

Impact of *in-feed* versus *in-water* chlortetracycline and tiamulin
administrations on fecal prevalence and antimicrobial susceptibilities of
Campylobacter and *Salmonella* in a population of nursery piglets

by

Victor Landelinus Ishengoma

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Approved by:
Major Professor
Dr. Raghavendra G. Amachawadi

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Abstract

Antimicrobial resistance (AMR) in bacteria is a growing public health concern in the US and around the world threatening the continual use of antimicrobials. Antimicrobial resistant *Campylobacter* and *Salmonella* are listed as serious level threats to the public health by the Centers for Disease Control and Prevention. *Campylobacter* and *Salmonella* are common foodborne pathogens that reside in the gut of pigs and are shed in feces with the potential to be transmitted to humans. In pigs, oral route, either in-feed or in-water, is by far the most common route of administration of antimicrobials. Because the distribution of the antibiotic in the gut and the dosages are different, the impact of in-feed vs. in-water administration of antibiotics on the development of AMR is likely to be different. Therefore, a study was conducted to compare in-feed vs. in-water administrations of chlortetracycline (CTC) and or tiamulin on fecal prevalence and AMR profiles of *Campylobacter* and *Salmonella* in nursery piglets. A total of 1,296 weaned piglets, allocated into 48 pens (27 piglets per pen), were assigned randomly to six treatment groups: Control (no antibiotic), in-feed CTC, in-water CTC, in-feed tiamulin, in-water tiamulin, or in-feed CTC and tiamulin. Fecal samples were collected randomly from 5 piglets from each pen during pre-treatment (days -7, 0), treatment (days 7, 14) and post-treatment (days 21, 28) phases. Bacterial isolations and species identifications were done by culture and PCR, respectively. The microbroth dilution method with SensititreTM plates were used to determine antimicrobial susceptibility and resistance of *Campylobacter* and *Salmonella* strains. The results on resistance were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for *Salmonella* and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cutoff values for *Campylobacter*. Whole genome sequencing (WGS)

of *Campylobacter* and *Salmonella* isolates was done on an Illumina HiSeq platform. Overall prevalence of *Campylobacter* and *Salmonella* were 18.2% (262/1,440) and 3.0% (43/1,440) respectively. Speciation of *Campylobacter* isolates by PCR indicated *C. hyointestinalis* (17.9%; 258/1,440) and *C. coli* (0.3%; 4/1,440), while WGS indicated that all the *Salmonella* strains were serovar Typhimurium. *Campylobacter* isolates were resistant to tetracycline (98.5%), ciprofloxacin (89.3%), and nalidixic acid (60.3%). *Salmonella* isolates were susceptible to azithromycin and totally resistant (100%) to ampicillin, streptomycin, sulfisoxazole, tiamulin, and tetracycline. Also, WGS revealed a high correlation of phenotypic and genotypic AMR in both *Campylobacter* and *Salmonella*. Most of the *Campylobacter* and *Salmonella* isolates were multidrug resistant. Neither antibiotic nor the route of administration had an effect ($P > 0.05$) on the occurrence of resistant *Salmonella* or *Campylobacter* in the feces of piglets.

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Chapter 1 - LITERATURE REVIEW

Antimicrobial usage in Animal Agriculture

The increase in global population coupled with the increase in demand for livestock products has coincided with increased consumption of antimicrobials in food animal agriculture (Van Boeckel et al., 2015; Schar et al., 2018). Efforts to meet the rising demand for animal-source nutrition have driven a shift in animal production from smallholder, mixed crop, and livestock operations to increasingly intensive, large-scale, and specialized commercialization. Historically, intensive animal production included administration of antimicrobials for treatment and prevention of diseases and growth promotion (Van Boeckel et al., 2015; Kimera et al., 2020). As a consequence, tons of antimicrobials have been used in animal agriculture (Silbergeld et al., 2008). In total, 73% of all antimicrobials consumed globally are used in food-producing animals (Van Boeckel et al., 2017). Approximately 93,309 tons of antimicrobials were consumed in food animal production globally in 2017 (Tiseo et al., 2020). These figures are expected to rise 1.5% to 104,079 tons by 2030 (Tiseo et al., 2020).

Data on antimicrobial consumption in animal agriculture is well documented in high income countries as opposed to low and middle income countries (Schar et al., 2018). In the US, the U.S. Food and Drug Administration (FDA) estimates a grand total of 11,468,357 kg of both medically important and not medically important antimicrobials for use in food-producing animals were actively marketed in 2019 (FDA, 2019). Medically important antimicrobials are antimicrobials in classes used for human medicine (WHO, 2017). Swine and cattle consume the majority of these antimicrobials, 42% versus 41% for swine and cattle respectively. This highlights the potential role the two species may play in the selection for resistant bacteria.

Nevertheless, the 2019 FDA report indicated a significant food producing animal antimicrobial consumption decrease of 25% from 2010 through 2019 and 36% from 2015 through 2019 in the US (FDA, 2019). The decrease was seen across all food producing animals in the 2016-2019 period. Chickens displayed the leading 62% decrease, followed by cattle and swine at 32% and 30% decrease respectively. The FDA guidance requires the use of medically important antimicrobials in animals to be limited to uses that are considered necessary for assuring animal health and should only be used under veterinary oversight or consultation (FDA Guidance for Industry #209; 2017). Evidence suggesting the association of antimicrobials usage in animal agriculture and development of AMR in commensal and pathogenic bacteria exists in literature (Funk et al., 2006; Marshall & Levy, 2011; Landers, 2012; Liebana et al., 2013; M. J. Martin et al., 2015).

Antimicrobial usage in the swine production

Pork is the most widely consumed meat in the world (FAO, 2014). In the US, the size swine herds and specialization in swine production have been increasing for the past several years (USDA-ERS, 2007). Based on the USDA hog inventory survey released in March 2021, the US has 74.8 million heads (hogs and pigs) (<https://downloads.usda.library.cornell.edu/usda-esmis/files/rj430453j/7p88db205/mw22w1890/hgpg0321.pdf>) indicating a 13.7% increase from 65.9 million head as of December 2011 (<https://downloads.usda.library.cornell.edu/usda-esmis/files/rj430453j/9p290c11x/4x51hm06m/HogsPigs-12-23-2011.pdf>). Larger swine operations are likely to use antimicrobials to control infections (Macdonald & McBride, 2009). When compared to other food animals swine have the highest antimicrobial consumption in the US (FDA, 2019). Approximately, 2,582,399 kg³, 42% of all medically important antimicrobials

marketed for food-producing animals in 2019 in the US were consumed by swine indicating a 9% increase from 2,374,277 kg³ consumed in 2018 (FDA, 2019). According to Tiseo et al., (2020) the swine industry has the highest projected antimicrobial consumption globally, expected to contribute to 44% of the total increase in antimicrobials consumed in food producing animals by 2030. A wide array of antimicrobials are used in swine production systems in the US including tetracyclines, macrolides, pleuromutilins, lincosamides, beta lactams, aminoglycosides, fluoroquinolones and cephalosporins (Davies & Singer, 2020). But tetracyclines, macrolides and pleuromutilins are the most commonly used antimicrobials in swine production (Apley et al., 2012; Holman & Chénier, 2013). The macrolides were declared by the World Health Organization (WHO) as critically important antimicrobials, while tetracyclines and a pleuromutilin, tiamulin are declared highly important and important antimicrobials respectively (WHO, 2017). For antimicrobials to be classified as critically important, they must meet the WHO criterion 1 (C1), i.e. sole or one of limited therapies, to treat serious bacterial infections in people and criterion 2 (C2) of antimicrobials used to treat infections in humans caused by bacteria that could be acquired from non-human sources and could acquire AMR genes from sources other than humans (WHO, 2017). Highly important antimicrobials must meet either C1 or C2 category and important antimicrobials do not meet either of the categories (WHO, 2017). According to Davies & Singer, (2020), critically important antimicrobials contributed 4.5% and 5.3% of the total antimicrobial consumption in growing pig production in 2016 and 2017, respectively, while important antimicrobials constituted 80.7% and 78.4%, respectively.

Tetracyclines

Tetracyclines were discovered in the 1940s and were found to be highly effective against a wide range of bacteria, thus becoming a class of broad-spectrum antibiotics (Griffin et al., 2010). Chlortetracycline was the first tetracycline discovered and introduced in human and animal medicine in 1948 (Griffin et al., 2010). Years later other analogues were discovered which include the naturally occurring tetracyclines and demethylchlortetracycline and semisynthetic analogues, doxycycline, and minocycline (Chopra & Roberts, 2001). To date, tetracyclines are widely used in animal agriculture with chlortetracycline (CTC) and oxytetracycline (OTC) being the two most consumed antimicrobial members of the tetracycline class in swine production (Fritz & Zuo, 2007).

Tetracyclines inhibit protein synthesis by interacting with the 30S bacterial ribosome and preventing the association of aminoacyl-tRNA and ribosomal acceptor (A) site (Chopra & Roberts, 2001). Tetracycline structural features that confer activity against bacteria have been established (Mitscher, 1978; Rogalski, 1985; Dax, 1997). Tetracyclines are bacteriostatic at low doses but become lethal to bacteria at high doses. Tetracyclines are broad spectrum antimicrobials with inhibitory activity against gram-positive and gram-negative bacteria, mycoplasmas, chlamydia, rickettsia, and protozoans (Chopra & Roberts, 2001). Since their discovery, tetracyclines have been used all over the world for therapeutic and non-therapeutic uses in a multitude of species including food producing animals. In the US, tetracyclines are approved for use in food animals and frequently used due to their broad-spectrum nature and low cost (Granados-chinchilla & Rodríguez, 2017). This is evidenced by 2019 FDA estimates where 4,117,031 kg³ of tetracyclines were sold and distributed for use in food producing animals with a 4% increase from 2018 (FDA, 2019).

Chlortetracycline is used in swine for the treatment of bacterial enteritis caused by *Escherichia coli* and *Salmonella choleraesuis* and pneumonia caused by *Pasteurella multocida*. In their study, Apley et al. (2012), reported chlortetracycline having the highest estimated annual use in swine production in the United States. The study estimated the annual consumption of 533,973 kg, out of which 204,580 kg (38.3) were used in grower/finisher phase pigs and 329,393 kg (61.7%) used in nursery piglets. Nursery piglets are more prone to enteric diseases (Laine et al., 2008), which justifies the large amount of antimicrobials used in the nursery period as compared to the grower/finisher stages. Similarly, Davies & Singer, (2020) reported tetracyclines to contribute 60% of all antimicrobial consumption in swine in 2016 and 2017 with chlortetracycline constituting 49.3% and 40.9% in 2016 and 2017 respectively. It has also been used in combination with tiamulin. Chlortetracycline is only available as an oral formulation for use in food animals in the US.

Tiamulin

Tiamulin is a semi-synthetic pleuromutilin, a class of antimicrobial substance, produced by the fungus, *Pleurotus mutilus* (EMA, 2013). It is bacteriostatic to gram-positive bacteria, anaerobic bacteria and Mycoplasma (EMA, 2013, Giguere, 2006).) Tiamulin hydrogen fumarate is used in swine to control dysentery associated with *Brachyspira hyodysenteriae* and swine pneumonia caused by *Actinobacillus pleuropneumoniae* (NAHMS Swine Study, 2017; Davies & Singer, 2020). It has also been proven to be effective against porcine proliferative enteropathy (PPE) associated with *Lawsonia intracellularis*. According to FDA, the dosage administered depends on the targeted pathogen. For the treatment of swine dysentery associated with *B. hyodysenteriae*, a dosage of 3.5 mg/lb is used and for the treatment of *A. pleuropneumoniae*

infections, a dosage of 10.5 mg/lb is used. Tiamulin is compatible with many drugs used in swine including tetracyclines. But it was stipulated that pigs administered with tiamulin should not be fed feed containing polyether ionophores such as lasalocid, monensin, narasin, salinomycin, or semduramycin as their reaction causes adverse effects

(<https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=c968bfc7-3d69-4d3b-8b5d-4c41b880daf2&type=display>,

<https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=520.2455>).

Tiamulin has also been used for preventive measures in swine herds (Apley et al., 2012).

According to the European Medicines Agency, preventive treatment with tiamulin should only be initiated after confirmed infection with *B. hyodysenteriae* and then as part of a program including measures aiming to eradicate or control the infection in the herd (EMA, 2010b).

Approximately 1,470 kg of tiamulin is used in the swine nursery production cycle annually in the US (Apley et al., 2012). The number of days per production cycle Tiamulin is included in nursery piglets feed, averages 29.5 days for growth promotion and 35days for prevention and therapy purposes (NAHMS Swine Study, 2006).

Tetracycline resistance

Following the discovery of tetracyclines, most of the commensal and pathogenic bacteria were susceptible to these compounds (Levy, 1984). This was illustrated in a study conducted using 433 bacteria of the *Enterobacterales* family collected between 1917 and 1954, in which resistance to tetracycline was observed in only 2% of the isolates (Hughes & Datta, 1983). The first resistant bacteria was *Shigella dysenteriae* in 1953 (Chopra & Roberts, 2001). Currently, tetracycline resistance is among the most common AMR detected in bacteria. A wide array of

tetracycline resistance determinants (Roberts, 1996) have been reported in both gram-negative (Tuckman et al., 2007) and gram-positive bacteria (Huys et al., 2004). Tetracycline resistance is one of the most commonly encountered resistance phenotypes in gut bacteria of pigs (Tadesse et al., 2012) and often is co-selected with other antibiotic resistant determinants (Funk et al., 2006). Often, tetracycline resistance is used as a model to study the ecology of AMR in bacteria (Karami et al., 2006).

Resistance to tetracyclines among pathogenic and nonpathogenic species of bacteria is due to different tetracycline resistance (*tet*) genes (Roberts, 2005). All 26 English alphabet letters have been used to name tetracycline resistance determinants and now numerals are employed in naming the newly discovered determinants (Levy et al., 1999). More than 50 tetracycline resistance (*tet*) genes have been described (Wang et al., 2017). The majority of the tetracycline resistance determinants act by efflux-mediated mechanisms in both gram-positive and gram-negative bacteria (Chopra & Roberts, 2001). The efflux proteins that act to pump tetracyclines out from bacteria cells are located on the cytoplasmic surface of the cell membrane (Levy & McMurry, 1974). A few determinants code for ribosomal protection proteins and only one gene, *tetP* has been noted to act through both efflux pumps as well as ribosomal protection (Chopra & Roberts, 2001). A unique tetracycline gene is *tetX* that acts through enzymatic inactivation of tetracyclines (Roberts, 2005).

Tetracycline resistance determinants are widely distributed across various bacteria species which is partially explained by their association with conjugative or mobilizable elements (plasmids, transposons, conjugative transposons, and integrons) (Mendez et al., 1980; Jones et al., 1992). The gram-positive efflux tetracycline resistance determinants are usually associated with small mobilizable plasmids (Khan & Novick, 1983; Schwarz et al., 1992) as

opposed to the gram-negative efflux tetracycline resistance determinants that are associated with transposons inserted in a variety of plasmids (Jones et al., 1992; Mendez et al., 1980).

Tetracycline resistance is inducible and increases the propensity for horizontal gene transfer in the presence of sub-inhibitory concentrations of tetracycline (Sapunaric & Levy, 2005).

Although it has also been reported that the selection of tetracycline resistance bacteria is not related to the dosage of the drug administered (Græsbøll et al., 2017).

Campylobacter

Four different tetracycline resistance genes, *tetA*, *tetB*, *tetO*, and *tetS* have been identified in different *Campylobacter* species (Abdi-Hachesoo et al., 2014). Tetracycline resistance in *Campylobacter* is mainly mediated by *tetO*, a gene coding for ribosomal protection protein (Moore et al., 2006). The *tetO* gene and a rarely found but yet important *tetS* determinant are only transferred as plasmid-encoded genes or in a chromosome where they are not self-mobile (Roberts, 1996). The *tetO* is not associated with conjugative plasmids (Roberts, 1996; Roberts et al., 1991) as opposed to *tetS* that is usually associated with conjugative plasmids or conjugative transposons (Levy et al., 1999) and the two determinants can easily be transferred between *Campylobacter* (Taylor & Courvalin, 1988). The two genes *tetA* and *tetB* are codes for efflux proteins and act by pumping out tetracyclines from the bacterial cells (Chopra & Roberts, 2001). *Campylobacter* spp. are also known for easy natural transformation, they can easily pick DNA from the environment which makes it easier to acquire resistance determinants (Wang & Taylor, 1990).

Salmonella

The *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* genes are more commonly reported among *Salmonella enterica* (Chopra & Roberts, 2001; Ng et al., 2001). All five resistance genes code for efflux proteins in *Salmonella* (Chopra & Roberts, 2001). The four *tet* genes, *tet* (A, B, C, and D) are transferred between bacteria through mobile and conjugative plasmids and hence have a wide host range (Chopra & Roberts, 2001). The *tetE* gene differs from the rest of the group as it is associated with large, non-mobile and non-conjugative plasmids which explains its relatively low host range and prevalence (Roberts, 1996). Plasmids facilitate horizontal gene transfer influencing the spread of resistance in Enterobacterales (order) including *Salmonella* (Carattoli, 2003; Gillings, 2014; Brown-Jaque et al., 2015). Plasmids are typed based on their compatibility, that is, the ability of two plasmids to coexist in the same cell line (Novick et al., 1976). Different compatibility groups have been found in different *Salmonella* serovars and no compatibility group is limited to one serovar (McMillan et al., 2020). Conjugative plasmids that are associated with tetracycline resistance in *Salmonella* include IncC, IncF, IncHI, and IncI1 and mobilizable plasmids include IncQ1 and IncR plasmids (McMillan et al., 2020).

Pleuromutilin resistance

Tiamulin inhibits protein synthesis by binding to the 50S subunit of the bacterial ribosomes and inhibiting peptidyl transferase (Poulsen et al., 2001; EMA, 2013). Tiamulin resistance is mediated by chromosomally-mediated *rplC* and *rplD* genes (Giguere, 2006). In some cases, tiamulin resistance genes are also carried on a plasmid or a transposon as in like *vga* and *cfr* genes, respectively (Long et al., 2006; Mendes et al., 2011). The actual mechanism of resistance to pleuromutilins varies among bacterial species (Malbruny et al., 2011; Wang et al.,

2012). Pleuromutilin resistance has in most cases been reported in gram-positive bacteria and was previously believed that gram-negatives are naturally resistant to this class of antimicrobials. This view was changed by the identification of the multidrug resistance *cfr* genes in *Proteus vulgaris* (Wang et al., 2011) and *E. coli* (Wang et al., 2012) isolated from porcine nasal swabs in China. In the US, the *cfr* gene, *cfr*(C) was identified in multiple *C. coli* isolated from different cattle farms in different states (Tang et al., 2017). The gene is known to confer resistance to five different classes of antimicrobials including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A.

Campylobacter species have demonstrated a very high susceptibility to tiamulin (Giacomelli et al., 2014; Hlashwayo et al., 2020) but it has shown to enhance cross resistance of *C. coli* to macrolides (azithromycin and erythromycin) in swine (Quintana-hayashi & Thakur, 2012). In 2017, *cfr* determinants were reported for the first time in *C. coli* isolated from cattle that demonstrated significantly minimal inhibitory concentrations to tiamulin (Y. Tang et al., 2017). Since then, *cfr* determinants in *Campylobacter* have been reported in other studies (Liu et al., 2019; Moser et al., 2020). *Salmonella* has demonstrated complete resistance to tiamulin (Jones et al., 2002) but information on susceptibility/resistance is limited. Also, pleuromutilin resistance genes have not been reported in *Salmonella*.

***Campylobacter* in swine**

Campylobacter are gram-negative, microaerophilic, non-spore forming, slender, curved or spiral bacilli that are motile with single or a pair of polar flagella (Padungton et al., 2003; Alfredson & Korolik, 2007). The bacterium is indole negative, catalase positive, oxidase positive, nitrate positive, hippurate positive and glucose utilization negative (Pal, 2017). They

belong to the family *Campylobacteriaceae* which consists of *Campylobacter*, *Arcobacter*, and *Helicobacter* genera. The genus *Campylobacter* comprises 25 verified species (Kaakoush et al., 2015). *Campylobacter* species have a wide ecological range and have been isolated from humans, domesticated and wild animals, poultry, wild birds as well as the environment.

Campylobacter was the second leading causative agent of food transmitted diseases in the European Union and the United States, preceded by norovirus (European Food Authority Safety, 2017). The CDC estimates 1.5 million *Campylobacter* infections that cost \$270 million in direct medical costs every year (<https://www.cdc.gov/drugresistance/pdf/threats-report/Campylobacter-508.pdf>). About 29% of all infections have shown resistance to fluoroquinolones such as ciprofloxacin or macrolides such as azithromycin, the antimicrobials used for the treatment of *Campylobacter* infection. Consequently, drug resistant *Campylobacter* is estimated to cause 448,400 infections and 70 deaths every year in the US (CDC, 2019). Considering the threat posed by the alarming increase of resistance, drug resistant *Campylobacter* was categorized as a serious threat by the CDC (<https://www.cdc.gov/drugresistance/biggest-threats.html#cam>). Similarly, *Campylobacter* isolates obtained from different animal species have been demonstrating high resistance to fluoroquinolones (ciprofloxacin and nalidixic acid) and tetracyclines (European Food Authority Safety, 2017).

Swine are considered as a major reservoir for *Campylobacter*, the majority of which are commensals. Some of the *Campylobacter* species found in swine including *C. coli*, *C. jejuni*, *C. lari*, and *C. hyointestinalis* have public health importance (Facciola et al., 2017). *C. Jejuni* is very rare in pigs and is the primary pathogen in humans. This indicates that pigs as a source of human *Campylobacteriosis* are low on the source hierarchy. *C. coli* is the most isolated species in pigs worldwide (Thakur & Gebreyes, 2005a; Harvey et al., 2007; Varela et al., 2007; Dorr et al.,

2009; Fosse et al., 2009). Higher *C. coli* prevalence ranging from 66% to 100% have been obtained in pig feces worldwide while *C. jejuni* is rarely isolated (Gue´vremont et al., 2004). The difference in prevalence could be because pigs carry a large amount of *C. coli* as compared to *C. jejuni* which makes it more likely to detect *C. coli* than *C. jejuni*. Selective enrichment for *C. jejuni* might be required for its optimal detection in swine fecal samples (Gharst et al., 2006). Along with *C. coli*, *C. hyointestinalis* is also most commonly isolated from swine (Allos et al., 2015). It is frequently isolated in pigs with proliferative enteritis and less so in pigs with other enteric diseases (Gebhart et al., 1983).

Pigs usually acquire *Campylobacter* infections at a young age in the multiplier farms. Studies suggest that piglets acquire infections from their mothers in their early days of life. The prevalence of 25% and 75% were recorded in one week old piglets in two farms with the sows having a 90% prevalence (Weijtens et al., 1997). The prevalence in piglets continued to rise reaching 80% and 90% at four and eight weeks of age respectively. Restriction Fragment Length Polymorphism (RFLP)-typing of *Campylobacter* showed similarity between sows and their piglets suggestive of sow to piglet transfer of the bacteria (Magras et al., 2005; Weijtens et al., 1997). Stress caused by parturition increases the shedding of *Campylobacter*, increasing the chances for transmission to piglets (Weijtens et al., 1997). Although eliminating *Campylobacter* from pig breeding populations would be a promising control strategy (Weijtens et al., 2000). The higher prevalence of *Campylobacter* in sows and early infection in piglets severely limits the possibility for success of this intervention. Infection can also be acquired from other piglets sharing the same pen. Contaminated environments can potentially act as a source of *Campylobacter* infection in swine (Weijtens et al., 2000). Contact with rodents and birds, vehicles, clothes and boots also plays a role in transmission. Currently, the transmission of

Campylobacter to swine through feed and water has not been reported (Leblanc-maridor et al., 2008; Fosse et al., 2009). *Campylobacter* infections are correlated with the level of management in the farm, where larger farms with intensive management have been reported to have lower prevalence compared to small farms with less management (Fosse et al., 2009). Separating pigs in stalls has also been shown to minimize transmission risk.

Some of the *Campylobacter* spp. found in swine are zoonotic and are implicated to cause disease in humans. *C. jejuni* and *C. coli* are best known and are frequently implicated in causing gastritis in humans. *C. jejuni* is associated with most of the gastritis cases in humans as compared to *C. coli* (Varela et al., 2007; Facciola et al., 2017). But *C. coli* has also been pointed out as major zoonotic pathogen due to the higher prevalence of antibiotic resistant strains when compared to *C. jejuni* (Luangtongkum et al., 2009). *C. hyointestinalis* subsp. *hyointestinalis* and *C. lari* are considered emerging and have occasionally been associated with gastrointestinal illness in humans (Fitzgerald & Nachamkin, 2011; Man, 2011; Corry, 2014; Facciola et al., 2017).

***Salmonella* in swine**

Salmonella are gram-negative, aerobic and facultative anaerobic, bacilli shaped bacteria of the order *Enterobacterales*. *Salmonella* was first discovered in 1955 in the intestine of pigs with classical swine fever (Eng et al., 2015). Based on the WHO nomenclature system, the genus *Salmonella* is classified into *Salmonella enterica* and *Salmonella bongori* species (Popoff et al., 2003). *S. bongori* is mainly found in the environment and cold blooded animals (Brenner et al., 2000). *S. enterica* consists of six subspecies namely, *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp.

houtenae, and *S. enterica* subsp. *indica* (Popoff et al., 2003). Within *S. enterica* subspecies, several serovars/serotypes have been assigned based on phylogeny, a White-Kauffmann-Le Minor scheme which identifies serovars based on three major antigenic determinants: somatic (O), capsular (K) and flagellar (H) (Brenner et al., 2000). There are more than 2,500 serovars identified, half of which belong to *S. enterica* subsp. *enterica* (Guibourdenche et al., 2010). *Salmonella* species are also classified as typhoidal and non-typhoidal (NTS) based on their ability to cause specific diseases in humans (Pinna et al., 2014). *S. enterica* is ubiquitous, found in the environment colonized in warm and cold blooded animals (Holt, 1994). *Salmonella* has a predilection for the gastrointestinal tract of food producing animals making it easy to disseminate to the environment through feces, water and food products (Ashbolt, 2004; Hutchison et al., 2005).

Salmonella species produce positive catalase, oxidase, lysine decarboxylase, methyl red, Simmons citrate, and ornithine decarboxylase tests and negative indole, urea and Voges-Proskauer tests (Holt, 1994). Except for *S. Gallinarum* and non-motile mutants, most of the serovars are motile (Holt, 1994). Some of the serovars produce hydrogen sulfide (Holt, 1994). *Salmonella* species are facultative intracellular pathogens.

Salmonella spp. are the leading causes of gastroenteritis in humans in the world and its burden is substantial (Majowicz et al., 2010). Playing a major role, contaminated food and to a relatively lower extent, direct contact with clinically ill or carrier animals are responsible for human infections (Hoelzer et al., 2011). Five out of the top 20 *Salmonella* serovars isolated in clinically ill human beings are commonly isolated in animals (Griffith et al., 2019). The WHO-Foodborne Disease Burden Epidemiology Reference Group estimates that 93.8 million cases of gastroenteritis due to *Salmonella* species occur every year, resulting in 155,000 deaths

(Majowicz et al., 2010). In the US, the CDC reports 1.35 million *Salmonella* infections in humans every year, causing 26,500 hospitalizations, and 420 deaths costing an estimated \$400 million in direct medical costs (<https://www.cdc.gov/Salmonella/index.html>). The estimates may be lower than actual, as not all cases are confirmed in the lab and reported to CDC (Chalker & Blaser, 1988). The CDC estimates that 16% of all NTS are resistant to one or more antimicrobials (CDC, 2019). Drug-resistant NTS causes 212,500 estimated infections and 70 estimated deaths every year (CDC, 2019). *Salmonella* is developing resistance at an alarming rate to critically important antimicrobials such as ciprofloxacin, azithromycin, and ceftriaxone which are used to treat *Salmonellosis*, prompting the CDC to categorize drug resistance *Salmonella* as a serious level threat (<https://www.cdc.gov/drugresistance/biggest-threats.html#non>). Nalidixic acid which is usually precursor to fluoroquinolone resistance has been demonstrating higher resistance incidence in many studies (Ochiai et al., 2008; Ryan et al., 2011).

Swine are infected with *Salmonella* in their early days of life and fecal shedding increases at weaning in both sows and piglets (Nolleta et al., 2005). This is explained by stress caused by the change of feed and decrease in sow colostral antibodies (Kranker et al., 2003). Studies have also found evidence of feed contamination with *Salmonella*, indicating that feed could also be a source of infection (Hurd et al., 2001; Langvad et al., 2006). Other risk factors associated with acquiring infection in swine include antibiotic treatment, dry feed, crowding, poor sanitation and lack of biosecurity. In a study conducted in the USA, antibiotic free farms were found to have low *Salmonella* prevalence when compared to farms that used antibiotics (Gebreyes et al., 2005). Increased *Salmonella* shedding was also observed when swine were fed dry feed as compared to wet feed (Fablet et al., 2003).

Salmonella has been isolated from clinically healthy pigs (Wilcock & Schwartz, 1992). These asymptomatic carrier pigs serve as a major source of infections to other pigs. Rodents and contaminated feed and premises also play a role in spreading the infection (Harris, 2013). *S. Choleraesuis* is among the most commonly isolated *Salmonella* species in swine (Gray et al., 1996; Harris, 2013). It is host adapted and known to cause a septicemic disease characterized by hepatitis, pneumonia, and cerebral vasculitis and sometimes produces necrotizing enterocolitis (Harris, 2013). *S. Choleraesuis* causes disease in pigs of all ages but primarily affects piglets below five months of age (Wilcock & Schwartz, 1992). *S. Typhimurium*, *S. Derby*, *S. Agona*, and *S. Heidelberg* are the only non-host adapted serotypes that have only been associated with clinically ill pigs (Erdman et al., 2001). Usually, non-host adapted serovars result in enterocolitis and rarely result in severe disease (Reed et al., 1986; Wilcock & Schwartz, 1992).

Antimicrobial resistance in general with public health significance

The discovery and commercialization of antimicrobials stand as a defining achievement of 20th-century medicine. But the antimicrobial use in animal agriculture has received a great deal of public attention concerning the occurrence of AMR (Avgustin, 2012). The use of antimicrobial agents in food animals constitutes an important contributing factor for the development of AMR and their subsequent entry into the food chain may be major source of human exposure (Marshall & Levy, 2011). This use of antimicrobials in animals is a key driver of selection pressure on both gut commensals and pathogenic bacteria that can spread to humans (Liebana et al., 2013).

AMR has emerged as a global public health threat estimated to cause over 700,000 deaths globally (Jasovsky et al., 2016). AMR was declared among the top world threats requiring a

collaborative approach across countries (<https://www.cdc.gov/drugresistance/solutions-initiative/stories/ar-global-threat.html>). AMR directly touches the human, veterinary and agricultural sectors as well as the biological, ecological, economic, political and social aspects of human beings which requires a collaborative global approach across sectors. In 2018, the US government conducted a yearlong AMR challenge that encouraged a one health approach in combating AMR (<https://www.cdc.gov/drugresistance/intl-activities/amr-challenge.html>). The challenge was focused on five commitment areas, which included tracking AMR data, reducing the spreading of germs, improving antimicrobial use, decreasing AMR in the environment and investing in the development and improvement of vaccines, therapeutics and diagnostics. In total 32 countries committed, making the first breakthrough step toward combating AMR.

AMR bacteria-caused infections are associated with increased burdens in terms of morbidity, mortality and expense than the infections due to susceptible bacteria (Barza, 2002; Martin et al., 2004). Multidrug resistant bacteria are currently considered as an emerging global public health concern (Munir & Xagorarakis, 2010; Roca et al., 2015). If not tackled, AMR is estimated to claim over 10 million lives globally and cost \$100 trillion in economic output by 2050 (O'Neill, 2016). In the United States, at least 2.8 million people get sick from drug resistant bacterial infections every year and it is estimated that 35,000 die from those infections (<https://www.cdc.gov/drugresistance/biggest-threats.html>).

Bacteria and fungi have the ability to mutate and acquire resistance determinants that render them resistant to antimicrobial drugs. Every time the antimicrobial is used it exerts a selective pressure that favors the growth of the resistant organism (Marshall & Levy, 2011). Every antibiotic, including the newly developed, are followed by selection for resistance by these organism leaving us without options (Kouyos et al., 2011). Also, AMR determinants can be

shared among the bacteria and resistant bacteria can be shared between animals, humans and the environment making resistance widespread (Subbiah et al., 2020). AMR is a significant problem that should be addressed by transforming the threat into a manageable problem

(<https://www.cdc.gov/drugresistance/pdf/public-health-action-plan-combat-antimicrobial-resistance.pdf>). The Public Health Action Plan to Combat Antimicrobial Resistance suggests measures to address dangers associated with AMR, which include improving surveillance of emerging AMR problems, improve diagnostics, vaccines and infection control measures and encouraging, conducting and support AMR research

(<https://www.cdc.gov/drugresistance/pdf/public-health-action-plan-combat-antimicrobial-resistance.pdf>).

Route of antibiotic administration as an alternative to mitigate AMR

The use of antimicrobials to keep animals healthy and to increase productivity has been an integral part of swine production and management. Antimicrobials are administered to swine through parenteral (e.g. intramuscular, and subcutaneous), and oral medication (in-feed and in-water). The choice of antibiotic administration route is guided by choice for antibiotics, the aim for antibiotic administration and the number of animals being handled. Also, the swine production stage can influence the route used, where oral administration is frequently used in weaners and fatteners whereas parenteral is applied more in sows (Merle et al., 2014; Lekagul et al., 2019). The parenteral route is used when treating individual sick animals, small numbers of animals, or under circumstances where the drug indications are strictly parenteral (Trauffler et al., 2014; Sjölund et al., 2015). Injections are associated with the handling of individual animals which requires significant labor resources and induces stress to the animals. The oral route is by

far the most common route of administration of antibiotics in pigs (Apley et al., 2012; Callens et al., 2012; Lekagul et al., 2019) which is more convenient and can be carried out in a large number of animals. In-feed antibiotics have traditionally been used to prevent bacterial enteric infections, improve body weight gain and improve production efficiency (Muhl and Liebert, 2007). Several studies have reported more than 90% of antimicrobials are administered orally, in-feed and in-water (Apley et al., 2012; Merle et al., 2012; Van Rennings et al., 2015). According to Davies & Singer (2020), 70.5%, 67.8% of the antimicrobials administered in swine in 2016 and 2017 respectively were through in-feed administrations while only 27.5%, 30.1% were in-water administrations in the respective years.

AMR mitigation has been focused on limiting antimicrobial use in human and animals (Casewell et al., 2003; Zhang et al., 2013) despite the multitude of factors that plays the role in its development, amplification and dissemination. Risk factors such as the use of antimicrobials for prolonged periods, under-dosing, and dust containing antimicrobials may favor the selection of bacterial resistance (Burrow et al., 2014). Similarly, the way antimicrobials are used also plays a significant role in AMR development (Wang et al., 2015). In a study, a group of mice given tetracycline through the oral route displayed increased tetracycline determinants in feces as compared to the intravenous-treated group (Zhang, 2011). Similar results were observed by Zhang et al., (2013) where oral administration of antibiotics had a greater amplification of AMR in gut microbiota compared to intravenous injection. The practical implications of these results need to be validated in humans and other animals. In a study in humans, it was concluded that parenteral antibiotic administration is preferred over oral antibiotic administration as the gut commensal and pathogenic bacteria are less exposed to the antimicrobials leading to less chance of resistance selection (Rafii et al., 2008). Another study in chickens demonstrated a fourfold and

tenfold increase in ampicillin resistance in the orally treated group versus the sham and intravenous respectively (Zhou et al., 2020). In a similar study, the oral route was observed to promote for selection of fluoroquinolone resistance in commensal *E. coli* in broiler chickens (Chantziaras et al., 2017). In a swine study, both in-water and intramuscular administrations displayed increase in the AMR of enterococcus when compared to the in-water treated group (Wu et al., 2019). They also found that antibiotic administration increased AMR regardless of the route of administration. These studies suggest that modifying the way we administer antimicrobials could help in tackling AMR.

Summary

The overall goal of this thesis is to evaluate the route of administration of antimicrobials as a mitigation strategy towards controlling the development of AMR in the foodborne pathogens *Campylobacter* and *Salmonella* in swine. Studies have shown that the method of administering antimicrobials influences the selection for AMR and the extent of AMR promotion differs by the route used to administer these antimicrobials. Most studies have used lab animals to prove this theory. Even the studies done in other food animals were focused on commensal bacteria with none focused on foodborne pathogens. *Campylobacter* and *Salmonella* are the second and third leading foodborne pathogens following noroviruses. They are known to easily acquire multiple resistance determinants, share the determinants within and between bacterial species and develop resistance to multiple drugs which threaten global public health. As a consequence, these organisms are categorized as serious level threat that requires collaborative and immediate efforts to address this global challenge. Most efforts put forward are on limiting

antimicrobial use in humans and more in food producing animals, but an important risk factor, route of administration, was not emphasized which justifies our study to look for its applicability.

Chapter 2 - In-water and in-feed antimicrobial administration did not influence development and persistence of antimicrobial resistance of *Campylobacter* in a population of nursery piglets

Introduction

Antimicrobial Resistance (AMR) has emerged as the global threat to human and animal health (ECDC/EFSA/EMA, 2015). In 2020, the World health Organization (WHO) declared AMR among the top health challenges in the world (WHO, 2020). According to the Centers for Disease Control and Prevention (CDC) more than 2.8 million AMR bacterial and fungal infections and 35,900 million deaths occurs every year in the United States (CDC, 2019). The increased AMR infections in humans are linked to the use of antimicrobials in animal agriculture (O'Neill, 2015). Antimicrobials have been used in swine production on a routine basis as a management tool for many years (Aarestrup, 2015). According to the United States Food and Drug Administration (FDA, 2019), approximately six million kilograms of medically important antimicrobials were used for food animal production, with the swine industry utilizing 42% of these antimicrobials. The overconsumption of antimicrobials has posed great public health risks which has prompted the FDA to ban the use of medically important antimicrobials for production purposes (FDA Guidance for Industry #209, 2017).

In their joint report, the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) reported the positive association of antimicrobial consumption in food producing animals and AMR of bacteria including *Campylobacter* (ECDC/EFSA/EMA, 2015). *Campylobacter* is one of the

leading causes of foodborne diarrheal illnesses in the US and poses a significant public health threat (Scallan et al., 2011). The thermophilic *Campylobacter* species are among the pathogens that are increasingly becoming resistant to important and critically important antimicrobials. In 2019, the CDC categorized drug-resistant *Campylobacter* under serious level threat. In the US, it is estimated to cause approximately 448,400 infections and about 70 deaths every year (CDC, 2019). The CDC's National Outbreak Reporting System (NORS) reported a total of 647 *Campylobacter* outbreaks between 2008 and 2018 (<https://wwwn.cdc.gov/norsdashboard/>). Pigs are reservoirs for *Campylobacter* spp. therefore playing a pivotal role in transferring the bacteria to human beings (Ono et al., 1977; Weijtens et al., 1999; Thakur & Gebreyes, 2005a; Fosse et al., 2009). However, the primary pathogen in humans is *C. Jejuni* which is very rare in pigs indicating that pigs as a rare source of human *Campylobacteriosis*.

Worldwide, several antimicrobials including medically important antimicrobials are used in swine production for therapeutic and non-therapeutic purposes. Tetracyclines and tiamulin are the most commonly used antibiotics in swine (Holman & Chénier, 2013). Tetracyclines are widely employed either alone or in combination with other antibiotics for prevention, control, and treatment of bacterial diseases in swine (Phillips et al., 2004; Apley et al., 2012). Tiamulin is extensively used in the swine industry to control and treat dysentery (caused by *Brachyspira hyodysenteriae*), respiratory diseases, and for growth promotion in some countries (Bøsling et al., 2003; NAHMS Swine Study, 2017). Tiamulin, an antibiotic that is used exclusively in swine and does not require a veterinary feed directive for its usage, so likely to be frequently used or even overused by producers. Previous studies have shown that tiamulin and its metabolites select for resistance among *C. coli* (Lykkeberg et al., 2007).

Oral medication is the most commonly used route of antimicrobial administration in pig production (Sjölund et al., 2015; Van Rennings et al., 2015; NAHMS Swine Study, 2017). Several studies have reported oral medication of antimicrobials as a driving factor for development of AMR (Varga et al., 2009; Lutz et al., 2011; Burow et al., 2014). Risk factors such as prolonged antimicrobial use periods, under-dosing, and environment contamination with antimicrobials may favor the selection of bacterial resistance (Burrow et al., 2014). Despite of a broad array of AMR risks associated with oral route of antimicrobial administration, its impact on the amplification and persistence of AMR in *Campylobacter* is a largely unexplored area. Therefore, the objective of this study was to investigate the effect of oral route of administration antimicrobials (in-feed vs in-water) on AMR profiles of *Campylobacter* spp., a foodborne pathogen shed in the feces of piglets.

Materials and Methods

Animal and Experimental Design

The Kansas State University Institutional Animal Care and Use Committee approved all experimental protocols used in these studies (IACUC # 4033; Manhattan, KS). Experiments were conducted at a commercial research nursery facility located in Pipestone Minnesota. A total of 1440 weaned piglets (L337×1050, PIC, Hendersonville, TN) were used in a 35-day trial. The piglets were randomly allocated into 48 pens, with each pen containing 27 piglets. Distribution of piglets in pens was made to ensure the average weight between pens was approximately equal. The piglets were provided with 7 days of acclimatization after which they were randomly assigned to six treatment groups. The treatments included the control (no medication), in-water

chlortetracycline @ 22 mg/kg BW (CTC) (CTC-hydrochloride, Elanco Animal Health, Indianapolis, IN), in-feed CTC @ 22 mg/kg BW, in-water tiamulin @ 23 mg/kg BW (Denagard®, Elanco, Animal Health, Indianapolis, IN), in-feed tiamulin @ 5 mg/kg BW and a CTC @ 22 mg/kg BW and tiamulin @ 5 mg/kg BW combination in feed. These treatments were applied for 14 days. All 48 pens were used in the trial allowing for eight replications per treatment.

Fecal Samples

Weekly fresh fecal sample collection was carried out by gentle rectal massage of the first 5 piglets caught out of 27 piglets in the pen (convenience sampling). Fecal samples from each piglet were collected into individual sterile plastic bags (Whirl-Pak® bags, Nasco, Ft. Atkinson, WI) and transported on ice in a cooler to the Pre-harvest Food Safety laboratory, College of Veterinary Medicine, Kansas State University. Samples were stored at 4°C and processed within 24 hours of collection.

Isolation of *Campylobacter* from fecal samples

All fecal samples were assigned to laboratory-specific IDs. Approximately, 1 g of fecal sample was mixed in 9 ml of Mueller-Hinton broth (MH broth; BD Difco, MD, USA) containing *Campylobacter* selective and two growth supplements, Oxoid™ *Campylobacter* Growth Supplement (SR0232E) and Oxoid™ Modified Preston *Campylobacter* Selective Supplement (SR0204E) (Thermo Fisher Scientific, Fair Lawn, NJ, USA) (MH+SS agar) to 50 ml filter centrifuge tubes (TubeSpin® Bioreactor, TPP Techno Plastic Products AG, Switzerland). The tubes were then incubated in a Tri-Gas incubator (BINDER, Germany) at 42°C for 48 hours. A

sterile loopful (~10 µl) of the enriched fecal suspension was streaked onto MH+SS agar and incubated in a Tri-Gas incubator at 42°C for 48 hours. Three well-isolated, circular, transparent and, glassy colonies were picked and streaked onto MH agar to confirm growth and obtain pure cultures. Plates were incubated in a Tri-Gas incubator at 42°C for 48 hours. Pure cultures from the MH agar plate were stored in MH broth with 30% glycerol for future use at -80°C.

DNA isolation and speciation

DNA was extracted as per the published procedure (Olah et al., 2006). Two colonies from MH agar were mixed with 40 µl of single cell lysis buffer (SCLB) (Thermo Fisher Scientific, Fair Lawn, NJ, USA) in a 200 µl PCR tube and mixed thoroughly. The SCLB solution contained 1ml Tris-EDTA (1X TE) (Thermo Fisher Scientific, Fair Lawn, NJ, USA) and 10µl proteinase K (5 mg/ml) (SIGMA-ALDRICH, Co., St. Louis, MO, USA). The 200 µl PCR tubes were kept in a PCR machine at 80⁰ C for 10 min and 55⁰ C for another 10 min. Then, 40 µl of distilled water was added to the tube, mixed and centrifuged for 2 min at 4500 × g to get the DNA supernatant.

Speciation of *Campylobacter* was done using the multiplex polymerase chain reaction assay (Yamazaki-Matsune et al., 2007). The procedure involved simultaneous detection of 16S rRNA for genus *Campylobacter*, 23S rRNA for *C. hyointestinalis* subsp. *hyointestinalis*, *askD* for *C. coli*, *cstA* for *C. fetus*, *glyA* for *C. lari*, *cj0414* for *C. jejuni* and *lpxA* for *C. upsaliensis*. Genes, their sizes, and primer sequences are shown in table 2.1. The PCR reaction of 20 µl consisted of 10 µl of master mix (iQ™ Multiplex Powermix-Bio-Rad, Hercules, CA), 5 µl of nuclease-free water, 4 µl of template and 1µl of 7 pairs of primers (100 pM) mix (7 pM for each primer). Reactions were run on a master-cycler gradient thermal cycler (Eppendorf, Germany),

using the following conditions: initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 90 seconds and extension at 72°C for 60 seconds, and final extension at 72°C for 7 minutes. Analysis of PCR products was done by capillary gel electrophoresis in a QIAxcel Fast Analyzer system (Qiagen, Valencia, CA).

Antimicrobial Susceptibility Testing

Commercial premade antimicrobial panel plates (Sensititre™ CAMPY, Trek Diagnostic Systems, Thermo Scientific) consisting of nine antimicrobials were used to determine minimal inhibitory concentrations (MICs) of *Campylobacter* strains. The antimicrobials tested were azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin and tetracycline. Two to three single isolated *Campylobacter* colonies were mixed in demineralized water (Trek Diagnostic Systems, Thermo Scientific) to obtain 0.5 McFarland turbidity standards. Subsequently, 10 µl aliquot of the suspension was transferred to MH broth (Trek Diagnostic Systems, Thermo Scientific) and vortexed. Then, 50 µl of the culture was inoculated to the Sensititre plates and incubated in a Tri-Gas incubator at 42°C. The epidemiological cutoff values established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used to interpret the MIC values as either resistant or susceptible

(https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf).

PCR detection of tetracycline resistance genes

Campylobacter was tested for the presence of *tetA*, *tetB*, *tetO*, and *tetS* genes using a PCR protocol modified from published procedures (Abdi-Hachesoo et al., 2014). The genes were screened individually by regular PCR using a Master-cycler gradient thermal cycler (Eppendorf, Germany). The 20 µl reaction mixture consisted of 10 µl master mix, 4 µl nuclease-free water, 4 µl of DNA template and 1 µl of each forward and reverse primers. The reaction was carried out under the following conditions: 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at 56°C for 60 seconds and extension at 72°C for 60 seconds, and 1 cycle of final extension at 72°C for 10 minutes. Information about Primers and target gene size is found in table 2.2. Analysis of PCR products was done by capillary gel electrophoresis in the QIAxcel Advanced system (Qiagen, Germantown, MD). Tetracycline resistance *E. coli* strains used as positive controls were acquired from USDA (MARC, Clay Center, NE) and Dr. Marilyn C. Roberts at the University of Washington.

Whole genome sequencing (WGS)

Genomic DNA was extracted from cultures grown on MH agar in a Tri-Gas incubator for 48hrs using Invitrogen PureLink™ Genomic DNA Mini Kit (Catalog no. K1820-01 × 2, Thermo Fisher Scientific, Fair Lawn, NJ, USA). Preparation of DNA libraries was carried out using Illumina NextEra XT library preparation kit and the quality check done with Qubit (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Sequencing was performed on an HiSeq platform 2x150bp at CosmosID. The sequences were trimmed using BBduk (Bushnell, 2014) and the genomes sequences assembled using SPAdes (Bankevich et al., 2012). The quality of genomes was assessed using Check-M (Parks et al., 2015) and genome alignment and visualization done

using Harvest (Treangen et al., 2014). The CosmosID core genome SNP typing pipeline was used to evaluate phylogenetic placement and SNP-based phylogenetic trees constructed using FastTree2 (Price et al., 2010).

Statistical Analysis

Prevalence data were organized as a binomial response at the animal-level per pen, i.e. the number of '+' out of 5 sample per pen per sampling day. Binomial data was summarized by species and treatment at each sampling day. Due to low prevalence of *C. coli*, only *C. hyointestinalis* and the overall *Campylobacter* prevalence were subjected to statistical analysis. The prevalence data was analyzed using PROC GLIMMIX procedure in SAS (version 9.4; Cary, NC). Binomial response post treatment was analyzed under the logit linear mixed model with repeated measurements over time. Fixed effects of the model included treatment, sampling day, and their interactions. Random effects of the model included block and pen. The random pen effect corresponds to the model intercept term and accounts for the correlation of binomial responses from the same pen. Prevalence at sampling day 0 (i.e. baseline response) served as the model covariate. All tests were conducted at the 0.05 significance level. Comparison between two levels of a model fixed effect was carried out using a 2-sided test. Distributions of test statistics were approximated by Chi-square distributions. Treatment groups were compared within and across sampling day due to no differences observed between treatment and sampling day interaction.

The MIC data were summarized by species and treatment at each sampling day and analysis was performed using SAS PROC MIXED procedure. Due to the limited number of isolates tested for MIC, *C. coli* data at sampling day -7 and *C. hyointestinalis* data at sampling day 0 were excluded from the statistical analysis. The MIC data of *C. hyointestinalis* was analyzed

separately at every post-treatment sampling day, under the linear model with treatment being the fixed effect. To better achieve model assumptions, data underwent natural log transformation before statistical modeling. Treatment effect was assessed via back-transformed least squares (LS) means (and mean differences. Comparisons were carried out using the 2-sided test.

Results

Prevalence of *Campylobacter*

A total of 262 (18.2%) *Campylobacter* strains were recovered from 1,440 samples. The majority of these were *Campylobacter hyointestinalis*, (258; 17.9%) and 4 (0.3%) were *Campylobacter coli*. The prevalence distributions across different treatments, treatment phases and sampling day are summarized in table 2.3. Prevalence of *Campylobacter* was similar between in-feed tiamulin (53; 22.1%) and in-feed CTC (52; 21.7%) when compared to control (49; 20.4%), in-water CTC (37; 15.4%), in-feed CTC + tiamulin combination (37; 15.4%) and in-water tiamulin (34; 14.2%) treatment groups. Based on treatment phases, the post-treatment phase had a numerically higher number of isolates, (168; 35%) when compared to 75 (15.6%) and 19 (4.0%) recorded in treatment and pre-treatment phases, respectively. The four *C. coli* strains were all recovered in pre-treatment phase. Overall there was no significant effect of treatment on the prevalence of *Campylobacter* ($P > 0.05$) (table 2.4), although sampling day effect was significant ($P < 0.05$). No evidence of Sampling Day $\times$ Treatment interaction or the main effect of treatment was observed for the prevalence *Campylobacter* ($P > 0.05$) (table 2.5).

Prevalence of tetracycline resistance genes

Campylobacter strains (n=114) were screened for the presence of tetracycline resistance genes, *tetA*, *tetB*, *tetO*, and *tetS* by PCR. All 114 strains were positive for *tetO* gene. The *tetA*, *tetB*, and *tetS* genes were not detected in these strains

Antimicrobial susceptibilities

The resistance percentages and MIC distributions of *Campylobacter* strains (n=262) against the nine antimicrobials used are displayed in table 2.6. The strains (n=262) were highly resistant to tetracycline (98.5%), ciprofloxacin (89.3%) and nalidixic Acid (60.3%). Low resistance to gentamicin (8%), azithromycin (5%), erythromycin (3.4%), clindamycin (3.1%), telithromycin (1.9%) and florfenicol (8%) were also observed in this study. A total of six different resistance profile were observed in this study (table 2.7). The most common resistance profile was CIP_NAL_TET which was observed in 122 (46.6%) strains followed by CIP_TET (83, 31.7%), CIP_GEN_NAL_TET and NAL_TET, 14 (5.3%) strains each, and 8, (3.1%) strains resistance to tetracycline alone. A total of 31 (11.8%, n=262) strains were resistant to more than three antimicrobial classes, thus recorded as multidrug resistance. Four of these strains exhibited multidrug resistance to five or more antimicrobial classes with the highest resistance shown by two strains to seven antimicrobial classes. Three of the strains were resistant to four antimicrobial classes and the rest of the 24 strains were resistant to three antimicrobial classes.

Whole genome sequencing results

Five strains were identified as *Campylobacter* by whole genome sequencing. Three of these were *C. coli* and two were *C. hyointestinalis* subsp. *hyointestinalis*. AMR analysis (table 2.8) revealed all the five strains carrying determinants conferring resistance to tetracycline (*tetO*). In addition, the five strains carried aminoglycoside/ streptomycin (30S ribosomal protein S12 RpsL point mutation), and macrolide (23S ribosomal RNA point mutation) resistance determinants. Three strains revealed RpsL point mutation, *rpsL_K88R* that confers resistance to streptomycin. The OXA-61 family class D beta-lactamase OXA-489 gene conferring resistance to beta-lactams, aminoglycoside O-phosphotransferase APH(3')-IIIa gene conferring resistance to amikacin/kanamycin, 50S ribosomal protein L22 point mutations (50S_L22_A103A, 50S_L22_A84A, 50S_L22_A88A, and 50S_L22_G86G0 conferring resistance to macrolides , point mutations in the gyrase subunit A (*gyrA_T86I*, *gyrA_D90D* and *gyrA_P104P*) conferring resistance to fluoroquinolones, and aminoglycoside O-phosphotransferase APH(2'')-Ih genes conferring resistance to amikacin/gentamicin/kanamycin/tobramycin were carried by two strains each.

Discussion

Swine are major reservoirs of *Campylobacter* with *C. coli* as the major species (Thakur & Gebreyes, 2005b; Dorr et al., 2009; Fosse et al., 2009). Of public health importance are the *C. coli*, *C. jejuni*, *C. lari*, and *C. hyointestinalis* species that are associated with gastrointestinal illness in humans (Fosse et al., 2009). According to CDC, *Campylobacter* is among the four most important causative agents of foodborne illness in the US

(<https://www.cdc.gov/foodsafety/foodborne-germs.html#:~:text=The%20top%20five%20germs%20that,Clostridium%20perfringens>) with foods of animal origin being the most common source. In addition, *Campylobacter* spp., are increasingly becoming resistant to the antimicrobials used to treat the infections, posing an added threat to public health.

Several studies have reported the use of antimicrobials in conventional swine production farms in the US (Thakur & Gebreyes, 2005b; Rollo et al., 2010). Tetracycline and tiamulin are among the most commonly used antimicrobials in swine production (Apley et al., 2012; NAHMS Swine Study, 2017). Tetracyclines are the leading antimicrobial class used in all production stages of swine in the US (NAHMS Swine Study, 2017) and globally (Van Boeckel et al., 2015). Tiamulin is commonly used in the treatment of diseases in swine especially weaners and growers (Jensen et al., 2012). The use of antimicrobials including tetracycline and tiamulin is associated with AMR in *Campylobacter* (Rollo et al., 2010; Hlashwayo et al., 2020). A study by Quintana-hayashi & Thakur, (2012) recorded a clear association between use of tetracyclines and AMR of *Campylobacter* in nursery piglets. Similarly, Lykkeberg et al., (2007) observed that tiamulin metabolites select for AMR in *C. coli*.

The overall animal-level prevalence of *Campylobacter* in this study was 18.2% (262/1440). In one study, Thakur & Gebreyes, (2005b) found a prevalence of 27.6% in piglets reared in conventional farms as compared to 77.3% in antibiotic-free farms. But this prevalence is relatively lower than what has been reported elsewhere in grower pigs (Varela et al., 2007; Kempf et al., 2017) in contrast to Papadopoulos et al., (2020) findings that nursery piglets were more likely to get colonized with *Campylobacter* spp. than sows. The majority of the strains in

our study were *C. hyointestinalis*, 17.9% (258/14400) and *C. coli* was only 0.3% (4/1440). Results on *C. hyointestinalis* align with what is in the literature in that it is among the commonly isolated *Campylobacter* species in swine (Allos et al., 2015). Most prevalence studies in swine limit their reporting on the two *Campylobacter* spp., *C. jejuni* and *C. coli* that are considered to have public health potential and leave *C. hyointestinalis* unreported. But studies have pointed out *C. hyointestinalis* as an emerging pathogen responsible for gastrointestinal illness in humans (Gorkiewicz et al., 2002; Man, 2011; Corry, 2014; Facciola et al., 2017) which makes it equally important. Surprising is the low prevalence of *C. coli* recorded in this study. Many studies have shown *C. coli* to be the most prevalent *Campylobacter* spp., in swine (Gebreyes et al., 2005; Harvey et al., 2007; Varela et al., 2007; Di Donato et al., 2020; Papadopoulos et al., 2020). *C. coli* is an important human pathogen that causing gastroenteritis and second most important after *C. jejuni* (Varela et al., 2007; Facciola et al., 2017).

Campylobacter strains were highly resistant to the critically important class of antimicrobials, the fluoroquinolones (ciprofloxacin) and quinolones (nalidixic acid). Similar results were recorded in other studies that have reported high resistance to ciprofloxacin and nalidixic acid (Griggs et al., 2005; Thakur & Gebreyes, 2005). In addition, the majority of *Campylobacter* strains were resistant to a medically important antimicrobial, tetracycline. The presence of *tetO* in all the strains tested accounts for the high resistance to tetracyclines (98.5%) observed in this study. The *tetO* gene is both chromosomally and plasmid-associated and can be transferred between bacteria of the same and different species (Taylor & Courvalin, 1988; Pratt & Korolik, 2005). The ability of the *tetO* gene to be transferred intraspecies and interspecies explains the high prevalence of *Campylobacter* species reported (Thakur & Gebreyes, 2005a).

In this study, we evaluated the impact of in-feed versus in-water antimicrobial administration and non-treated controls, on the fecal prevalence and antimicrobial susceptibilities of a foodborne pathogen, *Campylobacter* in nursery piglets. We initially hypothesized that the oral route of administering antimicrobial has an impact on *Campylobacter* prevalence and resistance to antimicrobials. Indeed Zhang et al. (2013) observed increased resistance in gut microbiota when mice were orally treated with antibiotics than when parenteral routes were used. In our study, neither in-feed nor in-water routes of antibiotic administration had a significant impact on the prevalence and AMR of *Campylobacter* compared to untreated controls. Similarly, treating piglets with either tiamulin or tetracycline or the combination of both did not significantly influence prevalence and AMR in *Campylobacter*. Similar results were recorded by Juntunen et al. (2013), where tetracycline administration in pigs was not associated with a change in resistance of *Campylobacter* to tetracycline, ciprofloxacin and erythromycin. High *Campylobacter* resistances have been recorded even in antibiotic-free farms indicating that the development of resistance to the pathogen does not necessarily result from antimicrobial usage (Rollo et al., 2010; Quintana-hayashi & Thakur, 2012). In contrast, Asai et al. (2007) reported an increased tetracycline resistance of *Campylobacter* spp. following tetracycline treatment in animals. The absence of difference between treatment groups could be due to a dominant effect of the environment or, that the microbiota has stabilized in the presence of constant tiamulin and tetracycline use. It could also be attributed to cross-contamination across pens by personnel movements.

The effect exerted by antimicrobials on gut pathogens could be influenced by the dosage used, distribution and their ultimate concentration in the gut. In this study, we used the dosages as per FDA guidance for treatment or prevention of diseases (FDA, 2020a, 2020b). The same

dosage of chlortetracycline, 22 mg/kg BW was provided in both in-feed and in-water administration, while for tiamulin, a 23 mg/kg BW dosage was applied in-feed and 5 mg/kg BW in-water. Also, the dosage of antimicrobials has a linear relationship with their concentrations in the gut (Peeters et al., 2016). Therefore, the relatively same gut concentration in both in-feed and in-water for both tetracycline and tiamulin could explain the lack of difference observed in prevalence and AMR of bacteria. In addition, the two drugs, chlortetracycline and tiamulin give enough concentrations in the gut which explains their use in treating enteric infections. Tetracyclines have low bioavailability (Peeters et al., 2016), making a large amount of the drug to remain in the gut and exert its effects on gut bacteria. On the other end, tiamulin has high bioavailability, and is metabolized quickly and excreted in the gut via bile to exert its effect on the gut microorganisms (Burch, 2012).

Whole genome sequencing from the five strains showed a high correlation of phenotypic and genotypic data. Similar to PCR results, all the strains contained the tetracycline resistant gene, *tetO* which has been found in other studies (Crespo et al., 2012; Zhao et al., 2016). Also, all strains carried point mutations in the gyrase subunit A (GyrA) conferring resistance to quinolones and fluoroquinolones. Therefore, the correlation between genotypic and phenotypic data was 100% for both tetracyclines and quinolones. Similar results have been observed in a previous study using *C. jejuni* (Zhao et al., 2016). WGS also revealed all the five strains to be multidrug resistant consistent with what was observed when broth microdilution was used.

In summary, our study did not find the route of administration of CTC and tiamulin to significantly impact the prevalence and AMR of *Campylobacter* in feces of piglets. Similarly, the main effect of treatment did not have a significant effect. In addition, there was no evidence of sampling day $\times$ treatment interaction on the prevalence of *Campylobacter*. Although the

interaction effect of treatment $\times$ sampling day on the prevalence of *Campylobacter* was significant on some sampling days. In addition, we found a higher prevalence of *C. hyointestinalis* in piglets as compared to *C. coli*, in contrast to what is reported in many studies. Also, a large number of *Campylobacter* strains were found to be multidrug resistant and exhibited a wide range of resistance profiles that included resistance to a fluoroquinolone (ciprofloxacin) that is frequently used to treat *Campylobacter* infections. This study also observed a high correlation of phenotypic and genotypic AMR showing that WGS can accurately predict AMR profiles in bacteria.

Table 2.1: Gene targets and primers used for speciation of *Campylobacter* isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin

Species	Primer	Target gene	Sequence (5' to 3')	Amplicon size	Reference
Genus	C412F	16S rRNA	5'-GGATGACACTTTTCGGAGC-3'	816	Linton et al. (1996)
<i>Campylobacter</i>	C1228R		5'-CATTGTAGCACGTGTGTC-3'		
<i>C. hyointestinalis</i>	HYO1F	23S rRNA	5'-ATAATCTAGGTGAGAATCCTAG-3'	611	Inglis & Kalischuk (2003)
subsp. <i>hyointestinalis</i>	HYOFET 23SR		5'-GCTTCGCATAGCTAACAT-3'		
<i>C. coli</i>	CC18F	<i>askD</i>	5'-GGTATGATTTCTACAAAGCGAG-3'	502	Linton et al. (1997)
	CC519R		5'-ATAAAAGACTATCGTCGCGTG-3'		
<i>C. fetus</i>	MG3F	<i>cstA</i>	5'-GGTAGCCGCAGCTGCTAAGAT-3'	359	Hum et al. (1997)
	CF359R		5'-AGCCAGTAACGCATATTATAGTAG-3'		
<i>C. lari</i>	CLF	<i>glyA</i>	5'-TAGAGAGATAGCAAAAGAGA-3'	251	Wang et al. (2002)
	CLR		5'-TACACATAATAATCCCACCC-3'		
<i>C. jejuni</i>	C-1	<i>cj0414</i>	5'-CAAATAAAGTTAGAGGTAGAATGT-3'	161	Wang et al. (1992)
	C-3		5'-CCATAAGCACTAGCTAGCTGAT-3'		
<i>C. upsaliensis</i>	CU61F	<i>lpxA</i>	5'-CGATGATGTGCAAATTGAAGC-3'	86	Yamazaki-Matsune et al., (2007).
	CU146R		5'-TTCTAGCCCCTTGCTTGATG-3'		

Table 2.2: PCR target genes and primers used for detection of *tet*(A, B, O, and S) resistance genes in *Campylobacter* isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin.

Primer	Target Gene	Sequence (5' to 3')	Amplicon (bp)	Reference
<i>tetA</i> -F <i>tetA</i> -R	<i>tet</i> (A)	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	888	Abdi-Hachesoo et al. (2014), Ng et al.(2001)
<i>tetB</i> -F <i>tetB</i> -R	<i>tet</i> (B)	CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTCGCC	774	Abdi-Hachesoo et al. (2014), Ng et al.(2001)
<i>tetS</i> -F <i>tetS</i> -R	<i>tet</i> (S)	CATAGACAAGCCGTTGACC ATG TTT TTG GAA CGC CAG AG	667	Abdi-Hachesoo et al. (2014), Ng et al.(2001)
<i>tetO</i> -F <i>tetO</i> -R	<i>tet</i> (O)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	Abdi-Hachesoo et al. (2014), Ng et al.(2001)

Table 2.3: Animal-level fecal prevalence of *Campylobacter* in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=1,440)

Treatment group	Treatment Phases						Total (%)
	Pre-Treatment		Treatment		Post-Treatment		
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
Control	1	2	8	9	15	13	49 (20.4)
In-feed CTC	0	5	6	12	11	18	52 (21.7)
In-water CTC	2	3	4	3	12	14	37 (15.4)
In-feed Tiamulin	0	1	5	11	19	17	53 (22.1)
In-water Tiamulin	0	2	3	2	14	13	34 (14.2)
In-Feed CTC + Tiamulin	0	3	5	7	13	9	37 (15.4)
Weekly Total (%)	3 (1.3)	16 (6.7)	31 (12.9)	44 (18.3)	84 (35.0)	84 (35.0)	
Total (%)	19 (4.0)		75 (15.6)		168 (35)		262 (18.2)

Table 2.4: Fecal prevalence (main effects) of *Campylobacter* in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=1,440)

			Comparison to Control
Endpoint	Treatment	Prevalence+/- S.E.	Odds Ratio (<i>P</i>-value for Testing Odds Ratio⁼¹)
<i>C. hyointestinalis</i> (n=258)	Control	0.275+/-0.047	--
	In-feed CTC	0.268+/-0.049	0.97 (0.923)
	In-water CTC	0.174+/-0.04	0.56 (0.109)
	In-feed Tiamulin	0.308+/-0.052	1.17 (0.639)
	In-water Tiamulin	0.153+/-0.04	0.48 (0.056)
	In-feed CTC + Tiamulin	0.198+/-0.041	0.65 (0.221)
Overall (n=262)	Control	0.274+/-0.048	--
	In-feed CTC	0.273+/-0.049	0.99 (0.987)
	In-water CTC	0.172+/-0.04	0.55 (0.105)
	In-feed Tiamulin	0.306+/-0.052	1.16 (0.655)
	In-water Tiamulin	0.153+/-0.04	0.48 (0.057)
	In-feed CTC + Tiamulin	0.199+/-0.041	0.66 (0.235)

Table 2.5: Interaction effect Treatment vs. Sampling Day on the prevalence of *Campylobacter* in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=1,440)

				Odds Ratio (P-value for Testing Odds Ratio^Λ=1) Comparison to		
Endpoint	Treatment	Sampling Day	Prevalence+/- S.E.	Day 14	Day 21	Day 28
Overall (n=262)	Control	Day 7	0.198+/-0.068	0.86 (0.784)	0.41 (0.086)	0.52 (0.205)
		Day 14	0.223+/-0.071	--	0.48 (0.144)	0.60 (0.316)
		Day 21	0.375+/-0.085	--	--	1.25 (0.637)
		Day 28	0.324+/-0.082	--	--	--
	In-feed CTC	Day 7	0.143+/-0.058	0.41 (0.111)	0.46 (0.173)	0.21 (0.004)
		Day 14	0.292+/-0.08	--	1.13 (0.803)	0.52 (0.164)
		Day 21	0.267+/-0.077	--	--	0.46 (0.103)
		Day 28	0.444+/-0.089	--	--	--
	In-water CTC	Day 7	0.097+/-0.048	1.37 (0.692)	0.26 (0.031)	0.20 (0.011)
		Day 14	0.073+/-0.042	--	0.19 (0.016)	0.15 (0.005)
		Day 21	0.296+/-0.079	--	--	0.79 (0.630)
		Day 28	0.347+/-0.083	--	--	--
	In-feed Tiamulin	Day 7	0.125+/-0.055	0.37 (0.100)	0.16 (0.001)	0.19 (0.004)
		Day 14	0.276+/-0.078	--	0.42 (0.066)	0.51 (0.160)
		Day 21	0.479+/-0.089	--	--	1.23 (0.651)
		Day 28	0.428+/-0.088	--	--	--
	In-water Tiamulin	Day 7	0.074+/-0.043	1.54 (0.646)	0.15 (0.006)	0.17 (0.009)
		Day 14	0.049+/-0.035	--	0.10 (0.003)	0.11 (0.005)
		Day 21	0.35+/-0.084	--	--	1.12 (0.812)
		Day 28	0.324+/-0.082	--	--	--
	In-feed CTC + Tiamulin	Day 7	0.122+/-0.054	0.67 (0.531)	0.29 (0.037)	0.49 (0.242)
		Day 14	0.171+/-0.063	--	0.44 (0.123)	0.73 (0.575)
		Day 21	0.322+/-0.082	--	--	1.67 (0.315)
		Day 28	0.221+/-0.071	--	--	--
<i>C. hyointestinalis</i> (n=258)	Control	Day 7	0.199+/-0.068	0.86 (0.784)	0.41 (0.086)	0.52 (0.205)
		Day 14	0.224+/-0.071	--	0.48 (0.144)	0.60 (0.316)
		Day 21	0.375+/-0.085	--	--	1.25 (0.638)

		Day 28	0.325+/-0.082	--	--	--
	In-feed CTC	Day 7	0.14+/-0.057	0.40 (0.110)	0.46 (0.173)	0.21 (0.004)
		Day 14	0.287+/-0.079	--	1.13 (0.803)	0.52 (0.163)
		Day 21	0.262+/-0.076	--	--	0.46 (0.102)
		Day 28	0.438+/-0.089	--	--	--
	In-water CTC	Day 7	0.098+/-0.049	1.37 (0.692)	0.26 (0.031)	0.20 (0.011)
		Day 14	0.073+/-0.042	--	0.19 (0.016)	0.15 (0.005)
		Day 21	0.299+/-0.08	--	--	0.79 (0.631)
		Day 28	0.349+/-0.084	--	--	--
	In-feed Tiamulin	Day 7	0.126+/-0.056	0.37 (0.100)	0.16 (0.001)	0.19 (0.004)
		Day 14	0.278+/-0.078	--	0.42 (0.066)	0.51 (0.160)
		Day 21	0.481+/-0.088	--	--	1.23 (0.651)
		Day 28	0.431+/-0.088	--	--	--
	In-water Tiamulin	Day 7	0.074+/-0.043	1.54 (0.646)	0.15 (0.006)	0.17 (0.009)
		Day 14	0.049+/-0.035	--	0.10 (0.003)	0.11 (0.005)
		Day 21	0.35+/-0.083	--	--	1.12 (0.812)
		Day 28	0.325+/-0.082	--	--	--
	In-feed CTC + Tiamulin	Day 7	0.121+/-0.054	0.67 (0.531)	0.29 (0.037)	0.49 (0.242)
		Day 14	0.17+/-0.063	--	0.44 (0.123)	0.73 (0.574)
		Day 21	0.32+/-0.081	--	--	1.67 (0.315)
		Day 28	0.199+/-0.068	0.86 (0.784)	0.41 (0.086)	0.52 (0.205)

Table 2.6: Prevalence and MIC* distributions of *Campylobacter* strains in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=262)

Antimicrobials	Resistant breakpoints	% Resistant	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
Azithromycin	≥1	5	11	13	197	23	3	3	0	1	2	3	2	1	3		
Ciprofloxacin	≥1	89.3		1	0	11	10	6	2	4	8	204	16				
Clindamycin	≥2	3.1		28	10	44	163	4	5	1	1	3	3				
Erythromycin	≥16	3.4		22	10	9	39	153	16	4	1	1	1	1	4		
Florfenicol	≥8	1.2				1	18	213	22	4	1	1	1	1			
Gentamicin	≥4	8				28	9	25	109	70	4	2	1	1			
Nalidixic Acid	≥32	60.3										5	99	86	72		
Telithromycin	≥8	1.9	26	4	7	1	8	152	52	7	1	4					
Tetracycline	≥4	98.5					1	1	1	1	4	14	75	86	79		

MIC*=Minimal inhibitory concentration

Note: the top dilution tested for each drug should be interpreted as ≥ and the lowest dilution tested for each drug as ≤

Table 2.7: Resistance profiles of *Campylobacter* strains isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=262)

Resistance Profile	Number of strains (%)
CIP_NAL-TET	122 (46.6)
CIP_TET	83 (31.7)
CIP_GEN_NAL_TET	14 (5.3)
NAL_TET	14 (5.3)
TET	8 (3.1)
AZI_CIP_CLI_ERY_FFN_GEN_NAL_TEL_TET	2 (0.8)
AZI_CIP_TET	2 (0.8)
CIP_CLI_NAL_TET	2 (0.8)
CIP_ERY_TET	2 (0.8)
AZI_CIP_NAL_TET	2 (0.8)
CIP_GEN_TEL_TET	1(0.4)
GEN_TET	1(0.4)
AZI_CLI_ERY_FFN_GEN_TEL_TET	1(0.4)
CIP_GEN_TET	1(0.4)
AZI_CIP_GEN_NAL_TET	1(0.4)
AZI_CIP_CLI_ERY_TET	1(0.4)
NAL	1(0.4)
AZI_CIP_CLI_ERY_TEL_TET	1(0.4)
AZI_CLI_ERY_TEL	1(0.4)
AZI_ERY	1(0.4)
AZI	1(0.4)

Phenotypes – AZI = azithromycin, CIP = ciprofloxacin, CLI = clindamycin, ERY = erythromycin, FFN = florfenicol, GEN = gentamicin, NAL = nalidixic acid, TEL = telithromycin, TET = tetracycline.

Table 2.8: AMR genotypes from whole genome sequencing of *Campylobacter* in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin

Gene/Mutation	Gene/Mutation Name	Antimicrobial class	Antimicrobial
<i>tetO</i>	tetracycline resistance ribosomal protection protein <i>tetO</i>	Tetracycline	Tetracycline
<i>rpsL_K43K</i>	30S ribosomal protein S12 RpsL	Aminoglycoside	Streptomycin
23S_A2074A	23S ribosomal RNA	Macrolide	Macrolide
23S_A2075A	23S ribosomal RNA	Macrolide	Macrolide
23S_C2627C	23S ribosomal RNA	Macrolide	Macrolide
<i>rpsL_K88R</i>	<i>Campylobacter</i> streptomycin resistant RpsL	Aminoglycoside	Streptomycin
<i>bla</i> _{OXA-489}	OXA-61 family class D beta-lactamase OXA-489	Beta-Lactam	Beta-Lactam
<i>aph</i> (3')-IIIa	aminoglycoside O-phosphotransferase APH(3')-IIIa	Aminoglycoside	Amikacin/Kanamycin
<i>gyrA_T86I</i>	DNA gyrase subunit A GyrA	Quinolones and Fluoroquinolones	Quinolones and Fluoroquinolones
<i>aph</i> (2'')-Ih	aminoglycoside O-phosphotransferase APH(2'')-Ih	Aminoglycoside	Amikacin/Gentamicin/ Kanamycin/ Tobramycin
50S_L22_A103A	50S ribosomal protein L22	Macrolide	Macrolide
50S_L22_A84A	50S ribosomal protein L22	Macrolide	Macrolide
50S_L22_A88A	50S ribosomal protein L22	Macrolide	Macrolide
50S_L22_G86G	50S ribosomal protein L22	Macrolide	Macrolide
<i>rpsL_K88K</i>	30S ribosomal protein S12 RpsL	Aminoglycoside	Streptomycin
<i>gyrA_D90D</i>	DNA gyrase subunit A GyrA	Quinolones and Fluoroquinolones	Quinolones and Fluoroquinolones
<i>gyrA_P104P</i>	DNA gyrase subunit A GyrA	Quinolones and Fluoroquinolones	Quinolones and Fluoroquinolones

Chapter 3 - The impact of in-water vs. in-feed chlortetracycline and or tiamulin administration in piglets on the fecal prevalence and antimicrobial resistance of *Salmonella*

Introduction

Antimicrobial Resistance (AMR) is a global public health concern and is listed among the top health challenges in the world (WHO, 2020). In the United States, 2.8 million AMR bacterial and fungal infections are estimated to occur, resulting in approximately 35,900 deaths every year (CDC, 2019). The public health risks associated with antimicrobial use as growth promoters in animal production has led to regulations to restrict antimicrobial use in many countries, including in the US. Antimicrobials have been used in swine routinely as a management tool by farmers, (Aarestrup, 2015). The weaned (nursery) piglets consumes most of these antimicrobials (Davies & Singer, 2020) as they are more vulnerable to infectious diseases (Lekagul et al., 2019). Several studies have shown a clear association between antimicrobial use and development of AMR in food animals (Bronzwaer et al., 2002; Tang et al., 2017).

Tetracyclines, macrolides, and pleuromutilin are the most commonly used antimicrobials in swine (Holman & Chénier, 2013). Chlortetracycline (CTC), a tetracycline, is widely used in nursery pigs (Apley et al., 2012; NAHMS Swine Study, 2017; Davies and Singer, 2020) and is available for use by three routes of administration (in-feed, and in-water). Tiamulin, a pleuromutilin and another commonly used antibiotic in nursery pigs to treat swine dysentery associated with *Brachyspira hyodysenteriae* and pneumonia, is administered in-feed or in-water (NAHMS Swine Study, 2017; Davies and Singer, 2020).

Salmonella enterica is a foodborne pathogen of major public health concern. In the US, non-typhoidal *Salmonella* is estimated to cause annually about 1.35 million infections, 26,500 hospitalizations, and 420 deaths (<https://www.cdc.gov/Salmonella/index.html>). Based on the CDC's National Outbreak Reporting System (NORS), between 1998 and 2017, a total of 3,467 *Salmonella* outbreaks have been reported in the US (<https://wwwn.cdc.gov/norsdashboard/>). The US Department of Agriculture (USDA) Economic Research Service (ERS) reports the total annual cost of foodborne illnesses due to *Salmonella* is \$3.6 billion (USDA ERS, 2014). Antimicrobial resistant *Salmonella* is categorized under serious level threat by the CDC and causes about 212,500 infections, resulting in about 70 deaths every year (CDC, 2019).

Apart from implementing control systems to monitor and minimize antimicrobial use, optimizing dosage and route of administration can be an effective strategy to mitigate the development and dissemination of AMR without affecting the therapeutic efficacy of the antimicrobials. In swine, the oral route (in-feed and in-water) has been the most commonly used route of antimicrobial administration to treat pigs in groups (Sjölund et al., 2015; NAHMS Swine Study, 2017). Based on a systematic review, oral administration of antimicrobials is considered to be a critical risk factor in the development and propagation of AMR in swine (Burow et al., 2014). A study conducted in mice suggested that administering antimicrobials orally caused a notable increase of AMR in the gut microbiota compared to intravenous injection (L. Zhang et al., 2013). Another study in humans recommended that parenteral antibiotic administration is preferred over oral antibiotic administration as the gut commensal and pathogenic bacteria are less exposed to the antimicrobials leading to less chance of resistance selection (Rafii et al., 2008). Therefore, the route of administration of antimicrobials in food animal production is likely to be a significant contributing factor in the development of AMR. The impact of oral, in-

water vs. in-feed antibiotic administration on the development and persistence of AMR in the foodborne pathogen, *S. enterica*, has not been studied in nursery pigs. Therefore, the objective of this study was to assess the impact of the route of administration of chlortetracycline and tiamulin, in-feed vs in-water, on prevalence and AMR profiles of *S. enterica* in the feces of piglets.

Materials and Methods

Animals and Experimental Design

The experimental protocols used in these studies were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC#4033). The study was conducted at a commercial swine research facility in the Midwest. The facility is totally enclosed, environmentally controlled, and mechanically ventilated. Pens were equipped with slatted flooring and deep pits for manure storage. Each pen consisted of six-hole stainless steel self-feeder, and a pan waterer to provide ad libitum access to feed and water. A total of 1,296 weaned piglets (L337×1050, PIC, Hendersonville, TN) were used in the study. Piglets, weaned at 21 days of age, were allocated into 48 pens (27 piglets per pen) distributed in a single room so that average pen body weights were relatively equal among pens. After a 7-d acclimatization period, pens were randomly assigned to the following six treatment groups: a) Control: piglets fed a basal diet without antibiotic, b) In-feed CTC: piglets fed the basal diet supplemented with chlortetracycline to provide 22 mg/kg body weight (CTC-hydrochloride, Elanco- Eli Lilly and Company, Indianapolis, IN) for 14 days, c) In-water CTC: piglets fed the basal diet and CTC administered in drinking water to provide 22 mg/kg body weight for 14 days, d) In-feed tiamulin:

piglets fed the basal diet supplemented with tiamulin to provide 5 mg/kg body weight (Denagard®, Elanco Animal Health- Eli Lilly and Company, Indianapolis, IN) for 14 days, e) In-water tiamulin: piglets fed a basal diet with tiamulin administered in drinking water to provide 23 mg/kg body weight for 14 days, and f) a combination of CTC and tiamulin (In-feed): supplemented with CTC to provide 22 mg/kg body weight and tiamulin at 5 mg/kg body weight for 14 days. Each treatment had 8 pens and the treatment allocations to pens followed a blocked design that ensured adjacent pens alternated among treatment groups. Also, the treatment allocation ensured that each pen-treatment was in contact with an equal number of other pen treatments. The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements. Daily feed additions to each pen were accomplished through a robotic feeding system to provide measured amounts for individual pens. The duration of the study was 35 days, which was divided into three phases: pre-treatment (days -7 and 0), treatment (days 7 and 14), and post-treatment (days 21 and 28).

Fecal sample collection

Fresh fecal samples were collected randomly from 5 piglets from each pen by a gentle rectal massage with proper restraining. Fecal samples were placed in individual plastic bags (Whirl-Pak® bags, Nasco, Ft. Atkinson, WI) and transported on ice in a cooler to the Pre-harvest Food Safety laboratory, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, at Kansas State University and stored at 4°C before and processed within 24 h. The laboratory personnel were blinded to the treatments.

Isolation of *Salmonella*

Approximately, 10 g of fecal sample was added to 90 ml of Tetrathionate (TT) broth (Difco™, MD, USA) with iodine added just before processing in a high temperature Nasco Whirl-Pak plastic bags (Nasco, Ft. Atkinson, WI). The contents were mixed well and incubated at 37° C for 24 h. One milliliter of TT broth was transferred to 9 ml of Rappaport Vassiliadis (RV) broth (Difco™, MD, USA) and incubated at 42° C for 24 h. After enrichment, the RV broth was plated onto Hektoen Enteric (HE) agar (Difco™, MD, USA) and incubated at 37° C for 24 h. Presumptive *Salmonella* colonies (green colored with black center colonies) were picked and streaked onto a blood agar plate and incubated overnight at 37°C. The three isolates from each sample were subjected to a rapid slide agglutination test using *Salmonella* antisera (BD Difco™ *Salmonella* O Antiserum Poly A-I & VI, MD, USA) for *Salmonella* identification.

PCR identification of *Salmonella*

DNA was extracted using the boil-prep method by suspending two bacterial colonies in 150 µl of nuclease-free water, followed by boiling for 10 minutes. The boiled bacterial lysate was centrifuged at 16,000×g for 2 min and the resulting supernatant was used as a DNA template for PCR reactions. Species confirmation was done by detecting *invA* (codes for an inner membrane protein of the type III secretion system) and *pagC* (codes for a periplasmic nonspecific phosphatase) genes using a duplex real-time PCR assay (Bai et al., 2018).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) of *Salmonella* strains were determined using commercial Sensititre panel plates (Sensititre™ CMV3AGNF, Trek Diagnostic Systems, Thermo Scientific Microbiology, Oakwood Village, OH, USA, Table 3.3). Overnight bacterial colonies from the blood agar plates were suspended in demineralized water (Trek Diagnostic Systems, Thermo Scientific), vortexed, and adjusted to 0.5 McFarland turbidity standard (Trek Diagnostic Systems, Thermo Scientific Microbiology, Oakwood Village, OH, USA). Ten microliters of the suspension were transferred to cation-adjusted Mueller-Hinton (Trek Diagnostic Systems, Thermo Scientific Microbiology, Oakwood Village, OH, USA) broth and vortexed. Then, 50 µl of the culture were inoculated into Sensititre plates and incubated at 37° C for 24 h. The antimicrobials tested were amoxicillin/clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tiamulin, tetracycline, and trimethoprim/sulphamethoxazole. The Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines for antimicrobial breakpoints were used to interpret the MIC values as either resistant (including intermediate) or susceptible. To obtain tiamulin MICs, the commercially prepared panels (Sensititre™ BOPO7F, Trek Diagnostic Systems, Thermo Scientific Microbiology, Oakwood Village, OH, USA, and Table 3.3) were used. The veterinary standards were used to determine the resistance breakpoint for tiamulin since there are no values established by CLSI. Strains that showed resistance to three or more antimicrobial classes were classified as multidrug resistant (MDR) (Schwarz et al., 2010).

PCR detection of tetracycline resistance genes

The DNA extracted from *Salmonella* was screened for *tet* (A, B, C, D, and E) genes by PCR. *E. coli* positive control strains were procured from USDA (MARC, Clay Center, NE) and Dr. Marilyn C. Roberts at the University of Washington. Target genes and primers used are shown in table 3.1. The 20- μ l reaction volume consisted of 10 μ l master mix, 6 μ l of nuclease-free water, 1 μ l each of forward and reverse primers, and 3 μ l of DNA template. Reactions were run in a Master-cycler gradient thermal cycler (Eppendorf, city, Germany), and the conditions were 1 cycle of initial activation at 95° C for 3 minutes, 35 cycles of denaturation at 94° C for 30 seconds, annealing at 60° C for 90 seconds and extension at 72° C for 90 seconds, followed by a cycle of final extension at 72° C for 10 minutes. Analysis of PCR products was done by capillary gel electrophoresis in the QIAxcel Advanced system (Qiagen, Germantown, MD).

Whole genome sequencing and bioinformatics

DNA libraries from *Salmonella* strains were prepared using the Illumina NextEra XT library preparation kit, with a modified protocol. Library quantity was assessed with Qubit (ThermoFisher). Libraries were then sequenced on an Illumina HiSeq platform 2x150bp. Raw sequencing reads were trimmed and processed using BBDuk (Bushnell & Brian; 2014) with a read quality trimming parameter of 22 for further downstream analysis. The trimmed fastq sequences were assembled using SPAdes (Bankevich, *et al.*, 2012). The assembled contigs were then processed through CosmosID core genome SNP typing pipeline to evaluate the phylogenetic placement and SNP differences for meaningful epidemiological inferences. The CosmosID SNP typing pipeline uses Parsnp (Treangen *et al.*, 2014) to align the core genome of

bacterial genomes. While generating the core genome alignment with the default parameters, Parsnp considers the recombination events and the variation that might exist within the bacterial genomes. The final set of core-genome SNPs was then used by Parsnp to reconstruct the phylogenomic relationship among the genome using FastTree2 (Price *et al.*, 2010). Bioinformatics tools from the Center for Genomic Epidemiology (CGE) were used to detect AMR genes (ResFinder) (Zankari et al., 2012) and plasmids (PlasmidFinder) (Carattoli *et al.*, 2014) in the genomes.

GenBank Accession numbers

Assembled genomes of *Salmonella* strains have been deposited in the National Center for Biotechnology Information (NCBI) under BioProject Accession number PRJNA663574.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software (SAS version 9.4; Cary, NC) LOGISTIC procedure with sampling day in the STRATA statement and treatment group in the EXACT statement. Prevalence data were organized as binomial response at the pen level, i.e. the number of ‘+’ out of 5 sample per pen per sampling day. Bivariate descriptive statistics of *Salmonella* prevalence and or detection of tetracycline resistance genes by treatment group was carried out before model building. Because of the low prevalence, the exact conditional logistic regression approach was used to evaluate the treatment effect for Day 7 and Day 14 only. Block served as the stratifying variable for the conditional inference. Fixed effects of the model included treatment and the number of ‘+’ samples in the pen on Day 0. All tests were conducted at the 0.10 significance level. Comparisons between antibiotic treatments vs.

control were carried out using the 2-sided exact test when the treatment group had ‘+’ samples and 1-sided exact test when the treatment group had no ‘+’ samples (Hirji et al., 1989). The MIC data categorized as resistant or susceptible to 15 different antimicrobials were considered as binary responses and multidrug profiles. A likelihood ratio chi-square test was performed to assess the relationship between *Salmonella* prevalence and MDR profiles.

Results

Fecal prevalence of *Salmonella*

A total of 1,440 fecal samples were collected in six weeks (240 fecal samples per week) during the study period. The fecal prevalence of *Salmonella* within treatment groups, treatment phases and sampling week is displayed in Table 3.2. Neither antibiotic nor route of administration had an effect ($P > 0.10$) on the fecal prevalence of *Salmonella*. Similarly, there was no sampling week effect on the prevalence of *Salmonella* ($P > 0.10$). The overall prevalence of *Salmonella* was 3.0% (43/1,440). The prevalence of *Salmonella* was numerically higher among piglets in the in-water tiamulin (4.2%, n=240) group compared to the control (2.5%), in-feed tiamulin (1.7%), in-feed CTC (2.1%), in-water CTC (3.8 %), and in-feed combination of CTC and tiamulin (3.8 %). The treatment phase had the numerically highest prevalence of *Salmonella* (5.0%, 24/480) when compared to the pre-treatment (3.5%, 17/480) and post treatment phases (0.42%, 2/480), but the difference was not significant.

Prevalence of tetracycline resistant genes

Salmonella strains (n=43) were screened for the presence of *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* genes. The *tetB* gene was detected in all 43 strains (100%). Only 11(25.6%) and 4 (9.3%) strains were positive for *tetD* and *tetA* genes, respectively, while all strains were negative for *tetC* and *tetE* genes. Fifteen *Salmonella* strains were positive for two tetracycline resistance genes, with 11 positives for *tetB* and *tetD* and four strains positive for *tetA* and *tetD*. Only one strain harbored three tetracycline resistant genes (*tetA*, *tetB*, and *tetD*) genes.

Antimicrobial susceptibility phenotypes

The phenotypic resistance and MIC distributions are summarized in a squashtogram (Table 3.4). All strains were resistant (100%) to ampicillin, streptomycin, sulfisoxazole, tetracycline, and tiamulin, and the majority were resistant to ciprofloxacin (95.4%) and nalidixic acid (74.4%). Low level resistance to azithromycin (0%), cefoxitin (0%), trimethoprim/sulphamethoxazole (4.7%), ceftriaxone (7.0%), and ceftiofur (7.0%) was observed among the strains. Resistance to most antimicrobials decreased ($P < 0.10$) over time. Neither CTC nor tiamulin treatment affected AMR profiles of *Salmonella* strains. All strains expressed resistance to at least three antimicrobial classes, hence, were classified as MDR.

Six unique resistance phenotypes were identified in the strains. No strain was resistant to all antimicrobials used in the susceptibility testing. The most common phenotype belonged to MDR strains, which were resistant to six antimicrobial classes (90.7%;39/43), followed by resistance to seven antimicrobial classes (7.0%; 3/43) and only one strain exhibited resistance to

five antimicrobial classes (2%; 1/43). The most frequently observed (48.8%; n=43) AMR phenotype was hepta-resistance pattern AMP_CIP_FIS_NAL_STR_TET_TIA (ampicillin, ciprofloxacin, streptomycin, sulfonamides, and tetracycline) (Table 3.6).

Whole genome sequence analysis

The number of contigs ranged from 61 to 403 with the N50 ranging from 172,736-325,586 bp. The genome size ranged from 4,906,044 to 5,174,184 bp, with the GC content averaging 52.26 %. The Parsnp in CosmosID core genome SNP typing pipeline identified all 43 strains to be *Salmonella enterica* subsp. *enterica* serotype Typhimurium. ResFinder detected the presence of amikacin and tobramycin (aminoglycosides) (*aac(6')*-Iaa), amoxicillin, ampicillin, cephalothin, piperacillin and ticarcillin (*bla*TEM-1B), ciprofloxacin (*qnr*B19), streptomycin (*strA* and *strB*), sulfamethoxazole and sulfisoxazole (*sul2*), and tetracycline (*tetB*) genes in all 43 strains. None of the strains carried genes that encode for clavulanic acid, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, gentamicin, nalidixic acid, tiamulin and trimethoprim. Furthermore, Plasmid Finder revealed the presence of Col (pHAD28) and IncQ1 in all strains. High correlation between phenotypic and genotypic resistance to antimicrobials was over 90% for ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, streptomycin, sulfisoxazole, tiamulin, and tetracycline (Table 3.5). Low correlation was observed on amoxicillin/clavulanic acid (20.9%) and trimethoprim/ sulphamethoxazole (4.7%). Despite having the resistant genes to amoxicillin and sulphamethoxazole, the strains were rendered susceptible by the presence of clavulanic acid and trimethoprim in the two antimicrobials.

Discussion

In this study, we investigated the impact of in-feed and in-water antimicrobial administration on the prevalence of antimicrobial resistant *Salmonella* among piglets. *Salmonella* is a major foodborne pathogen that colonizes hindgut of swine (Crump et al., 2015) and is a widely known cause of non-typhoidal infections in humans. Antimicrobials are used to treat *Salmonella* infections in both humans and animals, therefore, an increase in AMR is of major concern. In the present study, we explored the possibility of using two methods of oral administration of antimicrobials in nursery piglets as a potential strategy to mitigate AMR. We tested our hypothesis using the two most commonly used antimicrobials in the swine industry, tetracyclines and tiamulin. Tetracyclines and tiamulin are administered mainly to treat respiratory diseases in nursery and grower pigs (NAHMS Swine Study, 2017). Chlortetracycline has a more broad-spectrum (Chopra and Roberts, 2001), whereas, tiamulin has a limited spectrum of activity against Gram-positive bacteria and mycoplasma.

At the global level, the swine industry utilizes the highest consumption of antimicrobials when compared to other agricultural animals (Van Boeckel et al., 2015). In USA, the swine industry utilized 42% by weight of all medically important antimicrobials approved for use in food-producing animals in 2019 (FDA, 2019). Over 90% of these antimicrobials are administered orally in swine (both in-feed and in-water) making the oral route the most commonly used route of administration of antimicrobials in swine (Merle et al., 2012). We hypothesized that the oral administration of antimicrobials has an impact on the fecal prevalence and antimicrobial susceptibility of *Salmonella* in swine.

The effects of antimicrobials on gut bacteria are dependent on the dose administered, distribution of the antimicrobial and concentration in the gut. Chlortetracycline forms insoluble

compounds with aluminum, calcium, iron, magnesium and binds with lipids and protein in the gut reducing its absorption (Neuvonen, 1976; Agwuh & MacGowan, 2006). In an experimental study, the bioavailability of orally administered CTC in fed pigs was only 6% (Nielsen & Gyrd-Hansen, 1996; Peeters et al., 2016). The low bioavailability indicates that most of the antibiotic remains in the gastrointestinal tract, at a concentration large enough to act on the gut microbes. Therefore, the dosage will also have a linear relationship with the concentration of CTC (Peeters et al., 2016). In contrast, tiamulin bioavailability is up to 90% when administered orally (https://www.hpra.ie/img/uploaded/swedocuments/LicenseSPC_10825-017-001_06012016144953.pdf). Since oral bioavailability has an inverse relationship with intestinal concentration of the antibiotic (Peeters et al., 2016), tiamulin was expected to be available at a minimal concentration (<10%) in the gut content. Interestingly, tiamulin is quickly absorbed into the blood, gets metabolized in the liver, and is excreted via bile into the gut to accumulate in the ileum and colon contents at concentrations sufficient to exert its effect on gut microbes (<http://www.octagon-services.co.uk/articles/therapeutics.pdf>). Similar to CTC, increasing the amount of tiamulin provided orally enhances bioavailability and its ultimate concentration in the gut contents (Riond et al., 1993).

Salmonella infections in pigs vary from subclinical to clinical enteritis, which is often self-limiting. Estimates on the prevalence of *Salmonella* in pig farms of different ages seem to differ considerably by production and management types, ranging from 3.5 to 33% in the USA (Davies et al., 1998; Rodriguez et al., 2006). This variation is also observed across different stages of development of pigs, where piglets have a low level of *Salmonella* shedding when compared to sows (Dorr et al., 2009; Wendy Wilkins et al., 2010). In our study, the prevalence of *Salmonella* in nursery piglets was 3%. All strains belonged to *S. Typhimurium*. This is the first

time a single serotype of *Salmonella* has been reported in a prevalence study. The study was conducted in an enclosed commercial farm which explains the possibility of a single serotype circulating within farm piglets. According to Arguello et al., 2013 and García-Feliz et al., 2007, *Salmonella* Typhimurium is the most widespread serotype with zoonotic importance in swine production systems.

Five genes encoding resistance to tetracycline, *tet* (A, B, C, D, and G) have been identified in *Salmonella*. The *tetB* was present in all *Salmonella* strains obtained from this study. Similar findings were obtained in previous studies, where *tetB* and *tetC* were the most identified tetracycline resistance determinants in *Salmonella* (Mendez et al., 1980; Levy., 1988). The *tetB* gene has the widest distribution among Gram-negative bacteria (Chopra and Roberts, 2001). Moreover, it was observed in this study that in-feed CTC, tiamulin, or the combination of both antimicrobials did not show evidence of association with AMR in *Salmonella*. All strains obtained in this study were resistant to tetracycline and tiamulin. Several other studies have recorded high resistance to tetracyclines. *Salmonella* is inherently not susceptible to tiamulin. In this study, both CTC and tiamulin did not influence the prevalence and AMR in *Salmonella* in piglets. Similarly, Delsol et al. (2003) found that pigs treated with CTC showed constant high shedding of *S. Typhimurium* during treatment and post treatment periods. They argued that CTC helped the establishment of *S. Typhimurium* in the gastrointestinal tract of pigs making it persist throughout the post-treatment phase. Moreover, there was no significant difference in the resistance to CTC when *Salmonella* strains were compared between antibiotic-free swine farms and farms using antimicrobials (Ebner & Mathew, 2000; Gebreyes et al., 2006). The lack difference in resistances observed in these studies could be due to the effect of carryover from

earlier times when antibiotics were used. Information on the influence of tiamulin on AMR *Salmonella* is limited and this study will provide the first insight.

According to Bakkeren et al. (2019), *Salmonella* tends to develop persisters that spread resistance to other bacteria through plasmids, making the spread of resistance possible even without the use of antimicrobials. Resistant *Salmonella* could have been passed vertically from dams, or horizontally from farm personnel and environment, that persisted throughout the length of the experiment. Moreover, all *Salmonella* strains recovered in the present study were MDR. In their analysis, Wang et al. (2019) observed persistence and increase of MDR *Salmonella* Typhimurium in the food chain throughout the 1996-2016 period. The increasing resistance to multiple drugs could potentially cause *Salmonella* to evolve into bacteria that are difficult to treat (Campioni et al., 2014). According to Hoelzer et al. (2011), pigs infected with *Salmonella* Typhimurium are often asymptomatic or develop mild gastroenteritis but can transmit the bacteria to humans through direct and indirect contacts. The high occurrence of multidrug resistant *Salmonella* recorded in this study warns of the possible risk of outbreaks in humans. Third generation cephalosporins, fluoroquinolones, and macrolides are recommended for antimicrobial therapy against salmonellosis in humans (Berbari et al., 2015). However, a high level of resistance to most of these important classes of antimicrobials observed in this study poses concern on their continual use. Most of the *Salmonella* strains exhibited no to low-level resistance to ceftiofur and ceftriaxone (third generation cephalosporins) and azithromycin (a macrolide) in our study.

In recent years, WGS has emerged as an essential tool to identify AMR determinants. The analysis has played a key role in providing superior information on epidemiological surveillance of AMR pathogens and study emergence of AMR in these pathogens, building new

and improving present diagnostic tools, and developing novel antimicrobials of therapeutic importance (Köser et al., 2014). Based on WGS, our results suggest that a similar clone of *Salmonella enterica* subsp. *enterica* serotype Typhimurium is circulating in the farm of origin for the pigs in this study. The clone may have been dispersed among piglets and between pens through farm personnel and pieces of equipment. Baloda et al. (2001) observed the persistence of the *Salmonella enterica* serotype Typhimurium DT12 in the pig farm environment for a long period of time that poses risk for transmission to animals and humans. According to Foley et al. (2008), *S. Typhimurium* has replaced *S. Choleraesuis* as the predominant serotype in pigs in the USA. When genome sequences were compared with their expected AMR genotypes obtained by PCR, only *tetB* matched at 100%. Both *tetA* and *tetD* genes were not found in their genomes. The observed discrepancy might be due the fact that PCR amplifies a small region in DNA that might be similar to the gene of interest but be different from the actual entire sequence identified by WGS. In addition, the genes could have been part of the gaps in the draft genomes obtained after sequencing. Also, the plasmid carrying the resistant genes could have been lost through multiple re-streaking of the bacterial cells. High correlations between phenotypic and genotypic resistance to other antimicrobials was observed in this study. The discrepancy observed between some of the phenotypic and genotypic resistances could be attributed to a non-functional resistance gene, intrinsic resistance of the bacterial strains to some antimicrobials, and the inclusion of intermediate MIC values as resistant.

In summary, our results revealed that in-feed and in-water route of antimicrobial administration did not influence the fecal prevalence and antimicrobial susceptibilities of *Salmonella* in this population of nursery piglets. The study also showed the presence of MDR

Salmonella with zoonotic potential, *S. Typhimurium*. The MDR *S. Typhimurium* were resistant to important and critically important antimicrobials.

Table 3.1: PCR target genes and primers used for detection of *tet* (A, B, C, D, and E) resistance genes in *Salmonella* isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin

Target gene	Primer Sequences (5' to 3')	Amplicon size (bp)	Source
<i>tet</i> (A)	TET A1: CGA GCC ATT CGC GAG AGC TET A2: CGA AGC AAG CAG GAC CAT G	2027	(Miranda et al., 2003)
<i>tet</i> (B)	TET BF: CAG TGC TGT TGT TGT CAT TAA TET BR: GCT TGG AAT ACT GAG TGT AA	576	"
<i>tet</i> (C)	TET C: TTG CAT GCA CCA TTC CTT GCG TET CR: TGG TCG TCA TCT ACC TGC C	521	"
<i>tet</i> (D)	TET D FW2: GGA TAT CTC ACC GCA TCT GC TET D(RV1): CAT CCA TCC GGA AGT GAT AGC	436	"
<i>tet</i> (E)	TET EF: TCC ATA CGC GAG ATG ATC TCC TET ER: CGA TTA CAG CTG TCA GGT GGG	442	"

Table 3.2: Animal-level fecal prevalence of *Salmonella* in piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=1,440)

Treatment group	Treatment Phases						Total (%)
	Pre-Treatment		Treatment		Post-Treatment		
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
Control	1	1	3	1	0	0	6 (2.5)
In-feed CTC	1	2	2	0	0	0	5 (2.1)
In-water CTC	0	3	4	1	0	1	9 (3.8)
In-feed Tiamulin	0	3	1	0	0	0	4 (1.7)
In-water Tiamulin	0	3	5	2	0	0	10(4.2)
In-Feed CTC + Tiamulin	0	3	3	2	0	1	9 (3.8)
Weekly Total (%)	2 (0.8)	15 (6.3)	18 (7.5)	6 (2.5)	0	2 (0.8)	
Total (%)	17 (3.5)		24 (5.0)		2 (0.42)		43 (3%)

Table 3.3: CLSI-NARMS-evaluated antimicrobial concentrations for Sensititre™ CMV3AGNF, and their resistance breakpoints (CLSI, 2018; WHO, 2019)

Antimicrobial agent	Concentration, µg/mL	Breakpoint, µg/mL ¹	WHO Classification ²
Amoxicillin/Clavulanic Acid	1/0.5–32/16	≥16/8	Critically important
Ampicillin	1–32	≥16	Critically important
Azithromycin	0.12–16	N/A ³	Critically important
Cefoxitin	0.5–32	≥16	Highly important
Ceftiofur	0.12–8	≥4	Critically important
Ceftriaxone	0.25–64	≥2	Critically important
Chloramphenicol	2–32	≥16	Highly important
Ciprofloxacin	0.015–4	≥0.12	Critically important
Gentamicin	0.25–16	≥8	Critically important
Nalidixic Acid	0.5–32	≥32	Critically important
Streptomycin	2–64	≥64 ⁴	Critically important
Sulfisoxazole	16–256	≥256	Highly important
Tetracycline	4–32	≥8	Highly important
Tiamulin	0.5–32	≥32 ⁵	Important
Trimethoprim/sulfamethoxazole	0.12/2.38–4/76	≥4/76	Highly important

¹Resistance breakpoints based on Clinical and Laboratory Standards Institute (CLSI) guidelines for breakpoint (CLSI, 2018) except for streptomycin, azithromycin and tiamulin which have no CLSI breakpoints.

²Categorization of antimicrobials for human medicine according to the World Health Organization (WHO, 2019)

³CLSI has not established resistance breakpoints for azithromycin.

⁴CLSI has not established resistance breakpoints for streptomycin; interpretive standards used are National Antimicrobial Resistance Monitoring System (NARMS)-established breakpoints for resistance monitoring.

⁵CLSI has not established resistance breakpoints for tiamulin, veterinary standards were used instead.

Table 3.4: Prevalence and MIC* distributions of *Salmonella* strains isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=43)

Antimicrobials	3 letter code	Resistant breakpoint	% Resistant	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
Amoxicillin/Clavulanic Acid	AUG	≥16/8	20.9									2	32	2	7			
Ampicillin	AMP	≥16	100												43			
Azithromycin	AZI	≥32	0									31	3	9				
Cefoxitin	FOX	≥16	0								30	11	2					
Ceftiofur	XNL	≥4	7				3	1	34	2	1	2						
Ceftriaxone	AXO	≥2	7					40	0	0	0	0	0	1	2			
Chloramphenicol	CHL	≥16	2.3									7	35	1				
Ciprofloxacin	CIP	≥0.12	95.4		1	1	0	0	21	20								
Gentamicin	GEN	≥8	20.9						27	3	2	2	2	7				
Nalidixic Acid	NAL	≥32	74.4									1	2	8	32			
Streptomycin	STR	≥32	100													43		
Sulfisoxazole	FIS	≥256	100															43
Tetracycline	TET	≥8	100												43			
Tiamulin	TIA	≥32	100												43			
Trimethoprim/sulphamethoxazole	SXT	≥4/76	4.7				34	3	1	2	1	2						

***MIC=** Minimal Inhibitory Concentration

Note: The top dilution tested for each drug should be interpreted as ≥ and the lowest dilution tested for each drug as ≤

Table 3.5: Presence (+) and absence (-) of WGS genotypic (G) and phenotypic (P) resistance in *Salmonella* strains isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=43)

Antimicrobial	Genotypic/Phenotypic resistant strain s (%)			
	G+/P+	G-/P+	G+/P-	G-/P-
Amoxicillin/Clavulanic Acid	9 (20.9)		34 (79.1)	
Ampicillin	43 (100)			
Azithromycin				43 (100)
Cefoxitin				43 (100)
Ceftiofur		3 (7)		40 (93)
Ceftriaxone		3 (7)		40 (93)
Chloramphenicol		1 (2.3)		42 (97.7)
Ciprofloxacin	41 (95.3)		2 (4.7)	
Gentamicin		9 (20.9)		34 (79.1)
Nalidixic Acid		32 (74.4)		11 (25.6)
Streptomycin	43 (100)			
Sulfisoxazole	43 (100)			
Tetracycline	43 (100)			
Tiamulin		43 (100)		
Trimethoprim/sulphamethoxazole	2 (4.7)		41 (95.3)	

Table 3.6: Association between Phenotypic and genotypic AMR of *Salmonella* strains isolated from piglets administered with in-feed or in-water chlortetracycline (CTC) and or tiamulin (n=43)

<i>Salmonella</i> strain	Phenotypic resistance profiles	PCR detection of resistance genes	Resistance genes detection by WGS
120 A_2019-5- <i>Salmonella</i>	AMP_AXO_CIP_FIS_GEN_NAL_STR_SXT_TET_TIA_XNL	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
157 A_2019-5- <i>Salmonella</i>	AMP_FIS_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
265 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_GEN_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
268 A_2019-5- <i>Salmonella</i>	AMP_AXO_CIP_FIS_GEN_NAL_STR_SXT_TET_TIA_XNL	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
286 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
288 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
309 A_2019-5- <i>Salmonella</i>	AMP_AUG_AXO_CIP_FIS_GEN_STR_TET_TIA_XNL	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
315 B_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_GEN_STR_TET_TIA	<i>tetB</i> , <i>tetD</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
317 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
325 A_2019-5- <i>Salmonella</i>	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i> , <i>tetD</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
329 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_GEN_STR_TET_TIA	<i>tetB</i> , <i>tetD</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
333 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i> , <i>tetD</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
337 A_2019-5- <i>Salmonella</i>	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	<i>tetA</i> , <i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
350 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>

352 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
356 A_2019-5-Salmonella	AMP_CIP_FIS_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
397 A_2019-5-Salmonella	AMP_CIP_FIS_GEN_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
502 A_2019-5-Salmonella	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	tetB, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
503 B_2019-5-Salmonella	AMP_CHL_FIS_GEN_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
541 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
565 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
569 A_2019-5-Salmonella	AMP_CIP_FIS_GEN_NAL_STR_TET_TIA	tetB, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
602 A_2019-5-Salmonella	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	tetB, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
612 A_2019-5-Salmonella	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
624 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetA, tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
633 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
637 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
642 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetA, tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
643 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
647 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
648 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tet B, tetD	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB
669 A_2019-5-Salmonella	AMP_CIP_FIS_NAL_STR_TET_TIA	tetB	aac(6')-Iaa, strA, strB, blaTEM-1B, qnrB19,sul2, tetB

685 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
693 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
720 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
815 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
867 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
884 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
888 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
900 A_2019-5- <i>Salmonella</i>	AMP_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
931 A_2019-5- <i>Salmonella</i>	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
1387 A_2019-5- <i>Salmonella</i>	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	<i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>
1399 A_2019-5- <i>Salmonella</i>	AMP_AUG_CIP_FIS_NAL_STR_TET_TIA	<i>tetA</i> , <i>tetB</i>	<i>aac(6')</i> -Iaa, <i>strA</i> , <i>strB</i> , <i>bla</i> TEM-1B, <i>qnrB19,sul2</i> , <i>tetB</i>

Phenotypes – AMP = ampicillin, CIP = ciprofloxacin, AUG = amoxicillin/clavulanic acid, AXO = ceftriaxone, CHL = chloramphenicol, FIS = sulfisoxazole, GEN = gentamicin, NAL = nalidixic acid, STR = streptomycin, SXT = trimethoprim/sulfamethoxazole, TET = tetracycline, TIA = tiamulin, XNL = ceftiofur.

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