positive isolates carried the *tet*(M) gene, which is not surprising given the high transferability of this gene and others by mobile genetic elements (Hegstad et al., 2010). Unlike former studies, we phenotypically detected five vancomycin resistant *Enterococcus* isolates whose average MIC (μg/ml) were 8, 8, 12, and ≥128 with those whose MIC fell in the CLSI intermediate category (8-16 μg/ml) considered as resistant (≥32). The isolates originated from pigs in different pens where one isolate belonged to pigs in the control group, two to pigs in the CTC treatment group, and two to pigs the copper with CTC treatment group. Generally, vancomycin resistance is not detected in fecal *Enterococcus* of swine, although the presence of *vanA* has been detected in *E. faecium* of swine residing in Michigan (Donabedian et al., 2010), and in swine in Denmark (Bager et al., 1997). The detection of vancomycin resistance may suggest either the natural development of a resistant population or the environmental selection of vancomycin resistance from feeding diets supplemented with copper or copper with CTC. A large portion of both day 0 and day 28 isolates were resistant to the macrolide antibiotics, erythromycin and tylosin tartrate, which is comparable to a former study (Amachawadi et al., 2010). The past use of tyosin as a growth promoter, and both past and present use to treat enteric infections may be an unintended contributing factor in selection of antimicrobial resistance. Most of the isolates were resistant to tetracycline as well which is not

surprising based on the former study that demonstrated co-selection of tetracycline resistance with macrolide resistance (Cauwerts et al., 2007). In this study, the phenotypic resistance of both macrolides and tetracycline is supported genetically as well.

Enterococci are commensal bacteria found in both animals and humans. Antimicrobial resistant of enterococci is a primary concern in human medicine (Moellering, 1992) because enterococci are important nosocomial and opportunistic pathogens (Puchter et al., 2018). Animals, like humans, can serve as a reservoir of resistance genes capable of transferring to other subjects (Kojima et al., 2010). Enterococci tend to become resistant to medically important antibiotics by acquiring resistance genes via intra- (Amachawadi et al., 2010) and inter-specie (Amachawadi et al., 2011) conjugal transfer. It has been shown that transfer of genetic elements can occur between *Enterococcus* and other gut bacteria (Bertram and Durre, 1991). Because of the propensity of *Enterococcus* to house and share AMR genes, human-human, animal-animal, and human-animal exposures to resistant enterococci are concerning and warrant investigation as we did in this study.

Conclusions

The results of this study show that supplementing copper, with or without a selection pressure of chlortetracycline, did not increase copper-resistant enterococci and did not co-select resistance to any other antibiotics.

Figure 2.1 Prevalence of transferable copper resistant gene, *tcrB*, in *Enterococcus* isolates collected from the feces of piglets

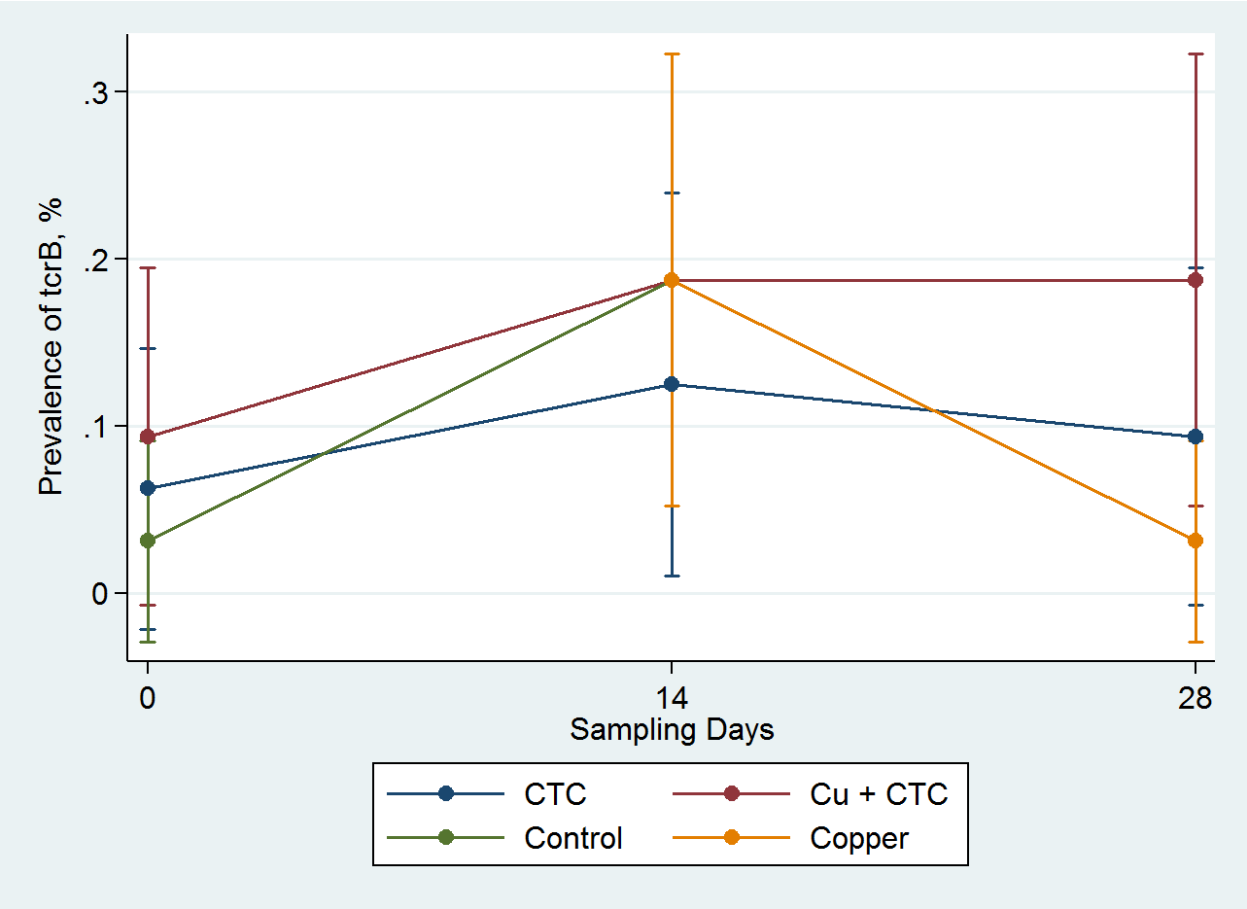

Figure 2.2 Prevalence of tetracycline resistant gene, *tet*(M), in *Enterococcus* isolates collected from the feces of piglets

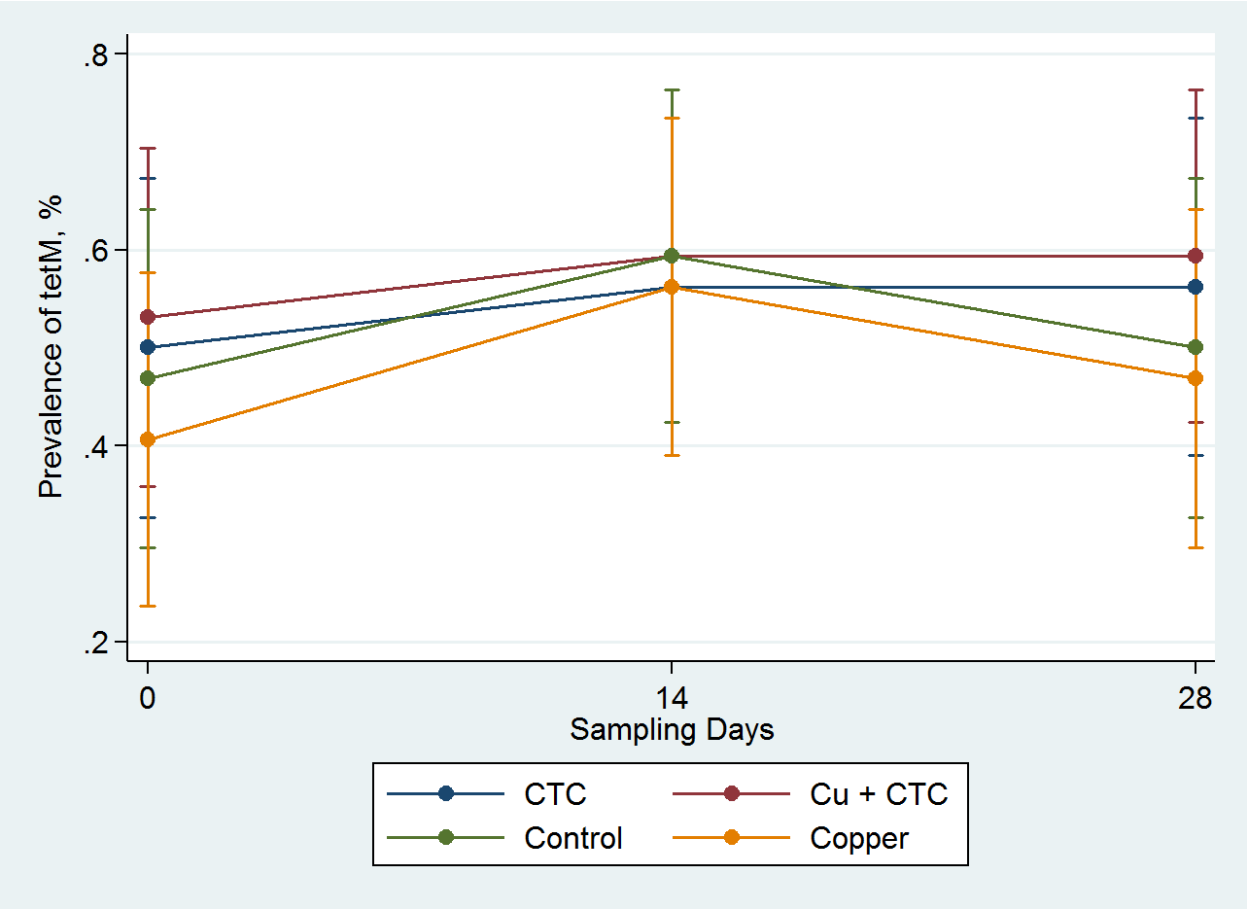

Figure 2.3 Prevalence of macrolide resistant gene, *erm*(B), in *Enterococcus* isolates collected from the feces of piglets

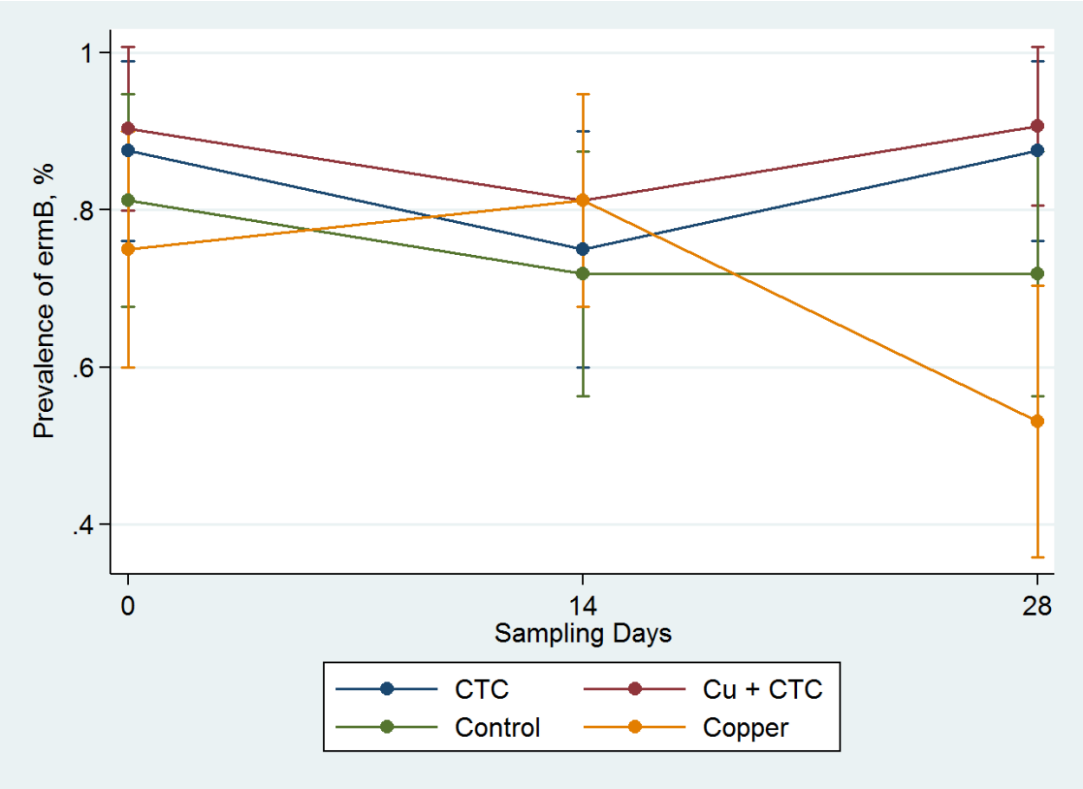

Figure 2.4 Comparison of copper minimum inhibitory concentrations of either transferable copper resistance gene (*tcrB*) negative or positive *Enterococcus* isolated from feces of piglets

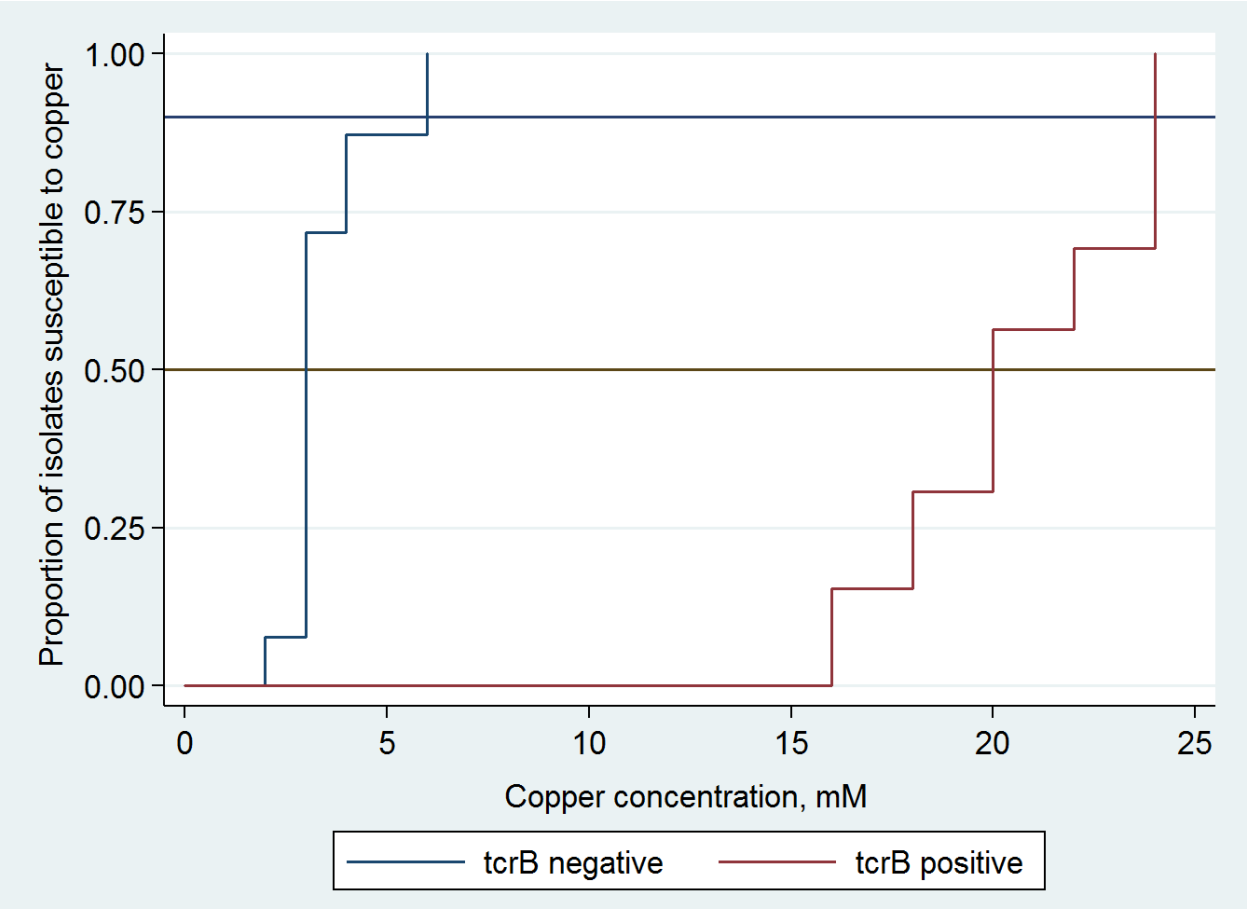

Table 2.1 Concentrations of enterococci, determined by spiral plate method, in weaned piglets fed diets supplemented with or without copper and or chlortetracycline

	Treatment groups and concentrations of fecal enterococci, CFU/g[1] of feces			
Sampling day	Control	Copper	Chlortetracycline	Copper + Chlortetracycline
0	$2.3x10^6$	$7.8x10^5$	$7.7x10^5$	$1.0x10^6$
14	$2.0x10^5$	$1.6x10^5$	$2.1x10^5$	$9.7x10^5$
28	$3.3x10^5$	$1.1x10^6$	$5.0x10^5$	$4.9x10^4$
Average	$9.5x10^5$	$9.3x10^5$	$6.1x10^5$	$1.0x10^6$

[1] Colony forming units per gram of feces

Table 2.2 Species identification of *Enterococcus* isolates from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment groups	Sample day	Total enterococcal isolates	*Enterococcus* spp.						
			E. faecalis	*E. faecium*	*E. hirae*	*E. casseliflavus*	*E. gallinarium*	*E. mundtii*	*E. vilorum*
Control	0	16	14	1	0	0	0	1	0
	14	16	6	3	3	4	0	0	0
	28	16	4	1	9	2	0	0	0
Total (%)		48	24[ab] (50)	5 (10.4)	12 (25)	6 (12.5)	0	1 (2.1)	0
Cu	0	16	13	2	0	1	0	0	0
	14	16	5	9	0	1	0	0	1
	28	16	0	1	13	1	0	0	1
Total (%)		48	18[a] (37.5)	12 (25)	13 (27.1)	3 (6.3)	0	0)	2 (4.2)
CTC	0	16	14	1	0	1	0	0	0
	14	15	6	3	2	2	1	0	1
	28	16	3	5	8	0	0	0	0
Total (%)		47	23[ab](48.9)	9 (19.1)	10 (21.3)	3 (6.4)	1 (2.1)	0	1 (2.1)
Cu + CTC	0	16	14	1	0	1	0	0	0
	14	16	8	4	1	1	1	0	1
	28	16	10	2	3	0	0	0	1
Total (%)		48	32[b] (66.7)	7 (14.6)	4 (8.3)	2 (4.2)	1 (2.1)	0	2 (4.2)

[a, b] Column means not sharing the same superscript differ at $P < 0.05$

Table 2.3 Prevalence of transferable copper resistance gene, *tcrB,* in speciated *Enterococcus* isolates (n=191) from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment groups	Sample day	Total enterococcal isolates	*Enterococcus* species							Total *tcrB*-positive (%)
			E. faecalis	*E. faecium*	*E. hirae*	*E. casseliflavus*	*E. gallinarium*	*E. mundtii*	*E. vilorum*	
Total No. strains	-	-	24	5	12	6	0	1	0	-
Control	0	16	0	0	0	0	0	1	0	1
	14	16	0	0	0	1	0	0	0	1
	28	16	0	0	0	0	0	0	0	0
Total *tcrB*-positive (%)		48	0/24	0/5	0/12	1/6 (16.7)	0/0	1/1 (100)	0/0	2/48 (4.2)
Total No. strains	-	-	18	12	13	3	0	0	2	-
Cu	0	16	0	0	0	0	0	0	0	0
	14	16	1	1	0	1	0	0	0	3
	28	16	0	0	0	0	0	0	0	0
Total *tcrB*-positive (%)		48	1/18 (5.6)	1/12 (8.3)	0/13	1/3 (3.3)	0/0	0/0	0/2 (0)	3/48 (6.3)
Total No. strains	-	-	23	9	10	3	1	0	1	-
CTC	0	16	0	0	0	1	0	0	0	1
	14	15	1	0	0	0	0	0	0	1
	28	16	0	0	0	0	0	0	0	0
Total *tcrB*-positive (%)		47	1/23 (4.3)	0/9	0/10	1/3 (3.3)	0/1	0/0	0/1	2/47 (4.3)
Total No. strains	-	-	32	7	4	2	1	0	2	-
Cu + CTC	0	16	0	0	0	1	0	0	0	1
	14	16	0	0	0	1	1	0	0	2
	28	16	1	2	0	0	0	0	0	3
Total *tcrB*-positive (%)		48	1/32 (3.1)	2/7 (28.6)	0/4	2/2 (100)	1/1 (100)	0/0	0/2	6/48 (12.5)

Table 2.4 Prevalence of tetracycline resistance gene, *tet*(M), in speciated *Enterococcus* isolates (n=191) from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment	Sample day	Total enterococcal isolates	*Enterococcus* spp.							Total *tet*(M)-positive (%)
			E. faecalis	*E. faecium*	*E. hirae*	*E. casseliflavus*	*E. gallinarium*	*E. mundtii*	*E. vilorum*	
Total No. strains	-	-	24	5	12	6	0	1	0	-
Control	0	16	13	1	0	0	0	1	0	15
	14	16	6	3	3	2	0	0	0	14
	28	16	4	1	9	1	0	0	0	15
Total *tet*(M)-positive (%)		48	23/24 (95.8)	5/5 (100)	12/12 (100)	3/6 (50)	0/0	1/1 (100)	0/0	44/48 (91.7)
Total No. strains	-	-	18	12	13	3	0	0	2	-
Cu	0	16	12	1	0	0	0	0	0	13
	14	16	5	8	0	1	0	0	1	15
	28	16	0	1	12	0	0	0	1	14
Total *tet*(M)-positive (%)		48	17/18 (94.4)	10/12 (83.3)	12/13 (92.3)	1/3 (3.3)	0/0	0/0	2/2 (100)	42/48 (87.5)
Total No. strains	-	-	23	9	10	3	1	0	1	-
CTC	0	16	14	0	0	1	0	0	0	15
	14	15	6	3	2	1	1	0	1	14
	28	16	3	5	7	0	0	0	0	15
Total *tet*(M)-positive (%)		47	23/23 (100)	8/9 (88.9)	9/10 (90)	2/3 (66.7)	1/1 (100)	0/0	1/1 (100)	44/47 (93.6)
Total No. strains	-	-	32	7	4	2	1	0	2	-
Cu + CTC	0	16	14	0	0	1	0	0	0	15
	14	16	7	4	1	1	1	0	1	15
	28	16	10	2	3	0	0	0	1	16
Total *tet*(M)-positive (%)		48	31/32 (96.9)	6/7 (5.7)	4/4 (100)	2/2 (100)	1/1 (100)	0/0	2/2 (100)	46/48 (95.8)

Table 2.5 Prevalence of macrolide gene, *erm*(B), in speciated *Enterococcus* isolates (n=191) from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment	Sample day	Total enterococcal isolates	*Enterococcus* spp.							Total *erm*(B)-positive (%)
			E. faecalis	*E. faecium*	*E. hirae*	*E. casseliflavus*	*E. gallinarium*	*E. mundtii*	*E. vilorum*	
Total No. strains	-	-	24	5	12	6	0	1	0	-
Control	0	16	13	1	0	0	0	1	0	15
	14	16	5	3	2	3	0	0	0	13
	28	16	3	1	4	1	0	0	0	9
Total *erm*(B)-positive (%)		48	21/24 (87.5)	5/5 (100)	6/12 (50)	4/6 (66.7)	0/0	1/1 (100)	0/0	37/48 (77.1)
Total No. strains	-	-	18	12	13	3	0	0	2	-
Cu	0	16	10	1	0	0	0	0	0	11
	14	16	5	7	0	1	0	0	1	14
	28	16	0	1	6	0	0	0	0	7
Total *erm*(B)-positive (%)		48	15/18 (83.3)	9/12 (75)	6/13 (46.2)	1/3 (3.3)	0/0	0/0	1/2 (50)	32/48 (66.7)
Total No. strains	-	-	23	9	10	3	1	0	1	-
CTC	0	16	13	0	0	1	0	0	0	14
	14	15	5	3	1	1	1	0	1	12
	28	16	3	5	6	0	0	0	0	14
Total *erm*(B)-positive (%)		47	21/23 (91.3)	8/9 (88.9)	7/10 (70)	2/3 (66.7)	1/1 (100)	0/0	1/1 (100)	40/47 (85.1)
Total No. strains	-	-	32	7	4	2	1	0	2	-
Cu + CTC	0	16	13	0	0	1	0	0	0	14
	14	16	8	3	0	1	0	0	0	12
	28	16	10	2	2	0	0	0	1	15
Total *erm*(B)-positive (%)		48	31/32 (96.9)	5/7 (71.4)	2/4 (50)	2/2 (100)	0/1	0/0	1/2 (50)	41/48 (85.4)

Table 2.6 Prevalence of copper (*tcrB*), macrolide, (*erm*B*)*, and tetracycline (*tet*M*)* resistance genes in *Enterococcus* isolates from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment	Sampling days	Total enterococcal isolates	Total *tcrB* positive	Total *erm*(B) positive	Total *tet*(M) positive
Control	0	32	1	26	15
	14	32	6	23	19
	28	32	1	23	16
Total (%)		96	8 (8.3)	72 (75)	50 (52.1)
Cu	0	32	0	24	13
	14	32	6	26	18
	28	32	1	17	15
Total (%)		96	7 (7.3)	67 (69.8)	46 (47.9)
CTC	0	32	2	28	16
	14	32	4	24	18
	28	32	3	28	18
Total (%)		96	9 (9.4)	80 (83.3)	52 (54.2)
Cu + CTC	0	32	3	28	17
	14	32	6	26	19
	28	32	6	29	19
Total (%)		96	15 (15.6)	83 (86.5)	55 (57.3)

Table 2.7 Detection limits of the quantitative PCR assay of serially diluted pure culture of *Enterococcuc faecium* (strain A17Sv1) for the detection of copper resistance gene, *tcrB*

Serial dilutions	Concentrations of *E. faecium,* CFU/ml	Average cycle threshold (Ct)
Undiluted	$4.0x10^{8}$	14.9
10^{-1}	$4.0x10^{7}$	19.9
10^{-2}	$4.0x10^{6}$	23.8
10^{-3}	$4.0x10^{5}$	27.4
10^{-4}	$4.0x10^{4}$	30.5
10^{-5}	$4.0x10^{3}$	34.1
10^{-6}	$4.0x10^{2}$	36.3
10^{-7}	$4.0x10^{1}$	ND
10^{-8}	$4.0x10^{0}$	ND

Table 2.8 Average cycle threshold (Ct) values of a serially diluted *tcrB*-positive culture (A17Sv1) used to generate a standard curve and calculate the 16S copy difference in a real-time PCR to determine the relative abundance of *tcrB* against total bacterial load in fecal DNA

	Average Ct's used to calculate the 16S copy difference								
Serial dilutions	Undiluted	-8	-7	-6	-5	-4	-3	-2	-1
Average *tcrB*-Ct	N/A	16.76	21.26	24.31	27.66	31.14	34.58	N/A	N/A
Average 16S-Ct	N/A	13.87	18.39	21.59	25.02	28.27	31.07	N/A	N/A

Table 2.9 Average percent of *tcrB* in total fecal DNA relative to the total bacterial load by quantitative PCR of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment	Sample Day	Average % *tcrB* in total fecal DNA relative to bacterial load of fecal sample	Average % *tcrB* in total fecal DNA relative to bacterial load of fecal sample
Control	0	0.46%	
	14	0.13%	0.24%
	28	0.13%	
Cu	0	0.28%	
	14	0.20%	0.19%
	28	0.10%	
CTC	0	0.14%	
	14	0.08%	0.09%
	28	0.04%	
Cu + CTC	0	0.45%	
	14	0.46%	0.34%
	28	0.11%	

Table 2.10 Minimum inhibitory concentrations (MIC) of copper towards *tcrB*-negative or -positive *Enterococcus* isolates from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Treatment groups	*tcrB*-negative enterococci		*tcrB*-positive enterococci	
	No. of isolates	MIC, µg/ml	No. of isolates	MIC, µg/ml
Control	8	3.1	8	20.5
Cu	7	3.7	7	21.4
CTC	9	3.4	9	20.7
Cu + CTC	15	3.5	15	20.1

Table 2.11 Number of antibiotic-resistant *Enterococcus* isolates from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Class and antimicrobial compounds	Treatment Groups									
	Control (n=32; %)		Cu (n=32; %)		CTC (n=32; %)		Cu + CTC (n=31; %)		Total (n=127; %)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
B-Lactams										
Penicillin	1/16 (6.3)	0/16 (0)	1/16 (6.3)	0/16 (0)	1/16 (6.3)	0/16 (0)	0/16 (0)	0/15 (0)	3/64 (4.7)	0/63 (0)
Glycopeptide										
Vancomycin	0/16 (0)	1/16 (6.3)	0/16 (0)	0/16 (0)	0/16 (0)	2/16 (12.5)	1/16 (6.3)	1/15 (6.7)	1/64 (1.6)	4/63 (6.3)
Lincosamides										
Lincomycin	16/16 (100)	16/16 (100)	15/16 (93.8)	16/16 (100)	15/16 (93.8)	16/16 (100)	16/16 (100)	15/15 (100)	62/64 (96.9)	63/63 (100)
Macrolides										
Erythromycin	16/16 (100)	11/16 (68.8)	14 /16 (87.5)	7/16 (43.8)	16/16 (100)	16/16 (100)	15 /16 (93.8)	13/15 (86.7)	61/64 (95.3)	47/63 (74.6)
Tylosin	12/16 (75)	9/16 (56.3)	9/16 (56.3)	8/16 (50)	16/16 (100)	12/16 (75)	14/16 (87.5)	12/15 (80)	51/64 (79.7)	41/63 (65.1)
Nitrofuran										
Nitrofurantoin	1/16 (6.3)	3/16 (18.8)	0/16 (0)	7/16 (43.8)	0/16 (0)	6/16 (37.5)	1/16 (6.3)	2/15 (13.3)	2/64 (3.1)	18/63 (28.6)
Oxazolidinones										
Linezolid	8/16 (50)	12/16 (75)	9/16 (56.3)	13/16 (81.3)	11/16 (68.8)	9/16 (56.3)	8/16 (50)	5/15 (33.3)	36/64 (56.3)	39/63 (61.9)
Phenicols										
Chloramphenicol	12/16 (75)	7/16 (43.8)	10/16 (62.5)	9/16 (56.3)	14/16 (87.5)	10/16 (62.5)	15/16 (93.8)	11/15 (73.3)	51/64 (79.7)	37/63 (58.7)
Quinolones										

Ciprofloxacin	1/16 (6.3)	2/16 (12.5)	2/16 (12.5)	6/16 (37.5)	3/16 (18.6)	7/16 (43.8)	3 /16 (18.6)	4/15 (26.7)	9/64 (14.1)	19/63 (30.2)
Streptogramins										
Synercid	16/16 (100)	9/16 (56.3)	15/16 (93.8)	6 /16 (37.5)	16/16 (100)	11/16 (68.8)	16/16 (100)	11/16 (73.3)	63/64 (98.4)	37/63 (58.7)
Tetracyclines										
Tetracycline	15/16 (93.8)	14/16 (87.5)	13/16 (81.3)	15/16 (93.8)	16/16 (100)	16/16 (100)	16/16 (100)	15/15 (100)	60/64 (93.8)	60/63 (95.2)

Table 2.12 Comparison of antibiotic susceptibilities between *tcrB*-positive (n=5) and *tcrB*-negative (n=5) *Enterococcus* isolates from feces of piglets fed diets supplemented with or without copper (Cu) and or chlortetracycline (CTC)

Antimicrobial compounds	[3]Mean minimum inhibitory concentration (MIC), μg/ml		Susceptible MIC, μg/ml	Intermediate MIC, μg/ml	Resistant MIC, μg/ml
	tcrB-negative	*tcrB*-positive			
Chloramphenicol	32	16	≤ 8	16	≥32
Ciprofloxacin	1.1	7.7	≤ 1	2	≥4
Daptomycin	5.2	3.3	≤ 4	N/A	N/A[1]
Erythromycin	42	33.8	≤ 0.5	1-4	≥8
Gentamicin	16.8	31.6	≤ 500	N/A	>500
Kanamycin	102.4	115.2	≤ 512	N/A	≥1,024
Lincomycin	128	37.6	≤ 2	4	≥8
Linezolid	16	52.8	≤ 2	4	≥8
Nitrofurantoin	25.6	11.2	≤ 32	64	≥128
Penicillin	1.3	2.9	≤ 8	N/A	≥16
Streptomycin	102.4	78.4	≤ 1,000	N/A	>1,000
Synercid	5.4	5.6	≤ 1	2	≥4
Tetracycline	89.6	76.8	≤ 4	8	≥16
Tigecycline	1.95	1.2	≤ 0.25	N/A	N/A[2]
Tylosin tartrate	102.8	45.4	≤ 8	16	≥32
Vancomycin	2.9	2.7	≤ 4	8-16	≥32

[1]According to the Clinical and Laboratory Standards Institute, only a susceptible breakpoint has been established for daptomycin (≤4 μg/ml)

[2]According to the Clinical and Laboratory Standards Institute, only a susceptible breakpoint has been established for tigecycline (≤0.25 μg/ml)

[3]For the purpose of averaging the *tcrB*-negative and *tcrB*-positive MICs, values that were ≥128 μg/ml were considered as 128 μg/ml

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Chapter 3 - *Salmonella enterica* in liver abscesses of cattle

Abstract

Liver abscesses in feedlot cattle have a significant economic impact because of reduction in cattle performance, and carcass yield and liver condemnation at harvest. *Fusobacterium necrophorum* is the primary causative agent of the liver abscesses. Recently, *Salmonella enterica* has been isolated from liver abscesses of cattle. Our objectives of this study were to determine prevalence of *Salmonella,* compare conventional (serological) and commercially available Check &Trace serotyping methods, and to describe the antimicrobial susceptibility patterns of *Salmonella* isolated from liver abscesses of feedlot cattle. In the 2014 study, the number of liver abscesses positive for *Salmonella* were higher ($P < 0.05$) in cattle fed no tylosin in the diet (66/200; 33%) compared to tylosin-fed cattle (31/183; 16.9%). In the 2015 study, *Salmonella* prevalence tended to be higher in liver abscesses categorized as severe (29/106; 27.4%) compared to mild liver abscesses (38/174; 21.8%), but the difference was not significant. Out of the 164 *Salmonella* isolated, 152 (92.7%) were used for serotyping and 164 strains were used for antimicrobial susceptibility testing. Serotyping was done by serological method, which is considered as the gold standard, and the commercial Check & Trace method, which is a molecular method based on differences in their DNA sequence. A total of 11 serotypes were identified with Lubbock (66/152; 43.4%) being the predominant serotype, followed by Agona (24/152; 15.8%), Anatum (20/152; 13.2%), and Montevideo (18/152; 11.8%). The commercial identified only a few serotypes correctly suggesting that the method requires further validation. Antimicrobial susceptibility testing was done by microbroth dilution method according to Clinical Laboratory Standards Institute guidelines. A majority of the *Salmonella* strains were pansusceptible to the antimicrobials included in the panel. Overall, 10 strains (10/164; 6.1%)

were resistant to one or more antibiotics and belonged to serotypes Agona, Anatum, Cerro, Lubbock, Mbandaka, and Reading. The top three of nine resistant antibiotics were chloramphenicol (5/10; 50%), streptomycin (5/10; 50%), and tetracycline (6/10; 60%). Whether *Salmonella* contributes to liver abscess formation or just happen to survive in an abscess initiated by the primary etiologic agent, *Fusobacterium necrophorum,* remains to be determined.

Keywords: Antimicrobial susceptibility, Feedlot cattle, Liver abscess, *Salmonella*, Serotype

Introduction

Approximately 67% of feedlot cattle livers are condemned at slaughter, with liver abscesses accounting for most abnormalities (Brown and Lawrence, 2010; McKeith et al., 2012). The economic impact of liver abscesses on the feedlot industry is largely dependent on severity of the abscess. Liver abscesses are categorized as mild (A-), moderate (A), or severe (A+) lesions (Brown et al., 1975). It has been well documented that only A+ abscesses have a significant influence on cattle performance, carcass grading, and carcass value (Brown and Lawrence, 2010; Brink et al., 1990; Davies et al., 2007).

Liver abscesses in feedlot cattle have been studied since the mid 1990's (Smith 1944; Jensen et al., 1954). Traditionally, the acidosis-rumenitis orchestrates the initiation and development of liver abscesses of feedlot cattle fed high concentrate diets. A few predisposing mediators include ruminal lesions (Jensen et al., 1954), acid-induced rumenitits by rapid diet change, consumption of unpalatable foodstuffs perforating of the ruminal wall (Elam, 1976; Grohn and Bruss, 1989), and hepatic artery access (Kelly, 1993). The bacteriology of liver

abscesses has remained somewhat constant over the last few decades in that *Fusobacterium necrophorum*, a normal rumianl bacterium, is the primary causative agent and *Truperella pyogenes* is the secondary causative agent (Amachawadi and Nagaraja, 2016).

Liver abscesses are controlled by inclusion of dietary tylosin (8 to 10 g/ton of feed as 60 to 90 mg/animal/day), which has been shown to reduce the incidence of liver abscesses in feedlot cattle by an average of 22% (Wileman et al., 2009), or with other approved dietary antibiotics (Lundeen, 2013). Although tylosin is used extensively for prevention, liver abscesses continue to occur. The occurrence of liver abscesses is not because of resistance of *F. necrophgorum* to tylosin and findings showed no difference in mean minimum inhibitory concentrations (MIC) of tylosin for *F. necrophorum* from cattle fed diets with or without tylosin (Nagaraja et al., 1999; Amachawadi eta l., 2016). In recent years, another potential etiological agent, *Salmonella enterica,* has been reported to occur in liver abscesses of cattle (Amachawadi and Nagaraja, 2015; Amachawadi et al., 2016).

Salmonella are ubiquitous bacteria with capabilities of thriving at 8-45°C, 4-9 pH, and up to 20% environmental salt concentration (Guthrie, 1991). *Salmonella* can be cultured both anaerobically and aerobically (Yamamoto and Doffner, 1985) and there is evidence that anaerobically grown *Salmonella* are more virulent (Lee and Falkow, 1990; Schiemann and Shope, 1991). Because *Salmonella* are present in gut contents, it can cross the epithelial barrier in either the small or large intestine, get phagocytosed and survive in phagocytes, and enter the lymphatic system into portal capillary circulation. In the liver, the organisms can reside and possibly causes infection. However, it is unknown if *Salmonella* is a causative agent or secondary invader in liver abscesses of cattle.

Among the serotypes of *Salmonella* identified in liver abscesses, the predominant serotype is *S. enterica* 6,7:g,m,s:e,n,z15, now known as *S. enterica* subsp. *enterica* serotype Lubbock (Amachawadi and Nagaraja, 2015). Classic serotyping method follows the original Kauffmann and White scheme using standardized animal antisera to test for lipopolysaccharide (O-antigen) and flagellar (H-antigen) proteins. Since 1934, this method has expanded to detect over 2,500 different serotypes by using 160 different antisera (Kauffmann, 1966; Ewing, 1986; Wattiau et al., 2008). While this serotyping method is considered as the "gold standard", there are many disadvantages including the dependency of phenotypic expression of O- and H-antigens which causes inconclusive serotyping of isolates that express rough O-antigens or have lost the ability to express either antigen, the cost of antisera, besides being time consuming and labor intensive. Check &Trace Salmonella by Check-Points (Check-Points, Wageningen, The Netherlands) is a commercially available serotyping method based on multiplex ligation detection reaction (LDR) targeting genetic markers with specific probes to generate DNA fragments that undergo amplification and detection to determine the serotype (Morningstar-Shaw, 2012). The objectives for this study were to compare conventional (serological) and commercially available LDR serotyping methods, and to describe the antimicrobial susceptibility patterns of *Salmonella* isolated from liver abscesses of feedlot cattle.

Methods and Materials

Study design and sample collection

Salmonella isolates used in the study originated from abscessed livers collected in two studies. The first study (2014 study) included liver abscesses from four groups of crossbred beef and Holstein steers that originated from 22 different feedlots located in the Central Plains, Desert

Southwest, and High Plains. Cattle were fed high-concentrate finishing diets with or without tylosin (Tylan® Elanco Animal Health, Greenfield, IN) in a 2 x 2 factorial design. Collection dates were between January and April of 2014 in six USDA-inspected abattoirs located in Arizona, California, Colorado, and Kansas. A total of 97 *Salmonella* strains isolated from liver abscesses were used from this study. The second study (2015 study) included liver abscesses from feedlot cattle that were categorized, based on the Elanco liver scoring system (Brown et al., 1975), as mild (A or A-) or severe (A+) liver abscesses. Collection dates were between the months of October 2015 and March 2016 in five USDA-inspected abattoirs located in Arizona, Kansas, Pennsylvania, Texas, and Wisconsin. This study resulted in isolation of 67 *Salmonella* strains. The procedures used for collection of liver abscesses, shipment, and abscess processing have been described before (Nagaraja et al., 1999; Amachawadi et al., 2016).

Salmonella isolation

Encapsulated abscesses, mostly with inflamed perimeters, were selected for bacterial isolation. The site of incision was seared with a red-hot spatula and lacerated with a sterile scalpel. Using a sterile cotton swab, the interior wall of the abscess was wiped and spot inoculated on two Hektoen-Enteric (HE) agar plates (Beckton and Dickson, Sparks, MD). To obtain isolated colonies, spot inoculations were streaked with a sterile inoculating loop. One plate was incubated in an anaerobic Glove Box (Thermo Fisher Scientific Inc., Waltham, MA) and the second plate was incubated aerobically. Additionally, an enrichment procedure to isolate *Salmonella* was performed. Ten grams of purulent material from each abscess were inoculated into 90 mL of tetrathionate broth (TT; Beckton and Dickson) and incubated at 37°C for 24 h; Next, 100 μL was inoculated into 10 mL of Rappaport-Vassiliadis broth (RV; Beckton and

Dickson) and incubated at 37°C for 24 h. Then, the inoculum was streaked onto an HE plate with a sterile loop and was incubated aerobically at 37°C for 24 h. Presumptive *Salmonella* colonies were sub-cultured onto blood agar plates and stored for further analysis.

Salmonella identification

The presumptive *Salmonella* colonies were re-streaked onto HE agar for phenotypic and purity confirmation. For genus confirmation, slide agglutination was performed on a single colony for each isolate using *Salmonella* polyvalent O antiserum (BD Diagnostics, Sparks, MD). Also, colonies were suspended in nuclease-free water, boiled at 95°C for 10 min, and centrifuged to obtain DNA. The DNA sample was subjected to PCR for detection of the *invA* gene (Alam et al., 2009). Running conditions of the assay with *Salmonella* Newport as a positive control were as follows: initial denaturation at 94° C for 7 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Isolates confirmed as *Salmonella* were stored in cryo-protection beads (Key Scientific Products Inc., Stamford, TX) at -80°C.

Salmonella Serotyping

Out of the 164 *Salmonella* isolated from the 2014 (n=97) and 2015 (n=67) studies, 152 were used for serotyping from the 2014 (n=87) and 2015 (n=65) studies. Isolates confirmed as *Salmonella* were subjected to serotyping by conventional (serological) and a commercial method, which were performed by the National Veterinary Services Laboratories (NVSL; Ames, IA), and Check-Points Laboratory (Netherlands, EU), respectively. The conventional serotyping, performed at the NVSL, was based on the use of standard antisera to test for lipopolysaccharide (O-antigen) and flagellar (H-antigen) proteins following the Kauffmann and White scheme

(Kauffmann, 1966; Ewing, 1986; Wattiau et al., 2008). The serological method is considered the "gold standard" method of serotyping.

The commercial serotyping was performed at Check-Points Laboratory using their Check & Trace *Salmonella* kit. This kit is certified by the World Organization for Animal Health (OIE) and Association of Official Analytical Chemists (AOAC-RI) for 22 and 102 *Salmonella* serotypes, respectively. The kit includes the required components and reagents for the test. The test is based on multiplex ligation detection reaction (LDR) that targets genetic markers. All materials were supplied by the manufacturer (Check-Points, Wageningen, The Netherlands). The following steps, outlined by the manufacturer, were followed for testing: For DNA extraction, isolates were streaked onto nutrient agar plates and incubated at 37° C for 16-20 h. A toothpick was used to pierce a single isolated colony and inoculated into 100 µL of lysis buffer, heated for 5 min at 95° C. For PCR amplification, 10 µL of DNA extract was added to a strip tube containing 2.5 µL probe solution and 5 µL Solution A. The sample was heated for 3 min at 95° C, followed by 25 cycles of denaturation at 95° C for 30 sec, primer extension at 65° C for 4 min, and a final step at 98°C for 2 min in a thermocycler. A total of 15 µL fresh B mix solution was made, added to the tube, and heated at 37° C for 30 min, followed by 95° C for 10 min. A total of 15 µL fresh C mix was added to the tube, heated at 95° C for 10 min, followed by 35 cycles of denaturation at 95° C for 5 sec, primer annealing at 55° C for 30 sec, and primer extension at 72° C for 30 sec, and final incubation at 95° C for 2 min. The detection step used a multiple oligonucleotide embedded ArrayTube with control spots to measure efficiency of critical steps and 14 specific ligation detection reaction (LDR) probes (Wattiau et al., 2008; 2008). The ArrayTubes was washed with 300 µL detection buffer for 1 min at 50°C and 400 rpm in a Thermo Mixer (Eppendorf, Hamburg, Germany). This step was repeated once using fresh

300 µL detection buffer. A total of 10 µL of amplified sample was added to fresh 300 µL of detection buffer in the ArrayTube and incubated at 50°C for 30 min and 400 rpm. A wash step was performed with 300 µL of blocking buffer at 50° C for 3 min and 400 rpm. This step was repeated by replacing and resupplying blocking buffer at 30° C, for 5 min and 400 rpm. Fresh conjugate was prepared by adding conjugate solution to detection buffer at given ratios depending on sample size. Blocking buffer was removed from the array tube, replaced with 150 µL of conjugate solution, and incubated for 12 min at 30° C and 400 rpm. Conjugate was removed, replaced with 600 µL of detection buffer, incubated at 30° C for 1 min and 400 rpm. Detection buffer was discarded, replaced with 600 µL of fresh detection buffer, and incubated at 30°C for 1 min and 400 rpm. Detection buffer was removed, 150 µL of staining solution was added to the array tube, and incubation occurred at room temperature for 15 min. Results were analyzed using Check-Points software.

Antimicrobial susceptibility testing

All of the *Salmonella* strains isolated from the 2014 (n=97) and 2015 (n=67) studies were used for antimicrobial susceptibility testing (n=164). Minimum inhibitory concentrations (MIC) of antimicrobials were determined by the micro-broth dilution method according to the procedure outlined by Clinical Laboratory Standards Institute (CLSI 2012). Individual bacterial colonies were suspended in demineralized water (Trek Diagnostic Systems, Cleveland, OH) to achieve a 0.5 McFarland standard turbidity. An aliquot of the bacterial inoculum (10 µL) was added to Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH), vortexed, and dispensed at 50 µL into the Gram-negative NARMS panel plate (CMV3AGNF, Trek Diagnostics Systems, Cleveland, OH). Plates were incubated for 18-24 h at 37°C. The breakpoints were recorded as resistant, intermediate or sensitive based on the CLSI guidelines (CLSI, 2012).

Escherichia coli, ATCC 25922, served as the reference quality control strains for MIC testing.

Statistical Analysis

Statistical analysis was carried out using STATA (Stata Version 14.2, College Station, TX). Bivariate descriptive statistics of *Salmonella* prevalence by tylosin supplementation was done before multivariate analyses. The likelihood chi-square test was performed to assess unadjusted differences in *Salmonella* prevalence proportions between the tylosin or no tylosin and mild and severe abscesses.

Results

Prevalence of Salmonella in liver abscesses

For the 2014 study, a total of 200 and 183 liver abscesses were cultured from no-tylosin fed and tylosin-fed cattle, respectfully. Anaerobically, by direct plate method, a total of 33 and 31 *Salmonella* were isolated from no-tylosin-fed and tylosin-fed cattle, respectfully. Aerobically, by direct plate method, no *Salmonella* were isolated. Anaerobically, by enrichment method, no *Salmonella* were isolated. Aerobically, by enrichment method, a total of 33 and 10 *Salmonella* were isolated from no-tylosin-fed and tylosin-fed cattle, respectfully. The prevalence was lower in tylosin-fed group (16.9%; 31/183) compared to no tylosin-fed group (33%; 66/200; $P < 0.01$). A total of 97 strains of *Salmonella* were used from the 2014 study. (Table 1).

In the 2015 study, a total of 174 and 106 mild and severe liver abscesses were cultured, respectfully. Anaerobically, by direct plate method, a total of 9 and 4 *Salmonella* were isolated from mild and severe abscesses, respectfully. Aerobically, by direct plate method, no *Salmonella* were isolated. Anaerobically, by enrichment method, a total of 33 and 21 *Salmonella* were

isolated from mild and severe abscesses, respectfully. Aerobically, by enrichment method, a total of 28 and 19 *Salmonella* were isolated from mild and severe abscesses, respectfully. There was no difference in the prevalence of *Salmonella* between mild and severe liver abscesses. A total of 67 strains of *Salmonella* were used from the 2015 study. (Table 1).

Serotyping of Salmonella

Serotyping by serological method

In the 2014 study, a majority of the 87 strains were identified as serotype Lubbock (52/87; 59.8%). The other serotypes identified included Agona (24/87; 27.6%), Cerro (9/87; 10.3%), Give (1/87; 1.1%), and Muenster (1/87; 1.1%). (Table 2). In the 2015 study, few strains were identified as serotype Lubbock (14/65; 21.5%). The other major serotypes identified included Anatum (20/65; 30.8%) and Montevideo (18/65; 27.7%). A small number of strains were identified as Give (3/65; 4.6%), Kentucky (5/65; 7.7%), Mbandaka (2/65; 3.1%), Reading (2/65; 3.1%), and Schwarzengrund (1/65; 1.5%). The distribution of the serotypes as a proportion of the total serotypes was not different between the treatment groups (Table 2).

Serotypes detected by Check & Trace Salmonella method

In the 2014 study, Check &Trace method identified four serotypes, Agona (13/87; 14.9%), Cerro (10/87; 11.5%), Give (1/87; 1.1%) and Lubbock (63/87; 72.4%). The predominant serotypes was Lubbock (72.4%) (Table 3). The distribution of the serotypes as the proportion of the total in each treatment group was not different. In the 2015 study, Check & Trace method identified multiple serotypes. The three predominant serotypes were Antum (22/65; 33.8%), Lubbock (17/65; 26.2%) and Montevideo (15/65; 23.1%). The other serotypes included Give

(3/65; 4.6%), Kentucky (4/65; 6.2%), Mbandaka (2/65; 3.1%), and Reading (2/65; 3.1%) (Table 3).

Comparison of the two serotyping methods: Serological method vs. Check & Trace method

Of the 152 serotypes identified by serological method, only 98 (65%) were correctly identified by the Check & Trace method. Of all the serotypes, only Mbandaka (2 vs. 2) and Reading (2 vs.2) were identified correctly by both methods. The Check & Trace method did not identify any of the strains in the serotypes of Cerro (0 vs. 19) and Muenster (0 vs. 1), and Schwarzengrund (0 vs. 1) identified by the serological method. A majority of the strains in the serotypes Anatum (20 vs 19), Give (4 vs. 3), Kentucky (5 vs 4), and Montevideo (18 vs. 15) identified by serological method matched the serotyping by Check & Trace method. The check & Trace method identified only 50 of 66 Lubbock strains identified by serological method correctly. On the other hand, serological method matched with only 50 out of 80 Lubbock strains identified by Check & Trace method. The poorest match in serotype identity by the two methods was with the Agona serotype. Only 3 of the 24 strains were identified correctly by Check & Trace method and only 3 of 13 strains were identified correctly by the serological method (Table 4).

Antimicrobial susceptibility testing

Salmonella enterica serotypes in the 2014 study

A total of 97 isolates were subjected to antimicrobial susceptibility testing. All but two strains in the no-tylosin group and two strains in the tylosin group were pansusceptible (Table 5). In the no-tylosin-fed group, the two resistant isolates represented serotypes Cerro and Lubbock. The Cerro isolate was resistant to chloramphenicol and streptomycin with MIC's of 32 µg/ml and 64 µg/ml, respectfully. The Lubbock isolate was resistant to ceftiofur with an MIC of 8

μg/ml. In the tylosin-fed group, the two resistant isolates represented serotypes Agona and Lubbock. The Agona isolate was resistant to chloramphenicol, streptomycin, tetracycline with MIC's of 32, 64, and >32 μg/ml, respectfully. The Lubbock isolate was resistant to chloramphenicol, streptomycin, tetracycline with MIC's of 32, 64, and 32 μg/ml, respectfully.

Overall, the average MIC's of all serotypes fell within the lower half of each antibiotic's tested concentration range (Table 7), the number of resistant serotypes represented less than half of all resistant isolates (4/10; 40%) in this study (2014 and 2015) (Table 8), and serotypes Agona and Lubbock were the most prevalent serotypes resistant to antibiotics (Table 9, 10).

***Salmonella enterica* serotypes in the 2015 study**

A total of 67 isolates were subjected to antimicrobial susceptibility testing. All but three strains in the mild abscess group and three strains in the severe abscess group were pansusceptible (Table 6). In the mild abscess group, the three resistant isolates represented serotypes Anatum (n=2) and Mbandaka (n=1). The two Anatum isolatess were resistant to tetracycline with MIC's of 32 μg/ml. The Mbandaka isolate was resistant to ceftiofur with an MIC of 32 μg/ml. In the severe abscess group, the three resistant isolates represented Lubbock (n=1) and Reading (n=2). The Lubbock isolate was resistant to ampicillin with an MIC of 32 μg/ml. One Reading isolate was resistant to ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, streptomycin, tetracycline with MIC's of 32, 32, 8, 16, 32, 4, 64, and 32 μg/ml , respectfully. The other Reading isolate was resistant to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, and tetracycline with MIC's of >32/16, 32, 32, 8, 8, 32, 64, and 32 μg/ml, respectfully.

Overall, the average MIC's of all serotypes fell within the lower half of each antibiotic's tested concentration range (Table 7), the number of resistant serotypes represented more than

half of all resistant isolates (6/10; 60%) in this study (2014 and 2015) (Table 8), and serotype Anatum was one of the most prevalent serotypes resistant to antibiotics (Table 9).

Discussion

Not much is known about *Salmonella enterica* in liver abscesses of cattle. In fact, the very first report was in a recent study by Amachawadi and Nagaraja (2015). There have been a number of studies on liver abscesses in cattle that have outlined prevalence, economic impact, etiological agents, pathogenesis, and prevention, but they are primarily associated with *Fusobacterium necrophorum* (Nagaraja and Lechtenberg, 2007). These studies contributed to the following: feedlot cattle abscesses occur at an average rate of 12% to 32% (Brink et al., 1990). Average daily gain and feedlot efficiency can be negatively affected up to 11% and 9.7%, respectively. Liver abscesses are poly-microbial in nature with *Fusobacterium necrophorum* being the primary infectious organism (Langworth, 1997; Scanlan and Hathcock, 1983; Tan et al., 1996). Ruminal acidosis is a predisposing factor of hepatic abscess (Jensen et al., 1954). Tylosin, among five antibiotics formerly accepted to prevent liver abscess, is the most effective (Brown et al., 1973; Rogers et al., 1995).

A previous study provided evidence that mean minimum inhibitory concentrations of tylosin for *F. necrophorum* isolated from cattle fed diets with or without tylosin did not differ and that the incidence of *F. necrophorum*, as part of the mixed infection, was lower in the tylosin (n=36 cattle) group than in the no-tylosin (n=41 cattle) group (33% vs 61%). Although there was reduced incidence of liver abscess in the group fed tylosin, liver abscesses were still present. That evidence in combination with the antimicrobial susceptibilities of each group suggests that there may be other bacteria that cause liver abscesses in feedlot cattle. This would not be

surprising seeing that we have known hepatic abscesses as poly-microbial infections (Scanlan and Hathcock, 1983).

The role of *Salmonella* in causing liver abscesses is unknown. Other organisms are thought to have a synergistic relationship with *F. necrophorum* in liver abscesses of cattle, one being *Trueperella pyogenes* (Nagaraja et al., 1999). Although unknown, a similar relationship is possible with *Salmonella enterica* as both *F. necrophorum* and *Salmonella enterica* are Gram-negative and are capable of growing under anaerobic conditions. There are reports of *Salmonella* causing liver abscesses in humans (Chaudhary et al., 2003; Qu et al**.,** 2013). The ability to survive intracellularly within phagocytic cells allows *Salmonella* to reach liver from any other location in the body (Ibarra and Steele-Mortimer, 2009). It is likely that *Salmonella* residing in the gut enters through the gut epithelium, which may be facilitated by inflammation of the gut epithelium associated with acidosis in the gut. Further studies are warranted to investigate whether *S. enterica* is one of the etiologic agents or entered, via blood or lymph, into an abscess initiated by *F. necrophorum* in the liver and survived.

In the present study, *Salmonella* isolation from liver abscess samples included direct plating of the sample on a selective medium and a conventional enrichment step in a broth before plating on a selective medium. In a few instances, samples that did not yield *Salmonella* by direct plating were positive for *Salmonella* after it was enriched in R-V broth, which suggests a much lower concentration of *Salmonella* in some abscesses compared to those that yielded by direct plating. Interestingly, direct plating of the abscess samples on HE agar plate, a medium that is selective and differential for *Salmonella,* yielded *Salmonella* only when the plates were incubated anaerobically, not when incubated aerobically. The anaerobic incubation was necessary for initial isolation only because subsequent isolates grew well under aerobic

condition. Although *Salmonella* is categorized as a facultatively anaerobic bacteria, it is capable of robust growth under anaerobic condition (Yamamoto and Droffner, 1985). In fact, there is evidence that *Salmonella* grown anaerobically is more invasive and virulent and adheres better to mammalian cells compared to aerobically-grown cells (Lee and Falkow, 1990; Schiemann and Shope, 1991). The prevalence of *Salmonella* was lower in tylosin-fed compared to no tylosin-fed cattle, which is somewhat surprising because tylosin is a Gram positive-spectrum antibiotic and has no inhibitory effect on *Salmonella.* It is likely that the effect of tylosin on *Salmonella* is mediated via changes in gut flora. This needs to be investigated.

Multiple serotypes were detected by serological and Check & Trace methods. Among all the serotypes detected, Lubbock (n=75), closely related to the serotype Mbandaka (Bugarel et al., 2015), represented most *Salmonella* isolates and has been isolated from lymph nodes of cattle at slaughter (Bugarel et al., 2015).

The serological method carried out in the National Veterinary Service Laboratory is based on the antigenic structures of the O polysaccharides and flagellar proteins. Because the method is the most commonly used method of serotyping it is considered as the gold standard. The Check & Trace *Salmonella* method is a molecular method that discriminates *Salmonella* serotypes based on differences in their DNA sequence. The genetic test is relatively rapid and is based on a microarray platform. Each position on the microarray represents a specific DNA marker associated with a unique *Salmonella* target sequence. Spots only become visible if the DNA markers exactly match the corresponding DNA sequences of the *Salmonella* isolate. The combination of present and absent spots yields a pattern known as a genovar. The software provided with the test translates these scores to the known serotypes.

A couple of serotypes (Mbandaka and Reading) were identified by both methods or were identified by the serological method (Cerro, Muenster, and Schwarzengrund) and not by Check & Trace method. Other serotypes (Anatum, Give, Kentucky, Montevideo, and Lubbock) were identified by both methods with exceptions of a few strains. If serological method is considered as the gold standard, it becomes obvious that Check & Trace is a good method for certain serotypes but not all. The discrepancy in serotyping between the two methods require further investigation. As stated in the Check&Trace *Salmonella* user manual, certain serotypes generate an overlapping score with another serotype. Because Check & Trace is a commercial method, further validation of the method is required.

In this study, only ten strains were resistant to certain antibiotics representing serotypes Agona, Anatum, Cerro, Lubbock, Mbandaka, and Reading. The reason for limited resistance is unknown. The serotype, Reading, represented two of the ten strains and was multidrug-resistant serving as a higher health threat to the animal, and in turn, to humans. *Salmonella enterica* serotype Reading has been reported as a causative agent of a multi-state and province salmonellosis outbreak from alfalfa sprouts and unknown food sources as reported by the Centers for Disease Control and Prevention in 2016 and the Public Health Agency of Canada in 2017, respectively. One of each of the serotypes Agona and Lubbock were also multidrug resistant. *Salmonella enterica* serotype Agona, Cerro, Lubbock, and Mbandaka have been found as agents in multi-state outbreaks linked to rice and wheat puff cereal (CDC, 2008), in dairy cattle herds with increasing cases of salmonellosis (Cummings et al., 2010), in beef cattle as a new serotype (Amachawadi and Nagaraja, 2015), and in diets containing a vegetable fat supplement causing illness in dairy cattle (Jones et al., 1982), respectively.

Conclusion

This study provides evidence that *Salmonella* is present in liver abscesses of cattle and initial isolation requires anaerobic condition. Multiple serotypes were involved with Lubbock being the dominant serotype. The commercial serotyping method of Check & Trace did not match with serotyping by the conventional serological method suggesting further validation of method is required. A majority of the strains were pansusceptible and a small number of strains exhibited antimicrobial resistance. Whether *Salmonella* is an etiologic agent of liver abscesses or just happen to survive in an abscess initiated by *Fusobacterium necrophorum,* considered as the primary etiological agent, remains to be determined.

Table 3.1 Prevalence of *Samlonella enterica* in liver abscesses of feedlot cattle

Culture method	2014 study[a]		2015 study[b]	
	No tylosin-fed cattle	Tylosin-fed cattle	Mild liver abscesses[c]	Severe liver abscesses[d]
No. of abscesses cultured	200	183	174	106
Isolation method				
Direct plating on Hektoen-Enteric (HE) agar				
No. aerobically isolated	0	0	0	0
No. anaerobically isolated	33	21	9	4
Plating on HE agar after enrichment[e]				
No. aerobically isolated	33	10[f]	28	19
No. anaerobically isolated	-	-	33	21
Total no. of abscesses positive for *Salmonella* (% of liver abscesses cultured)	66 **(33)**	31[f] **(16.9)**	38[c] **(21.8)**	29[d] **(27.4)**

[a] 2014 study: A total of 383 liver abscess samples were cultured (data adapted from Amachawadi et al., 2017).

[b] 2015 study: A total of 280 liver abscess samples were cultured.

[c] Liver abscess score of A or A- (Livers with a few [1 to 4] small abscesses)

[d] Liver abscess score of A+ (Livers with one large or multiple small abscesses).

[e] Abscess samples were inoculated into tetrathionate broth at 37° C for 24 h followed by enrichment in Rappaport-Vassiliadis broth at 42° C for 24 h before plating onto HE agar.

[f] Significantly different from cattle fed no tylosin

Table 3.2 Serotyping of *Salmoenlla enterica* isolated from liver abscesses of feedlot cattle by serological method[a]

Serotype	2014 study[a]		2015 study[c]	
	No tylosin-fed cattle (n=59)	Tylosin-fed cattle (n=28)	Mild Abscesse[d] (n=37)	Severe abscesses[e] (n=28)
Agona	15 (25.4)[f]	9 (32.1)	-	-
Anatum	-	-	11 (29.7)	9 (32.1)
Cerro	7 (11.9)	2 (7.1)	-	-
Give	1 (1.7)	-	1 (2.7)	2 (7.1)
Kentucky	-	-	1 (2.7)	4 (14.3)
Lubbock	36 (61.0)	16 (57.1)	10 (27.0)	4 (14.3)
Mbandaka	-	-	1 (2.7)	1 (3.6)
Montevideo	-	-	12 (32.4)	6 (21.4)
Muenster	-	1	-	-
Reading	-	-	-	2 (7.1)
Schwarzengrund	-	-	1 (2.7)	-

[a]Performed by the National Veterinary Services Laboratorb in Ames, IA.
[b]2014 study: A total of 383 liver abscess samples were cultured (data adapted from Amachawadi et al., 2017).
[c]2015 study: A total of 280 liver abscess samples were cultured.
[d]Liver abscess score of A or A- (Livers with a few [1 to 4] small abscesses)
[e] Liver abscess score of A+ (Livers with one large or multiple small abscesses).
[f]Numbers in parentheses are percentages of the total stains in the column.

Table 3.3 Serotyping of *Salmonella enterica* isolated from liver abscesses of feedlot cattle by Check &Trace *Salmonella* method[a]

Serotype	2014 study[a]		2015 study[c]	
	No tylosin-fed cattle (n=59)	Tylosin-fed cattle (n=28)	Mild Abscesse[d] (n=37)	Severe abscesses[e] (n=28)
Agona	7 (11.9)[f]	6 (21.4)	-	-
Anatum	-	-	11 (29.7)	11 (39.3)
Cerro	7 (11.9)	3 (10.7)	-	-
Give	-	1 (3.6)	1 (2.7)	2 (7.1)
Kentucky	-	-	1 (2.7)	3 (10.7)
Lubbock	45 (76.3)	18 (64.3)	12 (32.4)	5 (17.9)
Mbandaka	-	-	1 (2.7)	1 (3.6)
Montevideo	-	-	11 (39.3)	4 (14.3)
Muenster	-	-	-	-
Reading	-	-	-	2 (7.1)
Schwarzengrund	-	-	-	-

[a]Check-Points B. V., Wageningen, The Netherlands.
[b]2014 study: A total of 383 liver abscess samples were cultured (data adapted from Amachawadi et al., 2017).
[c]2015 study: A total of 280 liver abscess samples were cultured.
[d]Liver abscess score of A or A- (Livers with a few [1 to 4] small abscesses)
[e] Liver abscess score of A+ (Livers with one large or multiple small abscesses).
[f]Numbers in parentheses are percentages of the total stains in the column.

Table 3.4 Comparison of serotyping of *Salmonella enterica* isolated from liver abscesses of feedlot cattle by serological and Check & Trace *Salmonella* methods

Serotype	Serological[a] (n=152)	Check & Trace[b] (n=152)	Check & Trace[b] (n=152)	Serological[a] (n=152)
Agona	24	3	13	3
Anatum	20	19	22	19
Cerro	9	0	10	0
Give	4	3	4	3
Kentucky	5	4	4	4
Lubbock	66	50	80	50
Mbandaka	2	2	2	2
Montevideo	18	15	15	15
Muenster	1	0	-	-
Reading	2	2	2	2
Schwarzengrund	1	0	-	-

[a]Serological method was performed by National Veterinary Services Laboratory in Ames, IA.
[b]Check-Points B. V., Wageningen, The Netherlands.

Table 3.5 Antimicrobial susceptibilities of *Salmoenlla enterica* strains isolated from liver abscesses of feedlot cattle in the 2014 study (n=97)

Class and antimicrobial compounds tested	Concentration range tested (µg/ml)	Resistant breakpoints (µg/ml)	*Salmonella* isolates from:			
			No tylosin cattle (n=66)		Tylosin-fed cattle (n=31)	
			Average MIC[ab] (µg/ml)	Number resistant (%)	Average MIC[ab] (µg/ml)	Number resistant (%)
Aminoglycoides						
Gentamicin	0.25 – 16	≥ 16	0.4	0	0.7	0
Streptomycin	2 - 64	≥ 64	9.0	1 (1.5)	10.6	2 (6.5)
B-Lactams						
Amoxicillin/Clavulanic acid 2:1 ratio	1/0.5 – 32/16	≥ 32/16	1/0.5	0	1/0.5	0
Ampicillin	1 - 32	≥ 32	1	0	1	0
Cephalosporins						
Cefoxitin	0.5 - 32	≥ 32	2.6	0	2.6	0
Ceftiofur	0.12 - 8	≥ 8	0.6	1 (1.5)	0.5	0
Ceftriaxone	0.25 - 64	≥ 4	0.3	0	0.3	0
Macrolides						
Azithromycin	0.12 - 16	N/A[a]	3.2	N/A[c]	3.2	N/A[c]
Phenicols						
Chloramphenicol	2 - 32	≥ 32	4.7	1 (1.5)	6.1	2 (6.5)
Quinolones						
Ciprofloxacin	0.015 – 4	≥ 1	0.02	0	0.02	0
Nalidixic Acid	0.5 – 32	≥ 32	1.7	0	1.7	0
Sulfonamides						
Sulfisoxazole	16 - 256	≥ 512	94.1	0	107.4	0
Trimethoprim/Sulfamethoxazole	0.12-4.0/ 2.38-76.0	≥ 4/76	0.1/2.4	0	0.1/2.4	0
Tetracyclines						
Tetracycline	4 – 32	≥ 16	4	0	4.9	2 (6.5)

[a] Minimum inhibitory concentration.
[b] If the MIC included a symbol such as "≥ 32" or "< 1" the average was obtained by using the value "32" or "1".
[c] N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

Table 3.6 Antimicrobial susceptibilities of *Salmoenlla enterica* strains isolated from liver abscesses of feedlot cattle in the 2015 study (n=67)

Class and antimicrobial compounds tested	Concentration range tested (μg/ml)	Resistant breakpoints (μg/ml)	Category			
			Mild liver abscesses[a] (n=38)		Severe liver abscesses[b] (n=29)	
			Average MIC[cd]	Number resistant (%)	Average MIC[cd]	Number resistant (%)
Aminoglycoides						
Gentamicin	0.25 – 16	≥ 16	0.6	0	0.6	0
Streptomycin	2 - 64	≥ 64	10.4	0	14.4	2 (6.9)
B-Lactams						
Amoxicillin/Clavulanic acid 2:1 ratio	1/0.5 – 32/16	≥ 32/16	2.2/1.1	0	4.0/2.0	1 (3.4)
Ampicillin	1 - 32	≥ 32	1.4	0	4.7	3 (10.3)
Cephalosporines						
Cefoxitin	0.5 - 32	≥ 32	4.3	1 (2.6)	6.0	2 (6.9)
Ceftiofur	0.12 - 8	≥ 8	0.9	0	1.3	2 (6.9)
Ceftriaxone	0.25 - 64	≥ 4	0.3	0	1.1	2 (6.9)
Macrolides						
Azithromycin	0.12 - 16	N/A[a]	6.2	N/A[e]	5.8	N/A[e]
Phenicols						
Chloramphenicol	2 - 32	≥ 32	7.6	0	8.1	2 (6.9)
Quinolones						
Ciprofloxacin	0.015 – 4	≥ 1	0.02	0	0.2	1 (3.4)
Nalidixic Acid	0.5 – 32	≥ 32	3.2	0	3.4	0
Sulfonamides						
Sulfisoxazole	16 - 256	≥ 512	104.4	0	125.4	0
Trimethoprim/Sulfamethoxazole	0.12-4.0/ 2.38-76.0	≥ 4/76	0.1/2.4	0	0.1/2.6	0
Tetracyclines						
Tetracycline	4 – 32	≥ 16	5.5	2 (5.3)	5.9	2 (6.9)

[a] Liver abscess score of A or A- : Livers with 1 to 4 small abscesses.
[b] Liver abscess score of A+ : Livers with one large or multiple small abscesses.
[c] Minimum inhibitory concentration.
[d] If the MIC included a symbol such as "≥ 32" or "< 1" the average was obtained by using the value "32" or "1".
[e] N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

Table 3.7 Average minimum inhibitory concentrations of *Salmonella enterica* serotypes from liver abscesses of feedlot cattle in the 2014 (n=97) and 2015 (n=67). studies

Class and antimicrobial compound	Resistance breakpoint	Serotypes										
		Agona (26)	Anatum (20)	Cerro (10)	Give (4)	Kent-ucky (5)	Lubb-ock (75)	Mban-daka (2)	Monte -video (18)	Muen-ster (1)	Read-ing (2)	Schwar-Zengrund (1)
Aminoglycosides												
Gentamicin	≥ 16	0.56	0.45	0.58	0.44	0.60	0.56	0.75	0.56	0.25	0.38	1.0
Streptomycin	≥ 64	10.3	12.2	12.4	10.0	13.6	9.8	12	4.7	8	64.0	8.0
B-Lactams												
Amoxicillin/ Clavulanic acid (2:1)	≥ 32/16	1.0/ 0.50	1.8/ 0.90	1.0/ 0.50	1.0/ 0.50	2.4/ 1.2	1.0/ 0.51	1.0/ 0.50	3.4/ 1.7	1.0/ 0.50	32.0/ 16.0	4.0/ 2.0
Ampicillin	≥ 32	1	1.6	1	1	1.6	1.4	1.0	1.6	1.0	32.0	2.0
Cephalosporines												
Cefoxitin	≥ 32	2.7	4.2	3	2.5	4.8	2.9	18.0	2.6	2.0	32.0	4.0
Ceftiofur	≥ 8	0.5	0.83	0.48	0.63	0.90	0.68	1.0	0.86	0.50	8.0	0.50
Ceftriaxone	≥ 4	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	12.0	0.25
Macrolides												
Azithromycin	N/A[a]	3.3	5	3.6	3.5	4.8	3.9	6.0	7.3	2.0	4.0	4.0
Phenicols												
Chloramphenicol	≥ 32	5.1	6.6	7.6	6.0	7.2	5.2	8.0	8.4	4.0	32.0	16.0
Nalidixic Acid	≥ 32	1.6	3.5	1.6	3.5	3.6	1.9	2.0	3.8	1.0	4.0	2.0
Quinolones												
Ciprofloxacin	≥ 1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	2.0	0.02
Sulfonamides												
Sulfisoxazole	≥ 512	103.4	114.4	115.2	64.0	217.6	102.5	128.0	62.2	64.0	256.0	32.0
Trimethoprim/ Sulfamethoxazole	≥ 4/76	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.12/ 2.4	0.25/ 4.8	0.12/ 2.4
Tetracyclines												

Tetracycline	≥ 16	5.1	6.8	4.0	4.0	4.0	4.4	4.0	4.0	4.0	32	4.0

[a] N/A = not applicable: The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

Table 3.8 Number of antibiotic-resistant *Salmonella enterica* serotypes from liver abscesses of feedlot cattle in the 2014 (n=97) and 2015 (n=67) studies

	Serotypes											
Class and antimicrobial compound	Agona (26)	Anatum (20)	Cerro (10)	Give (4)	Kent-ucky (5)	Lubb-ock (75)	Mban-daka (2)	Monte -video (18)	Muen-ster (1)	Read-ing (2)	Schwar-Zengrund (1)	Total (164)
Aminoglycosides												
Gentamicin	0	0	0	0	0	0	0	0	0	0	0	0
Streptomycin	1	0	1	0	0	1	0	0	0	2	0	5
B-Lactams												
Amoxicillin/ Clavulanic acid 2:1	0	0	0	0	0	0	0	0	0	1	0	1
Ampicillin	0	0	0	0	0	1	0	0	0	2	0	3
Cephalosporines												
Cefoxitin	0	0	0	0	0	0	1	0	0	2	0	3
Ceftiofur	0	0	0	0	0	1	0	0	0	2	0	3
Ceftriaxone	0	0	0	0	0	0	0	0	0	2	0	2
Macrolides												
Azithromycin	0	0	0	0	0	0	0	0	0	0	0	0
Phenicols												
Chloramphenicol	1	0	1	0	0	1	0	0	0	2	0	5
Nalidixic Acid	0	0	0	0	0	0	0	0	0	0	0	0
Quinolones												
Ciprofloxacin	0	0	0	0	0	0	0	0	0	1	0	1
Sulfonamides												
Sulfisoxazole	0	0	0	0	0	0	0	0	0	0	0	0
Trimethoprim/ Sulfamethoxazole	0	0	0	0	0	0	0	0	0	0	0	0
Tetracyclines												
Tetracycline	1	2	0	0	0	1	0	0	0	2	0	6

Table 3.9 Antimicrobial susceptibilities of the most prevalent *Salmonella enterica* serotypes, Agona, Anatum, and Lubbock, isolated from liver abscesses

			Serotypes[a]					
			Agona (n=26)		Anatum (n=20)		Lubbock (n=75)	
Class and antimicrobial compounds tested	Resistant breakpoints (μg/ml)	Concentration range tested (μg/ml)	Avg. MIC[b]	No. Resistant[c]	Avg. MIC[b]	No. Resistant[c]	Avg. MIC[b]	No. Resistant[c]
Aminoglycoides								
Gentamicin	≥ 16	0.25 – 16	0.56	0	0.45	0	0.56	0
Streptomycin	≥ 64	2 – 64	10.3	1	12.2	0	9.8	1
B-Lactams								
Amoxicillin/ Clavulanic acid 2:1	≥ 32/16	1/0.5 – 32/16	1.0/ 0.50	0	1.8/ 0.90	0	1.0/ 0.51	0
Ampicillin	≥ 32	1 – 32	1	0	1.6	0	1.4	1
Cephalosporines								
Cefoxitin	≥ 32	0.5 – 32	2.7	0	4.2	0	2.9	0
Ceftiofur	≥ 8	0.12 – 8	0.5	0	0.83	0	0.68	1
Ceftriaxone	≥ 4	0.25 – 64	0.25	0	0.25	0	0.25	0
Macrolides								
Azithromycin	N/A[d]	0.12 – 16	3.3	0	5	0	3.9	0
Phenicols								
Chloramphenicol	≥ 32	2 – 32	5.1	1	6.6	0	5.2	1
Nalidixic Acid	≥ 32	0.5 – 32	1.6	0	3.5	0	1.9	0
Quinolones								
Ciprofloxacin	≥ 1	0.015 – 4	0.02	0	0.02	0	0.02	0
Sulfonamides								
Sulfisoxazole	≥ 512	16 – 256	103.4	0	114.4	0	102.5	0
Trimethoprim/ Sulfamethoxazole	≥ 4/76	0.12-4.0/ 2.38-76.0	0.12/2.4	0	0.12/2.4	0	0.12/2.4	0
Tetracyclines								
Tetracycline	≥ 16	4 – 32	5.1	1	6.8	2	4.4	1

[a] Serotypes Agona, Anatum, and Lubbock account for 121 of 164 *Salmonella* isolates in study 2014 and 2015 combined.
[b] Average minimum inhibitory concentration.
[c] Number resistant.
[d] N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

Table 3.10 Antimicrobial susceptibilities for *Salmonella* Lubbock isolated from liver abscesses

Class and antimicrobial compound tested	Resistant breakpoints (µg/ml)	Concentration range tested (µg/ml)	No-tylosin cattle (n=40)		Tylosin-fed cattle (n=19)		Mild liver abscesses[a] (n=11)		Severe liver abscesses[b] (n=5)	
			Avg. MIC[c]	No. Res[d]	Avg. MIC[c]	No. Res[d]	Avg. MIC[c]	No. Res[d]	Avg. MIC[c]	No. Res[d]
Aminoglycoides										
Gentamicin	≥ 16	0.25 –16	0.37	0	0.75	0	0.82	0	0.75	0
Streptomycin	≥ 64	2 - 64	8.2	0	9.7	1	15.6	0	10.8	0
B-Lactams										
Amoxicillin/ Clavulanic acid 2:1	≥ 32/16	1-32/ 0.5-16	1.0/ 0.5	0	1.0/0.5	0	1.0/0.5	0	1.2/0.6	0
Ampicillin	≥ 32	1 - 32	1.0	0	1.0	0	1.1	0	7.2	1
Cephalosporines										
Cefoxitin	≥ 32	0.5 - 32	2.3	0	2.7	0	4.6	0	4.2	0
Ceftiofur	≥ 8	0.12 - 8	0.69	1	0.53	0	0.84	0	0.82	0
Ceftriaxone	≥ 4	0.25 - 64	0.25	0	0.25	0	0.25	0	0.25	0
Macrolides										
Azithromycin	N/A[e]	0.12 - 16	3.1	0	3.2	0	6.6	0	8.0	0
Phenicols										
Chloramphenicol	≥ 32	2 - 32	4.3	0	5.7	1	6.9	0	6.8	0
Nalidixic Acid	≥ 32	0.5 – 32	1.9	0	1.6	0	2.2	0	2.8	0
Quinolones										
Ciprofloxacin	≥ 1	0.015 – 4	0.02	0	0.02	0	0.02	0	0.02	0
Sulfonamides										
Sulfisoxazole	≥ 512	16 - 256	88.0	0	107.8	0	139.6	0	116.0	0
Trimethoprim/ Sulfamethoxazole	≥ 4/76	0.12-4.0/ 2.38-76.0	0.12/ 2.4	0	0.12/ 2.4	0	0.12/ 2.4	0	0.15/ 2.9	0
Tetracyclines										
Tetracycline	≥ 16	4 – 32	4	0	5.47	1	4	0	4	0

[a] Liver abscess score of A or A- : Livers with 1 to 4 small abscesses.
[b] Liver abscess score of A+ : Livers with one large or multiple small abscesses.
[c] Average minimum inhibitory concentration. If the MIC included a symbol such as "≥ 32" or "< 1" the average was obtained by using the value "32" or "1".
[d] Number resistant.
[e] N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

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