Epidemiology and quinolone-susceptibilities of *Salmonella* and *Campylobacter* in feedlot cattle

by

ASHLEY B. THORNTON SMITH

B.S., Biology, Central Missouri State University, 2002
M.S., Veterinary Biomedical Sciences, Kansas State University, 2007

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Abstract

Salmonella and *Campylobacter* are two leading causes of human foodborne disease. Cattle can asymptomatically shed these organisms in their feces. Fluoroquinolones are antimicrobials used to treat both humans and animals. With concerns over antimicrobial resistance, antimicrobial use in livestock has become scrutinized. Data on prevalence and susceptibility of *Salmonella* and *Campylobacter* in feedlot cattle, particularly those exposed to fluoroquinolones, are sparse. The purpose of the research described in this dissertation was to determine the prevalence and quinolone susceptibility of *Salmonella* and *Campylobacter* isolated from feedlot cattle and to determine whether these outcomes were associated with fluoroquinolone use. First, an observational study was performed at five commercial feedlots that used enrofloxacin (a fluoroquinolone) as first-line treatment for bovine respiratory disease (BRD). Fecal samples were collected from cattle pens with various levels of BRD and exposure to enrofloxacin. *Salmonella* and *Campylobacter* prevalence and susceptibility to quinolones, nalidixic acid and ciprofloxacin, were evaluated. Prevalence of *Salmonella* and *Campylobacter* was highly variable among and within feedlots. All but one *Salmonella* isolate was susceptible to nalidixic acid and ciprofloxacin, whereas 49% (126/256) of the *Campylobacter* isolates were resistant to both antimicrobials. However, the number of enrofloxacin treatments was not associated with the prevalence or susceptibilities of either organism. A second, experimental study assessed prevalence and quinolone susceptibilities of *Salmonella* and *Campylobacter* in feces of feedlot cattle administered enrofloxacin for the control of BRD (metaphylaxis). Cattle with no history of fluoroquinolone exposure were randomly assigned to either an enrofloxacin treated pen or a non-treated, control pen. Cattle feces were repeatedly collected and cultured for *Salmonella* and *Campylobacter*, with isolates tested for susceptibilities to nalidixic acid and ciprofloxacin. Overall, *Salmonella* and *Campylobacter* prevalence estimates were relatively low and decreased over time. Resistance prevalence was negligible for *Salmonella*, but was high for *Campylobacter*. However, there was no evidence that enrofloxacin metaphylaxis impacted the prevalence of *Salmonella* or *Campylobacter*, nor did it significantly affect their susceptibility to human quinolones. In conclusion, enrofloxacin use in feedlot cattle does not appear to have a significant impact on the prevalence or resistance of *Salmonella* and *Campylobacter*.

EPIDEMIOLOGY AND QUINOLONE-SUSCEPTIBILITIES OF *SALMONELLA* AND *CAMPYLOBACTER* IN FEEDLOT CATTLE

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Approved by:

Major Professor
David G. Renter

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Preface

Antimicrobial resistance has become a global topic of discussion, particularly when antimicrobials, such as fluoroquinolones, are used to treat both human and animal diseases. For over a decade, scientists have been monitoring antimicrobial resistance trends for two major zoonotic pathogens, *Salmonella* and *Campylobacter*, in an attempt to evaluate the use of antimicrobials in livestock and their impact on human health. Numerous published documents describing the prevalence of these two microorganisms and their susceptibility to quinolones for both poultry and swine are available, while prevalence estimates for beef cattle are scarce.

The studies described in this dissertation provide pen prevalence estimates for *Salmonella* and *Campylobacter*, as well as quinolone susceptibility data in an effort to fill in key data gaps. The study described in chapter 2 was a cross-sectional observational study used to evaluate the prevalence and quinolone susceptibilities of *Salmonella* isolated from the feces of pre-harvest cattle that used the fluoroquinolone, enrofloxacin, to treat bovine respiratory disease. This study was published in the journal *Foodborne Pathogens and Diseases* in 2015. The study described in chapter 3 is additional data collected from the study described in chapter 2, yet focuses on the prevalence and quinolone susceptibilities of *Campylobacter*. My final study, which is discussed in chapter 4, evaluates the effect of enrofloxacin metaphylaxis on the prevalence and quinolone susceptibilities of *Salmonella* and *Campylobacter* isolated from the feces of feedlot cattle. Overall, the research described in this dissertation indicates that the use of enrofloxacin for the treatment or control of bovine respiratory disease does not significantly impact the prevalence of *Salmonella* or *Campylobacter*, nor does it significantly alter the susceptibility of these organisms to human quinolones.

Chapter 1 - Literature Review to Explore the Epidemiology of *Salmonella* and *Campylobacter*, their Antimicrobial Susceptibilities and the Use of a Fluoroquinolone in the Livestock Industry

Introduction

For many decades, antimicrobials have been used to treat and protect both human and animal populations. With the overuse and at times, inappropriate use of these antimicrobial agents, the emergence and transmission of antimicrobial resistance has become a global concern. In order to maintain a balance between keeping animals healthy and minimally impacting the health and well-being of our human population, we must understand what antimicrobial resistance is, how it develops, how it is spread, how to detect and monitor its existence and what data are needed to evaluate the human food safety risk when medically important antimicrobials are used in the livestock industry.

Antimicrobial Resistance

According to the Centers for Disease Control and Prevention (CDC), antimicrobial resistance is defined as the ability of microorganisms to resist the effects of drugs and, therefore, continue to grow causing untreatable infections that require expensive and sometimes toxic intervention (CDC, 2015a). An antimicrobial is an agent that kills microorganisms or inhibits their growth (Madigan *et al*. 2006). Antibiotics, which are a type of antimicrobial, were first discovered by Alexander Fleming in 1928 (Madigan *et al*. 2006). Penicillin, an antibacterial product of the fungus *Penicillium chrysogenum*, was the first clinically effective antimicrobial

commercially produced for the control of human staphylococcal and pneumococcal infections (Madigan *et*. *al*. 2006). Over the past 90 years, numerous antimicrobials have been introduced and mass produced to not only destroy disease-causing microorganisms in humans, but to also prevent, control and treat disease in animals. With the widespread use of antimicrobials in both the human and animal population increasing over the years, the development of antimicrobial resistance has become a major threat to our global healthcare system (CDC, 2013).

Antimicrobial resistant bacteria and fungi cause an estimated two million illnesses and 23,000 deaths in the United States, annually (CDC, 2015a). With penicillin-resistant *Staphylococcus* being identified as the first antimicrobial-resistant organism in 1940, resistance to other antimicrobials, such as tetracycline (1950), erythromycin (1953) and methicillin, soon followed (CDC, 2015a). Currently, resistant organisms that pose the most serious threat in the US are drug resistant *Campylobacter,* non-typhoidal *Salmonella, Salmonella Typhi, Shigella, Streptococcus pneumonaie, Staphylococcus aureus* and *Mycobacterium tuberculosis*, as well as multi-drug resistant *Acinetobacter* and *Pseudomonas aeruginosa* (CDC, 2013). These antimicrobial-resistant infections can require prolonged treatment, extended hospital stays and can cost up to $20 billion in excess direct healthcare costs (CDC, 2013). With antimicrobial use being one of the most important factors leading to antimicrobial resistance, judicious use of antimicrobials in both human and veterinary medicine is deemed necessary.

Foodborne Illness

Salmonella and *Campylobacter*, the pathogens studied in this dissertation, are considered two of the top five foodborne pathogens that cause human illness in the United States (Hoffmann *et al*., 2012). In 2009, the estimated annual cost of illness in the US for non-typhoidal *Salmonella*

was $3.3 million and $1.7 million for *Campylobacter* spp. (Hoffmann *et al*., 2012). In 2011, the CDC reported that there were an estimated 1 million illnesses, 19,000 hospitalizations and 380 deaths reported annually due to non-typhoidal *Salmonella*. The estimated annual number of illnesses, hospitalizations and deaths reported for *Campylobacter* spp. was 850,000, 8,500 and 76, respectively (CDC, 2014).

Salmonella and *Campylobacter* are shed in the feces of both beef and dairy cattle (Voetsch *et al*., 2004; Taylor *et al*., 2013). Human foodborne illness is often traced back to a food source of animal origin, such as raw (unpasteurized) milk, eggs, poultry, contaminated produce and undercooked meat (Taylor *et al*., 2013; Greig *et al*., 2009). Pathogenic organisms associated with foodborne illness can often carry antimicrobial resistance genes. Due to the use of antimicrobials in animal production, antimicrobial resistance has become a major concern, especially for those pathogenic organisms transmitted through food (Hoffmann *et al*., 2012).

Salmonella

Salmonella are Gram negative, rod-shaped facultative anaerobes with peritrichous flagella and inhabit the intestinal tracts of most invertebrates, including fish, reptiles, and mammals (Madigan *et al*. 2006). *Salmonella* can grow in cells along the lining of the intestine, as well as inside macrophages that normally ingest and kill pathogenic organisms (Madigan *et al*. 2006). *Salmonella* have several virulence factors such as enterotoxins, cytotoxins, lipopolysaccharide (LPS), and heat-shock proteins that allow the organism to escape the host's immune system and establish itself within its environment.

There are over 2500 serotypes of *Salmonella* currently identified (CDC, 2015b). *Salmonella* serotypes Enteritidis, Typhimurium and Newport are the most prevalent serotypes

reported in U.S human laboratories with Enteritidis being the most common serotype reported (FDA, 2014). A surveillance report illustrating *Salmonella* isolates recovered from human samples from 1968-2011 indicated that *Salmonella* Enteritidis infections increased from 1980 to 1995, but decreased by 30% from 1996 to 2006 (CDC, 2015b). The majority of the isolates recovered were from the stool samples of children ages 0-4 years and infections were the most prevalent during the late summer months (July-September) (CDC, 2015b). In 2014, 2,127 *Salmonella* were isolated from (U.S.) human stool samples and of the samples collected 438 (20.6%) were serotype Enteritidis, 262 (12.3%) were serotype Typhimurium and 235 (11.0%) were serotype Newport (FDAa, 2016).

Salmonella has been shown to be prevalent in cattle (Dargatz *et* al., 2003; Bosilevac *et al*., 2009; Haneklaus *et al*., 2012). In 2009, a study evaluated the prevalence of *Salmonella* in commercially produced ground beef (Bosilevac *et al*., 2009). Samples from seven different regions of the United States were collected over a 24-month period and analyzed for the presence of *Salmonella enterica*. A total of 4,136 samples were collected and of those samples, 172 tested positive for *Salmonella*, making the overall sample prevalence 4.2%. *Salmonella* was isolated more often in summer months (June-Sept.) than winter months (Oct.-Dec.) and regional monthly prevalence of *Salmonella* strains varied from 1.8% to 6.5%. The most common serotypes isolated were Dublin, Reading, and Typhimurium. Multi-drug resistant (MDR) strains accounted for 0.6% of the overall prevalence and resistance prevalence varied by region and species (Bosilevac *et al*., 2009).

Salmonella have been isolated from the feces, hides, and lymph nodes of cattle (Khaitsa *et al*., 2007; Kunze *et al*., 2008; Haneklaus *et al*., 2012). *Salmonella* fecal shedding was examined in North Dakota feedlot cattle during the finishing period (Oct 2003-May 2004) and

was found to be low at feedlot arrival (0.7%; 1/144), increased to 5.6% (8/144) 28 days later and increased to 62.0% (89/143) just prior to slaughter (Khaitsa *et al*., 2007). *Salmonella enterica* prevalence on hides and in the feces of harvest-ready cattle in the southern high plains of the United States was 69.6% and 30.3% (n=1,681 samples), respectively and had an average concentration of 1.82 $\log_{10}$ /100 cm^2 on hides and 0.75 $\log_{10}$/g in the feces (Kunze *et al*., 2008). Lymph nodes were examined in cattle harvested from seven different feedlots over a three-month period for the prevalence of *Salmonella* (Haneklaus *et al*., 2012). *Salmonella* prevalence within the lymph nodes of cattle was highly variable within feedlot and among feedlots and ranged from 4.0% to 88.2% (Haneklaus *et al*., 2012).

A study evaluating the prevalence and antimicrobial susceptibility of *Salmonella* spp. isolated from U.S. cattle feces in 1999 and 2000 indicated that isolates were most frequently resistant to tetracycline (35.9%, 252/702), streptomycin (11.1%, 78/702), ampicillin (10.4%, 73/702) and chloramphenicol (10.4%, 73/702). Multidrug resistance (isolates resistant to ≥ 2 antimicrobials) occurred in 11.7% (82/702) of the isolates (Dargatz *et al*., 2003). Ground beef samples, collected by 18 U.S. commercial ground beef producers between July 2005 and June 2007, were evaluated for *Salmonella* prevalence and antimicrobial susceptibility (Bosilevac *et al*., 2009). Results indicated that 4.2% (172/4,136) of the samples were positive for *Salmonella* and of the samples collected; the majority (21.5%, 37/172) of the isolates were resistant to tetracycline, chloramphenicol or ampicillin. Serotypes Agona, Dublin, Newport, Reading and Typhimurium were resistant to four or more antimicrobials and only one serotype (Agona) was resistant to a quinolone (nalidixic acid; Bosilevac *et al*., 2009).

According to the 2012-2013 National Antimicrobial Resistance Monitoring System (NARMS) interim surveillance report, non-typhoidal *Salmonella* were recovered from 0.9%

(15/1663) of the retail meat ground beef (0.9%; 15/1663) samples collected and the most common serotypes isolated were Montevideo (26.7%) and Dublin (26.7%; FDA, 2015b). A portion of the *Salmonella* isolates recovered from retail beef samples were resistant to Ceftriaxone (26.7%; 4/15), while 46.7% (7/15) of the *Salmonella* isolates were susceptible to all drugs tested (FDA, 2015a). The 2014 NARMS Integrated Report indicated that prevalence of non-typhoidal *Salmonella* was at an all-time low for retail ground beef (0.8%; FDA, 2016b). Ceftriaxone resistance for *Salmonella* Dublin isolated from cattle declined to 29% (9/31) in 2014 and ciprofloxacin resistance continued to be low (< 10%) for all *Salmonella* isolates tested (FDA, 2016b).

Campylobacter

Campylobacter are Gram negative, curved rod to spiral shaped organisms that have polar flagellum and thrive in microaerophilic (5% O2 10% CO2 85% N2) conditions (Madigan et. al, 2006). *Campylobacter* are able to invade colonic epithelial cells by a microtubule-dependent and actin filament-independent invasion mechanism. These pathogens produce cytolethal distending toxins (Cdt A, B, C), which cause cell elongation, swelling, and apoptosis (Dasti *et. al*, 2010). They contain lipopolysaccharide, which is involved in adherence and antigenic variation and some *Campylobacter* strains contain polysaccharide capsules, which help it escape the host's immune system (Madigan *et. al*, 2006).

The prevalence of *Campylobacter* has been characterized in both humans and animals. The Foodborne Diseases Active Surveillance Network (FoodNet) indicated that *Campylobacter* was a common pathogen isolated from humans and accounted for 41.7% (n=3,445) of the travel-associated infections during 2004-2009 (Kendall *et* al., 2012). There are numerous

Campylobacter species that are responsible for human campylobacteriosis which include: *C. jejuni, C. coli, C. fetus, C. hyointestinalis, C. upsaliensis,* and *C. ureolyticus* (Sanad, 2011). *Campylobacter jejuni* is the most common bacterial cause of gastroenteritis and has been associated with both waterborne and foodborne outbreaks (Taylor *et al*., 2013).

In 2009, fecal prevalence of *Campylobacter* species in beef cattle from seven large commercial feedlots in Alberta, Canada was evaluated. Out of 2,776 fresh pen-floor samples, 87% (2420 of 2776) were culture positive for *Campylobacter* (Hannon *et al*., 2009). The prevalence of *Campylobacter jejuni* in feedlot cattle was monitored throughout the feeding period and found to increase from 1.6% at first sampling to > 60% at the final sampling just prior to cattle harvest (Besser *et al*., 2005). *Campylobacter* isolates recovered from US feedlot cattle in 1999 and 2000 (N=448) were examined for antimicrobial resistance and of the *C. jejuni* tested, 49.1% were resistant to tetracycline, 10.2% were resistant to nalidixic acid, 8.4% were resistant to trimethoprim/sulfamethoxazole and 1.8% were resistant to ciprofloxacin. Approximately 20% (n=88) of the isolates tested in this study were resistant to two or more antimicrobials and 6% (n=25) were resistant to three or more antimicrobials (Englen *et al*., 2005).

According to the 2012-2013 NARMS interim surveillance report, *Campylobacter* was isolated from 42% (n=662) of the cecal samples collected from cattle (FDA 2015a). The majority of the isolates were *C. jejuni* (80.2%; 531/662), while the remaining isolates were *C. coli* (19.8%; 131/662). Of the *Campylobacter* isolates tested, 12% were resistant to ciprofloxacin and less than 5% were resistant to erythromycin (FDA 2015a).

The 2014 NARMS Integrated report revealed that *Campylobacter* prevalence in cecal samples collected from beef cows was similar to the prevalence reported in the 2012-2013 integrated report (42%; FDA 2016b). Erythromycin resistance remained low for these isolates

(0.3%) and ciprofloxacin-resistance increased (16% for *C. jejuni* (n=531); 62% for the *C. coli* (n=131; FDA, 2016b). Recovery of *Campylobacter* in retail ground beef during the 2002-2007 sampling period was low/negligible and therefore is no longer isolated or reported (FDA 2015a).

Antimicrobial Resistance Transmission

Antimicrobial resistance is often coded by genes which are spread through a process called horizontal gene transfer. Horizontal transfer of resistance genes among bacterial populations can occur by transduction, transformation, and conjugation (Giguere *et al*., 2006). Transduction is the transfer of DNA via a bacteriophage. The DNA is injected into a bacterial cell where it then becomes integrated into the chromosome of the recipient cell. Transformation is the uptake of naked DNA by a competent cell, so that recombination within the recipient's genome can take place. Conjugation is the transfer of a plasmid from a donor cell to a recipient cell where it can replicate and acquire transposon-carrying antimicrobial resistance genes (Giguere *et al*., 2006). The factors associated with the rate at which antimicrobial resistance genes are generated and transferred are still being investigated and are often found to be pathogen-specific (Giguere *et al*., 2006).

Salmonella and *Campylobacter*, which are resistant to multiple antimicrobials, have been found to be associated with the spread of antimicrobial resistance (Endtz *et al*., 1991; McDermott *et al*., 2002; Dechet *et al*., 2006; Doyle *et al*., 2015). A widespread outbreak of foodborne illness was documented in the mid-1990s in northeastern United States and was associated with *Salmonella* Typhimurium DT104. This pathogenic organism was highly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSUT) and is still a major threat to our food supply today (Dechet *et al*., 2006; Doyle *et al*., 2015).

Campylobacteriosis in humans is typically traced back to poultry (FDA, 2014). *Campylobacter coli* and *jejuni* are typically isolated from both turkeys and chickens and have been found to be co-resistant to tetracycline, doxycycline, erythromycin, nalidixic acid and ciprofloxacin (FDA, 2015). Fluoroquinolones sarafloxacin and enrofloxacin, approved for controlling *E. coli* infections in poultry, were removed from the market in the early 2000s. Surveillance data suggested that there was a temporal association between the approval/use of these drugs in poultry and the increased resistance in *Campylobacter* isolates recovered from human infections (Endtz *et al*., 1991; McDermott *et al*., 2002). Low levels of resistance to human quinolones (nalidixic acid and ciprofloxacin) are reported still today in both chicken and turkey, even after the removal of these products from the market (FDA, 2015).

Enrofloxacin (Baytril 100®), which is a broad spectrum antimicrobial manufactured by Bayer Animal Health, is approved in the U.S. for the treatment and control of bovine respiratory disease in beef cattle. Though resistance to human quinolones remains low for retail meat products, fluoroquinolone use in livestock continues to be highly scrutinized (Smith and Fratamico, 2010; Marshall and Levy, 2011).

Fluoroquinolones and Mechanisms of Drug Resistance

Fluoroquinolones are currently used to treat both campybacteriosis in humans as well as illnesses, such as respiratory disease, in food animals. DNA gyrase and topoisomerase IV are two enzymes targeted by fluoroquinolones to inhibit DNA syntheses in pathogenic bacteria (Hooper, 2001). These two enzymes are structurally related to one another showing homology in *gyr*A and *gyr*B subunits of DNA gyrase and *Par*C and *Par*E subunits of topoisomerase IV. Both DNA gyrase and topoisomerase IV act by breaking DNA strands apart, passing another segment

through the break, and then resealing the break. Fluoroquinolones have been shown to trap the enzymes during the topoisomerization reaction preventing movement of the replication fork, thus inhibiting protein synthesis. These trapped complexes trigger other unfavorable events, which eventually result in cell death (Hooper, 2001).

Mechanisms of quinolone resistance used by pathogenic microorganisms, such as *Salmonella* and *Campylobacter*, have been shown to include alterations in target enzymes (DNA gyrase and/or topoisomerase IV), alterations in drug permeation and the use of active efflux pumps (Giguere *et al*., 2006; Jesse *et al*., 2006; Chen *et al*., 2007). Acquisition of a broad-spectrum mechanism, such as the multi-drug efflux pump system, or multiple mechanisms acquired simultaneously, can lead to multiple drug resistance (Chen *et al*., 2007). Studies have shown that resistance mechanisms used by *Salmonella* and *Campylobacter* can cause decreased susceptibility to fluoroquinolones. Multidrug resistance efflux pumps and mutations in topoisomerase genes among naturally occurring fluoroquinolone-resistant *Salmonella enterica* serovar Typhimurium strains were examined by scientists and found that deletions in efflux pump genes (acrAB and tolC) increased fluoroquinolone susceptibility four-fold (Chen *et al*., 2007). These gene deletions, along with the replacement of *gyr*A with wild-type *gyr*A, increased susceptibility to fluoroquinolones greater than 500-fold. These data show that the acquisition of efflux pumps, in combination with topoisomerase gene mutations, play an important role in the development of fluoroquinolone resistance (Chen *et al*., 2007).

Another study showed that the overexpression of *ram*A in a susceptible strain of *Salmonella* increased expression of the AcrAB efflux pump (Abouzeed *et al*., 2008). Inactivation of a regulatory protein (*ram*R) upstream from *ram*A resulted in a four-fold increase in the minimum inhibitory concentration (MICs) results for nalidixic acid, flumequine, enrofloxacin,

ciprofloxacin, chloramphenicol, florfenicol, and tetracycline. This inactivation also resulted in four-fold increase in *ram*A gene expression and AcrAB efflux pump expression. This suggests that *ram*R plays a major role in the up regulation of the *ram*A and AcrAB genes, which are both responsible for multidrug resistance in *Salmonella enterica* serovar Typhimurium (Abouzeed *et al*., 2008).

Campylobacter coli and *Campylobacter jejuni* isolates recovered from chicken and beef cattle, with distinct mutations in gyrA (Thr86Ile and Thr86Ala), have been shown to be resistant to both ciprofloxacin and nalidixic acid (Jesse, *et al*., 2006). Minihan and others investigated the role of the Thr-86-Ile mutation in the *gyr*A gene and explored the involvement of the CmeABC efflux system in multi-drug resistant *C. jejuni* isolates (Minihan *et al*., 2006). Out of the 33 isolates tested, only five (15%) of the ciprofloxacin-resistant *C. jejuni* isolates were found to have the Thr-86-Ile point mutation and zero isolates had the efflux pump genes (CmeB or CmeR) present. Scientists have previously reported that both Thr-86-Ile point mutations and CmeABC efflux system are predominant mechanisms for ciprofloxacin resistance. However, because only the point mutation was present in the majority of the multi-drug resistant *Campylobacter* isolates tested for this study, authors concluded that further research is needed to identify the mechanisms of resistance associated with multi-drug resistant *Campylobacter* (Minihan *et al*., 2006).

Transmission of Fluoroquinolone Resistance

Fluoroquinolone resistance determinants have been shown to be acquired through horizontal gene transfer (Luangtongkum, *et al*, 2009). Horizontal gene transfer is mediated by transformation and transduction for both *Salmonella* and *Campylobacter*. As mentioned above,

transformation is the uptake of naked DNA by a competent cell, while transduction is the transfer of DNA via a bacteriophage. The most common method of horizontal gene transfer in enteric organisms, specifically *Campylobacter*, is via natural transformation (Luangtongkum, *et al*, 2009). Plasmids, which are circular chromosomal DNA that can replicate independently within a cell, are another way bacteria transfer resistance genes from one organism to the next (Tran and Jacoby, 2002). The plasmid pMG252 was discovered in a clinical strain of *Klebsiella pneumonia* in the U.S. in 1994 and contained the *qnr* gene responsible for quinolone resistance (Jacoby *et al*., 2003). Though plasmid-mediated quinolone resistance is often rare, isolates containing this plasmid were highly resistant to quinolones, as well as ampicillin (AmpC) and beta lactams (FOX-5 β-lactamase) (Jacoby *et al*., 2003).

Animals harboring enteric species such as *Salmonella* and *Campylobacter* have been found to be significant reservoirs for the transfer of resistance genes to humans and other animals (Sippy *et al*., 2012). Specifically, small mammals and birds, as well as insects have been shown to play a significant role in the transmission of quinolone-resistant *Campylobacter* to livestock (Sippy *et al*., 2012; Zurek *et al*., 2014). Commensal microflora, primarily from the gastrointestinal tract of humans and animals, along with soil microbiota, has been found to harbor antimicrobial resistance genes (Boerlin and Reid-Smith, 2008). Cattle, poultry and swine feces harbor antimicrobial resistance genes, which may spread to humans by the consumption of contaminated food (Endtz *et al*., 1991; Englen *et al*., 2005; Khaitsa *et al*., 2007; Tadesse *et al*., 2011). Another potential source of transmission of multidrug-resistance is through contact with family pets (Acke, 2009). *Campylobacter jejuni* isolates (n=51) obtained from 179 dogs and cats in Ireland were found to be resistant to nalidixic acid (37.3%), ciprofloxacin (19.6%), tetracycline (13.7%), ampicillin (13.7%), erythromycin (11.8%) and chloramphenicol (5.9%).

Resistance to two or more antimicrobials was detected in 31.4% of the *Campylobacter jejuni* isolates recovered, which illustrates the importance of proper hygiene in households that contained pets (Acke, 2009).

Studies have also shown that antimicrobial resistance can be present and transmitted even in the absence of antimicrobial selection pressure. Fecal (n=838) and carcass (n=1173) samples from both conventional and antimicrobial-free (ABF) swine production farms were collected and examined for the presence of *Campylobacter*. The minimum inhibitory concentration (MIC) of each sample was determined and results indicated that 3.7% and 1.2% of the *Campylobacter coli* isolates collected from both conventional and ABF swine, respectively, were resistant to ciprofloxacin (Tadesse *et al*, 2011). A similar phenomenon was seen when "specific pathogen-free" chickens were colonized with fluoroquinolone- resistant *Campylobacter* in an environment free of antimicrobials (Luo *et al*., 2004). Chickens shed *Campylobacter* in their feces at concentrations ranging from 10^5 to 10^8 cfu/g and by the end of the experiment all isolates recovered had genomic DNA similar to that of the resistant (challenge) isolate. Even without antimicrobial selection pressure, isolates failed to lose their *gyr*A mutations and associated fluoroquinolone resistance (Luo *et al*., 2004).

Surveillance and Detection

The National Antimicrobial Monitoring System (NARMS) was established in 1996 as a national public surveillance system to track changes in antimicrobial resistance in human, retail meat, animal and environmental samples (FDA, 2015). NARMS is a collaborative effort among state and local public health departments, the Centers for Disease Control (CDC), the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) (CDC, 2015a).

NARMS, the Foodborne Diseases Active Surveillance Network (FoodNet) and the National Animal Health Monitoring System (NAHMS) are all active participants in helping to better understand antimicrobial resistance.

A variety of test methods have been developed to assist in evaluating antimicrobial susceptibility. In vitro methods that are currently being used include: the disk diffusion assay, the micro broth dilution assay, the agar dilution assay and the mismatch amplification mutation assay (MAMA) (Giguere *et al*., 2006, Zirnstein *et al*., 1999). The disk diffusion assay is the susceptibility assay most widely used by scientists and clinicians (Giguere *et al*., 2006). This assay is cost effective and allows for numerous drugs to be tested at once. A commercially prepared disk containing the antimicrobial agent of choice is placed on the surface of the agar which is inoculated with approximately $2x10^8$ CFU/ml of pure culture. As growth of the bacteria occurs, the diffusion of the drug spreads across the media. When the concentration of the drug becomes too diluted to inhibit bacterial growth, a zone of inhibition is formed. The larger the zone of inhibition, the larger the concentration of drug required to inhibit pathogenic growth (Giguere *et al*., 2006).

The micro broth dilution assay is another susceptibility test that is comprised of micro titer trays that contain several antimicrobial agents of known potency in progressive two-fold dilutions. The microorganism of interest is transferred to the micro titer tray, so that approximately $5x10^4$ CFU/ml is suspended into each well. The tray is incubated overnight and the minimum inhibitory concentration (MIC) is determined by the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the well (Giguere *et al*., 2006). Sensititre™ (TREK Diagnostic Systems) is an automated version of the microbroth

dilution assay and is a fast, efficient way of testing susceptibility to multiple antimicrobials in one micro titer tray.

The agar dilution assay is similar to the micro broth dilution assay, except varying concentrations of the antimicrobial agent, in two-fold dilutions, are added to the agar medium. Bacterial inoculum (~$5x10^8$ CFU/ml) is added to the surface of the agar and once all plates are incubated, the plate that contains the lowest concentration of antimicrobial agent to inhibit growth is considered the MIC for the isolate tested (Giguere *et al*., 2006).

A mismatch amplification mutation assay (MAMA), which was developed to investigate the type and frequency of *gyr*A mutations in ciprofloxacin-resistant *C. jejuni* isolates, is yet another way of confirming antimicrobial resistance (Zirnstein *et al*., 1999). Chromosomal DNA that contains the mutated genes from a resistant isolate are amplified using polymerase chain reaction (PCR) and primers are chosen using GenBank®. PCR products are visualized using electrophoresis and the bands (or lack thereof) are compared to the wild-type strain to identify mutated genes (Zirnstein *et al*., 1999).

Data Gaps

Data generated by organizations all over the world have helped to evaluate antimicrobial resistance trends for human pathogenic organisms. These data are not only used to assist in investigating foodborne illness outbreaks, but are also used to help evaluate the human food safety risk associated with the use of antimicrobials in food animals.

Data necessary to generate valid and informative human food safety risk assessment models, however, are often lacking, leaving potentially overly conservative parameters to be used in the model; specifically data regarding the prevalence of *Salmonella* and *Campylobacter* and their susceptibility to human quinolones in relation to the use of a fluoroquinolone in the

feedlot industry. Research designed to determine the prevalence and susceptibility of these organisms at different time points during feedlot production, in pens of cattle with various levels of BRD morbidity and in pens of cattle with different levels of exposure to a fluoroquinolone would assist in filling in these data gaps. Data generated from these studies would not only assist in estimating the human food safety risk of consuming quinolone-resistant bacteria, but would also help to evaluate the epidemiology of *Salmonella* and *Campylobacter* isolated from cattle administered a fluoroquinolone for the treatment and control of bovine respiratory disease.

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Chapter 2 - Prevalence and quinolone susceptibilities of *Salmonella* isolated from the feces of pre-harvest cattle within feedlots that used a fluoroquinolone to treat bovine respiratory disease

Ashley B. Smith[1,2], David G. Renter[1], Natalia Cernicchiaro[1], Xiaorong Shi[1], and Tiruvoor G. Nagaraja[1]

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[1]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506

[2]Bayer Healthcare, LLC, Animal Health Division, Shawnee, Mission, KS 66216

Address correspondence to:

David G. Renter, DVM, PhD

Department of Diagnostic Medicine and Pathobiology

College of Veterinary Medicine

309 Coles Hall, 1620 Denison Avenue

Kansas State University,

Manhattan, KS 66506

Phone: 785-532-4801, Fax: 785-532-4851

drenter@vet.k-state.edu

Abstract

Salmonella is an important foodborne pathogen and antimicrobial resistance can be a human health concern. The objectives of this cross-sectional study were to 1) determine the prevalence and quinolone susceptibility of *Salmonella* in feces of pre-harvest commercial feedlot cattle, and 2) determine if the prevalence and susceptibility of *Salmonella* isolates were associated with previous fluoroquinolone use within pens. Five feedlots in western Kansas and Texas were selected based on their use of a commercially licensed fluoroquinolone for initial treatment of bovine respiratory disease (BRD). Twenty pen-floor fecal samples were collected from each of ten pens from each feedlot during early summer of 2012. *Salmonella* isolation was performed and microbroth dilution was used to determine susceptibility of isolates to nalidixic acid and ciprofloxacin. Prior antimicrobial treatment data were retrieved from feedlots' operational data. Generalized linear mixed models were used to assess associations between *Salmonella* prevalence and the number of fluoroquinolone treatments within pens, while taking into consideration cattle demographic and management factors, as well as the hierarchical structure of the data. Overall cumulative fecal prevalence of *Salmonella* was 38.0% (380/1,000), but prevalence varied significantly ($P < 0.01$) among the five feedlots: 0.5%; (1/200), 17.5%; (35/200), 37.0%; (74/200), 58.5%; (117/200), and 76.5%; (153/200). *Salmonella* serogroups included C1 (49.3%), E (36.4%), C2 (13.8%), and D (0.6%). There was no significant association ($P = 0.52$) between *Salmonella* prevalence and the frequency of fluoroquinolone treatments within a pen. All *Salmonella* isolates ($n = 380$) were susceptible to ciprofloxacin, while one isolate exceeded the human breakpoint [≥ 32 μg/mL] for nalidixic acid. In conclusion, *Salmonella* fecal prevalence in pre-harvest cattle was highly variable among feedlots. Nearly all

Salmonella isolates were susceptible to quinolones despite the fact that a fluoroquinolone was used as the primary therapeutic antimicrobial to treat BRD in these feedlot populations.

Key words: fluoroquinolone; cattle; susceptibility; resistance; prevalence; *Salmonella*

Introduction

The Center for Disease Control and Prevention (CDC) reports that *Salmonella* is one of the leading causes of foodborne illnesses in the United States (CDC, 2012). *Salmonella* outbreaks usually occur due to the consumption of contaminated food, water, and raw milk (CDC, 2012). Though beef is not generally considered a primary source of human salmonellosis, feedlot cattle can be asymptomatic carriers of *Salmonella* and shed these bacteria in their feces (Fedorka-Cray *et al*., 1998; Dargatz *et al*., 2003; Dodd *et al*., 2011). In addition, the recovery of *Salmonella* from lymph nodes in beef carcasses has become a significant concern for the U.S. beef industry (Arthur *et al*., 2008; Haneklaus *et al*., 2012; Gragg *et al*., 2013). The average prevalence of *Salmonella* in feces of feedlot cattle and beef cows in the U.S is generally low, but it can vary tremendously both within and among feedlots or herds (Fedorka-Cray *et al*., 1998; Dargatz *et al*., 2000; Dargatz *et al*., 2003; Dodd *et al*., 2011).

Antimicrobials are effectively used to treat and control diseases in feedlot cattle. Some classes of antimicrobials, such as quinolones, are used to treat bovine respiratory disease (BRD) in cattle as well as foodborne illnesses in humans. With the growing concern over antimicrobial resistance, the use of antimicrobial drugs in livestock is highly scrutinized (Emmerson *et al*., 2003; Page *et al*., 2012). Risk assessments are often used to evaluate antimicrobial use in livestock and its risk to humans; however, data necessary to develop valid and informative

quantitative risk assessment models are often lacking. Current knowledge regarding the prevalence of *Salmonella* in U.S. beef cattle and their antimicrobial susceptibilities, particularly to fluoroquinolones, is limited. National studies of feedlots and beef cows have shown that resistance of *Salmonella* to fluoroquinolones is extremely rare, but may cluster within a few herds for reasons not fully defined (Fedorka-Cray *et al*., 1998; Dargatz *et al*., 2000; Dargatz *et al*., 2003; Dodd *et al*., 2011). The objectives of this study were to 1) determine the prevalence and quinolone susceptibility of *Salmonella* in feces from pre-harvest pens of commercial feedlot cattle and to 2) determine if the prevalence and susceptibility of *Salmonella* in fecal isolates differ among feedlots or among pens with different fluoroquinolone treatment histories.

Materials and Methods

Study Population

A convenience sample of five commercial feedlots in western Kansas and Texas that used a fluoroquinolone (Baytril 100® (enrofloxacin), Bayer HealthCare LLC, Animal Health, Shawnee Mission, KS) as first-line therapy for the treatment of BRD were selected for this research. Feedlots were selected based on previous fluoroquinolone use and their willingness to participate in our study. Ten study pens within each feedlot were selected in consultation with feedlot managers based on the pens' projected slaughter dates, allowing for pens with both high and low respiratory disease morbidity (thus antimicrobial use), to be selected when available. Health and treatment data on study pens, including the number of animals treated with a fluoroquinolone or any other antimicrobials, were retrieved from the feedlots' operational database.

Twenty, freshly voided fecal samples were collected from the pen floors of each of the ten pens of cattle (approximately 1-2 weeks prior to slaughter) within each feedlot (total samples per feedlot =200) during May-July 2012. Cattle fecal samples were collected using plastic spoons and were placed in Whirlpak bags (Nasco, Inc., Fort Atkinson, WI). The samples were then transported on ice to the Kansas State University (KSU) Pre-harvest Food Safety laboratory for isolation of *Salmonella*.

Isolation of *Salmonella*

Ten grams of feces from each sample was placed in 90 mL Tetrathionate (TT) broth (Becton-Dickinson, Sparks, MD) and incubated for 24 h at 37°C. From the TT broth, 100 µL of the inoculum was transferred to 10 mL Rapport-Vassiliadis broth (Becton-Dickinson) and incubated at 42°C for 24 h. The enriched sample was then streaked for isolation on Hektoen Enteric (HE) agar plates (Becton-Dickinson) and incubated at 37°C for 24 h. Three presumptive colonies with morphology consistent with *Salmonella* (blue-green colony with black center) were streaked onto blood agar (Remel, Lenexa, KS) and incubated at 37°C for 24 h. Isolates were subjected to slide agglutination with the *Salmonella* polyvalent O antisera for *Salmonella* serogroups B, C1, C2, D1, D2, and E. Isolates were considered *Salmonella* based on hydrogen sulfide production on HE agar and agglutination with the polyvalent O antisera. These isolates were stored in cryo-protection beads (Hardy Diagnostics, Santa Maria, CA) at -80°C until further tested. Serotyping of isolates found to be resistant based on microbroth dilution methods (described below) was performed by the National Veterinary Services Laboratory (Ames, IA).

Susceptibility testing

Minimum inhibitory concentrations of ciprofloxacin and nalidixic acid were determined for each isolate by micro-broth dilution method (CLSI, 2008) using human *Salmonella* breakpoints (CLSI, 2010). Stock solutions of ciprofloxacin and nalidixic acid (Sigma-Aldrich, St. Louis, MO) were prepared in sterile, distilled water at a concentration of 1 mg/mL based on the potency of the antibiotic. Nalidixic acid was tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 µg/mL and ciprofloxacin was tested at concentrations of 3.125, 1.56, 0.78, 0.39, 0.195, 0.098, 0.024, 0.012, and 0.006 µg/mL. For the preparation of the inoculum, a single colony picked from a plate was inoculated into 10 mL Mueller Hinton II broth (Becton- Dickinson) and incubated for 6 h and the cell turbidity was adjusted using the 0.5 McFarland turbidity standard. The antimicrobial susceptibilities were performed in 96-well microtiter plates (Becton-Dickinson). Plates were incubated at 37° C for 24 h and results were recorded as growth or no growth within each well.

This process was performed separately for each antibiotic (ciprofloxacin and nalidixic acid) and each *Salmonella* isolate was tested in quadruplicate. Quality control organism, *Escherichia coli* (ATCC 25922), was used to ensure validity of the test. The minimum inhibitory concentration (MIC) was determined by the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism. *Salmonella* isolates were considered susceptible to nalidixic acid and ciprofloxacin, if the MIC was $\leq$ 16 µg/mL and $\leq$ 1 µg/mL, respectively and considered resistant if the MIC was $\geq$ 32 µg/mL and $\geq$ 4 µg/mL, respectively (CLSI, 2010). Isolates considered resistant were tested for susceptibility using Sensititre® plates that contained 17 antimicrobial drugs and a semi-automated testing system (Sensititre; TREK Diagnostics, Westlake, OH).

Statistical Analysis

Associations between antibiotic use and demographic characteristics with *Salmonella* pen-level prevalence were evaluated using generalized linear mixed models which were fitted using a binomial distribution, maximum likelihood estimation, complimentary-log-log link, Kenward-Roger degrees of freedom and Newton-Raphson and Ridging optimization procedures (Proc GLIMMIX SAS 9.3, SAS Institute, Inc., Cary, NC). A random intercept for feedlot was included to account for the clustering effect of pens nested within feedlots. Overdispersion was adjusted for by including a multiplicative parameter (scale parameter).

The outcome variable was the number of *Salmonella* positive samples in a pen (events)/number of samples collected per pen (trials). Independent variables included the number of BRD fluoroquinolone treatments administered, body weight at arrival, days on feed, sex (steers/heifers), and antimicrobial metaphylaxis use in the pen (use/no use). Due to the variability in pen size, the variable pertaining to the number of fluoroquinolone treatments was coded as the number of treatments per 100 cattle. All continuous variables (number of fluoroquinolone treatments per 100 cattle, body weight and days on feed) were categorized in quartiles to avoid violation of the linearity assumption.

Pearson's and Spearman's correlation analyses were performed to identify highly correlated variables ($\geq |0.80|$) prior to initiating a multivariable model building process. Initially, variables unconditionally associated with the outcome in the univariable screen ($P < 0.40$) were included in the main effects model. The number of fluoroquinolone treatments per 100 cattle, considered our exposure variable of interest, was forced in our multivariable model regardless of its significance. The following variables, considered *a priori* confounders were tested: sex, metaphylaxis use, and body weight: if their removal caused a 20% or greater change in the

coefficient of significant variables, they were considered analytical confounders and were kept in the model. Two-way interactions between potential *a priori* confounders that were deemed significant at the 5% level ($P < 0.05$) were kept in the model. Following forward selection, non-significant variables ($P > 0.05$), that were not acting as confounders or effect modifiers were removed from the multivariable model. Furthermore, to evaluate whether the within-pen prevalence of *Salmonella* differed among feedlots, feedlot was included as a fixed effect in a logistic regression model. Residual diagnostics included the evaluation of the best linearized unbiased predictors for feedlot-level residuals and Pearson and Deviance residuals for observations at the pen-level.

Descriptive statistics were used to evaluate the MIC data for both nalidixic acid and ciprofloxacin for samples collected within each pen across the five feedlots. Since each *Salmonella* isolate was tested in quadruplicate, the mode MIC value was reported for each sample. When only two MIC values were available and the mode could not be computed, the MIC value with the highest concentration was used. If four different MIC values were reported, the isolate was re-tested and either the mode or the highest MIC value was reported.

Results

Cattle Demographics

The mean number of cattle per pen and the mean number of days on feed for cattle across all feedlots are shown in Table 1. The mean body weight of cattle in study pens upon arrival at the feedlot ranged from 289 kg to 317 kg across all five feedlots. The majority (56.0%; 28/50) of the cattle pens was composed of steers and one pen contained both steers and heifers. The frequency of fluoroquinolone treatments across all feedlots ranged from 5 to 23 treatments

within a pen (Table 1). All feedlots had at least one pen where all cattle were treated metaphylactically for bovine respiratory disease with a range of 2 to 10 study pens receiving metaphylaxis (Table 1).

Salmonella Prevalence and Susceptibility Results

Overall sample-level prevalence of *Salmonella* across all feedlots and pens was 38.0% (380/1,000). Sample-level prevalence ranged from 0.5% (1/200) to 76.5% (153/200) across all pens within feedlots and ranged from 0.0% (0/20) to 100.0% (20/20) within pens. The prevalence of *Salmonella* varied significantly ($P < 0.01$) among the five feedlots (Table 2). The most common *Salmonella* serogroups isolated from pen-floor fecal samples across the five commercial feedlots were C1 (49.3%; 179/363), followed by E (36.4%; 132/363), C2 (13.8%; 50/363), and D (0.6%; 2/363) (Table 2). Of the 380 isolates tested, 17 did not test positive for any of the six serogroups evaluated.

Potential associations between pen-level *Salmonella* prevalence and the number of fluoroquinolone treatments per 100 cattle, sex, metaphylaxis use, arrival body weight, and days on feed were evaluated and these data are shown in Table 3. No significant unconditional associations were identified, except for arrival body weight ($P = 0.02$; Table 3). The variable pertaining to the number of fluoroquinolone treatments per 100 cattle was not statistically significant ($P = 0.52$), but was forced in the multivariable model as it was considered our main variable of interest. The multivariable model included body weight and fluoroquinolone treatments and provided very similar results to the univariable model results (data not shown). Arrival body weight was the only variable significantly associated with *Salmonella* prevalence ($P = 0.02$). Pens of cattle that weighed 266 to 298 kg at arrival had significantly lower prevalence of *Salmonella* (15.5%; 95% CI = 2.4 to 70.0) than those that weighed 215 to 265 kg

(28.4%; 95% CI = 4.7 to 90.4; $P = 0.01$) or 320 to 382 kg (32.3%; 95% CI = 5.5 to 93.4; $P < 0.01$). No other differences were found among other weight categories.

Out of the 380 isolates tested for susceptibility to nalidixic acid and ciprofloxacin, only one isolate (1/380; 0.3%) was resistant (MIC ≥32 µg/mL) to nalidixic acid and all isolates were susceptible (MIC ≤ 1 µg/mL) to ciprofloxacin (380/380; 100.0%). As indicated previously, further testing was only performed on the resistant isolate, which was found to be pan susceptible to: Amoxicillin/Clavulanic Acid, Ampicillin, Azithromycin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Doxycycline, Gentamicin, Kanamycin, Minocycline, Streptomycin, Sulfisoxazole, Tetracycline, and Trimethoprim/Sulphamethoxazole. Most of the isolates (81.6%; 310/380) had a MIC value for nalidixic acid of 6.25 µg/mL. However, some isolates had nalidixic acid MIC values of 1.56 µg/mL (n=1), 3.125 µg/mL (n=64), 12.5 µg/mL (n=3), 25 µg/mL (n=1) and > 32 µg/mL (n=1). The majority (83.2%; 316/380) of the *Salmonella* isolates had a MIC value for ciprofloxacin of0.012 µg/mL. Whereas other isolates had MIC values of ≤ 0.006 µg/mL (n=31), 0.024 µg/mL (n=31), 0.098 µg/mL (n=1) and 0.39 µg/mL (n=1).

Discussion

The results of our study indicated that the overall prevalence of *Salmonella* in feedlot cattle feces prior to harvest was highly variable across the five feedlots (0.5 to 76.5%) and across pens within feedlot (0 to 100.0%). These data are consistent with previously reported *Salmonella* prevalence data obtained from commercial feedlot cattle (Dargatz *et al*., 2000; Alam *et al*., 2009; Dodd *et al*., 2011). *Salmonella* isolates recovered from pens of cattle that were

treated with fluoroquinolones were susceptible to both nalidixic acid and ciprofloxacin, regardless of the number of fluoroquinolone treatments administered to the cattle within pens.

There are numerous factors that have been shown to be associated with *Salmonella* prevalence in feedlot cattle including: geographic location, season, environmental stress, number of days in the feedlot, and diet (Losinger *et al*., 1997; Barham *et al*., 2002; Dargatz *et al*., 2003; Edrington *et al*., 2010; Dodd *et al*., 2011). In the current study, the *Salmonella* prevalence varied tremendously among feedlots, with the highest sample-level prevalence (76.5%) in a Texas feedlot and the lowest prevalence (0.5%) in a western Kansas feedlot. Having lower prevalence in the most northern region is consistent with the literature (Dargatz *et al*., 2003; Green *et al*., 2010); however, there were too few feedlots (5) to determine potential feedlot-level risk factors as this was not a primary objective of this current study.

At the pen-level, it is important to note that the number of fluoroquinolone treatments per 100 cattle, the number of days the cattle were in the feedlot, metaphylaxis use (whole pen use of an antimicrobial to control respiratory disease), and sex were not found to be associated with *Salmonella* prevalence within pens. However, there was a significant association between cattle body weight at feedlot arrival and *Salmonella* prevalence within pens. Specifically, pens of cattle with a mean weight range of 266 to 298 kg had a significantly lower *Salmonella* prevalence than the smallest body weight category (215 to 265 kg) and the heaviest body weight category (320 to 382 kg). Cattle that are lighter at feedlot arrival generally require a longer feeding period and may have increased morbidity due to higher probability of stress, suboptimal immunity, and exposure to pathogens in the feedlot environment (Losinger *et al*., 1997; Fedorka-Cray *et al*., 1998; Bancock *et al*., 2010,). Heavier cattle in our study, spent fewer days on feed, received fewer antimicrobial treatments, and had fewer pens given metaphylaxis. However, the

mean prevalence of *Salmonella* was approximately 30% for both the lightest and heaviest weight categories. A previous study evaluating *Salmonella* in 30 commercial feedlot cattle cohorts found that mean body weight at feedlot arrival was not associated with pre-harvest fecal prevalence of *Salmonella* (Dodd *et al*., 2011), so it seems that cattle body weight at feedlot entry is not a consistent predictor of *Salmonella* prevalence at harvest.

Despite the fact that all five feedlots used a fluoroquinolone as the primary line of therapy for the treatment of BRD (the only legal indication for fluoroquinolone use at the time of the study), nearly all of the *Salmonella* isolated for this study (379/380) were susceptible to both nalidixic acid and ciprofloxacin, with only one isolate found to be resistant to nalidixic acid. These results are consistent with previous studies demonstrating an extremely low prevalence of quinolone-resistant *Salmonella* isolated from feedlot cattle (Beach *et al*., 2002; Sorensen *et al*., 2002; Edrington *et al*., 2010). However, the results reported here are unique as this is the first study in which the use of fluoroquinolones was an inclusion criterion to select feedlots for the study population.

Several studies completed over the last decade have shown that less than 1.0% of the *Salmonella* isolates recovered from cattle feces, hides and carcasses have been resistant to nalidixic acid and ciprofloxacin (Dargatz *et al*., 2000; Beach *et al*., 2002; Dargatz *et al*., 2003; Fluckey *et al*., 2007; Alam *et al*., 2009). The 2011 National Antimicrobial Resistance Monitoring System (NARMS) Meat Retail Report showed a similar trend in the number of quinolone resistant isolates recovered from ground beef (FDA, 2014). *Salmonella* isolates recovered from retail ground beef (n = 1,320) collected from eleven U.S. states were all susceptible to both ciprofloxacin and nalidixic acid (FDA, 2014). These results, and those first reported here, are important as *Salmonella* is a leading cause of foodborne illnesses in the United

States (CDC, 2012), and quinolones are considered a critically important class of antimicrobials for human health (FDA, 2003).

Follow-up serotype testing of the one resistant isolate recovered during our study revealed that it was *Salmonella* serotype Cerro. This serotype has been isolated from both US beef and dairy cattle and has been associated with human salmonellosis (Kunze *et al*., 2008; Cummings *et al*., 2010; Hoelzer *et al*., 2011). However, the incidence of human salmonellosis cases due to resistant *Salmonella* Cerro has been extremely low, with the majority of the resistance being attributed to non-fluoroquinolone drugs (Kunze *et al*., 2008; Cummings *et al*., 2010; Hoelzer *et al*., 2011).

Cross-sectional, pen-level studies designed to evaluate the prevalence of foodborne pathogens in pre-harvest feedlot cattle and their antimicrobial susceptibilities may be useful in assessing the potential human health risks. However, data collected from this type of study have limited value for causal inferences because the sequence of events that lead to the observed outcome cannot be determined (Mann, 2003). Lack of history of fluoroquinolone use prior to cattle's entry into the feedlot and lack of knowledge regarding the temporal dynamics of *Salmonella* within cattle and within their environment are limitations of the current study. However, this study provides estimates of the pre-harvest fecal prevalence of *Salmonella* and the susceptibility of recovered isolates to two quinolones that are used for human therapy. Importantly, the results also demonstrate no evidence of an association between the number of previous fluoroquinolone treatments administered to cattle and the within pen-level fecal prevalence of *Salmonella* prior to harvest.

This cross-sectional study indicates that *Salmonella* prevalence in pre-harvest feedlot cattle is highly variable among feedlots and across pens within feedlots. The prevalence of

quinolone-resistant *Salmonella* in this study population was extremely low, with all but one of the 380 recovered isolates being susceptible to both nalidixic acid and ciprofloxacin. Given that we purposely selected feedlots that used a fluoroquinolone for treating cattle with BRD, this study population could have been considered potentially higher risk for resistance; yet the level of *Salmonella* resistance was negligible. While *Salmonella* as a foodborne pathogen, as well as the use of antimicrobial drugs in livestock both remain critically important issues, this study found no evidence of an association between fluoroquinolone treatments administered to feedlot cattle and the within pen-level fecal prevalence of *Salmonella* prior to harvest.

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Disclosure Statement

The first author (A.Smith) is a graduate student of Kansas State University and an employee of Bayer Healthcare, LLC, who produce a commercially licensed fluoroquinolone for the treatment of BRD.

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Table 2-1: Summary of Demographic Characteristics for Pens of Cattle within Feedlots that were tested for *Salmonella*

Feedlot	Number of Pens (steers/heifers)	Mean # of Cattle per Pen	Mean # of Days on Feed at Sampling (range)	Mean Cattle Body Weight, in kg, at Feedlot Arrival (range)	Number of Pens that Received an Antimicrobial for Metaphylaxis[a] (product)	Mean # of Fluoroquinolone[b] Treatments per 100 Cattle (range)
A	10 (9/1)	158.4	150.0 (139-162)	317.0 (273-362)	2 (Tilmicosin)	5.3 (1-22)
B	10 (10/0)	125.2	183.0 (148-209)	290.5 (248-318)	10 (Tulathromycin)	19.2 (4-32)
C	10 (4/6)	96.3	178.2 (137-240)	288.9 (215-344)	2 (Streptomycin Sulfate)	10.1 (0-36)
D	10 (3/7)	198.6	163.9 (102-203)	300.0 (259-352)	8 (Tulathromycin)	22.8 (5-66)
E	10 (2/7; one mixed)	117.3	155.5 (118-210)	288.7 (226-382)	10 (Tulathromycin)	7.7 (1-15)

[a]Used for control of bovine respiratory disease in high risk cattle

[b]Cattle treated for respiratory disease were treated with the same fluoroquinolone.

Table 2-2: Sample-Level Fecal Prevalence and Presumptive Serogroups of *Salmonella* for Five U.S. Commercial Feedlots.

Feedlot	Prevalence	95% CI[a]	Serogroup C1[b]	Serogroup C2[b]	Serogroup D[b]	Serogroup E[b]
A	0.5% (1/200)	0.1-3.6%	0.0% (0/1)	0.0% (0/1)	0.0% (0/1)	100.0% (1/1)
B	17.5% (35/200)	12.7-23.6%	33.3% (11/33)	3.0% (1/33)	0.0% (0/33)	63.6% (21/33)
C	37.0% (74/200)	30.4-44.1%	28.2% (20/71)	28.2% (20/71)	2.8% (2/71)	40.8% (29/71)
D	58.5% (117/200)	51.4-65.3%	52.4% (55/105)	3.8% (4/105)	0.0% (0/105)	43.8% (46/105)
E	76.5% (153/200)	70.0-82.0%	60.8% (93/153)	16.3% (25/153)	0.0% (0/153)	22.9% (35/153)
Total	38.0% (380/1000)		49.3% (179/363)	13.8% (50/363)	0.6% (2/363)	36.4% (132/363)

[a]CI = Confidence Interval

[b] Out of the 380 isolates tested, 17 isolates did not test positive for any of the serogroups (B, C1, C2, D1, D2, and E) evaluated.

Table 2-3: Results from Univariable Mixed Models[a] of Associations between Pen-Level Risk Factors and Within-Pen Prevalence of *Salmonella*

Variable, units	Number of Pens	Mean Prevalence (%)	95% Confidence Intervals (%)	*P*-value
Number of fluoroquinolone treatments for bovine respiratory disease per 100 cattle (in quartiles)				0.52
0-2	13	20.3	3.5-76.8	
3-8	12	26.7	4.6-87.0	
9-23	13	26.9	4.6-87.3	
24-66	12	31.4	5.6-91.6	
Sex[b]				0.35
Steer	28	24.3	4.1-84.2	
Heifer	21	29.3	5.1-90.0	
Metaphylaxis use				0.33
No	18	21.9	3.9-78.8	
Yes	32	28.9	5.0-89.5	
Body weight at arrival, kg (in quartiles)				0.02
215-265	12	31.2[c]	5.0-93.5	
266-298	13	15.7[d]	2.4-70.5	
299-319	11	25.7[cd]	4.1-88.0	
320-382	14	30.2[c]	4.8-92.7	
Days on feed (in quartiles)				0.94
102-145	11	25.3	4.1-86.7	
146-161	14	25.3	4.2-86.0	
162-192	12	25.5	4.1-87.0	
193-240	13	28.4	4.7-90.2	

[a]Models included a random effect to account for lack of independence among pens within feedlots

[b]One pen was a mixed of heifers and steers, and was removed from this analysis

[c,d]Row values with different superscripts differ ($p < 0.05$).

Chapter 3 - *Campylobacter* prevalence and quinolone susceptibility in feces of pre-harvest feedlot cattle pens exposed to various levels of enrofloxacin for the treatment of bovine respiratory disease

Abstract

Campylobacter spp., which are commensal to cattle and can be pathogenic to humans, often harbor antimicrobial resistance genes. Data on quinolone resistance in relation to antimicrobial use in beef cattle are scarce. Therefore, the objective of our study was to evaluate *Campylobacter* prevalence and susceptibility to nalidixic acid and ciprofloxacin in pens of cattle at five different feedlots that were administered a fluoroquinolone as the primary treatment for bovine respiratory disease (BRD). Twenty, fresh, pen-floor samples were collected from each of 10 pens from each feedlot, 1-2 weeks prior to cattle being harvested. Fecal samples were cultured for *Campylobacter* using enrichment and selective isolation methods and confirmed by PCR. Minimum inhibitory concentrations (MICs) of ciprofloxacin and nalidixic acid were determined using a micro-broth dilution method and human quinolone breakpoints. The number of fluoroquinolone treatment used within each pen were recorded and summarized. Data analyses were performed using generalized linear mixed-models (prevalence) and survival analysis (MICs). Overall sample prevalence of *Campylobacter* was 27.2% (272/1000) and was significantly different among feedlots ($P < 0.01$). *Campylobacter coli* was the most prevalent species (55.1%; 150/272), followed by *Campylobacter hyointestinalis* (42.6%; 116/272). Prevalence was not significantly associated with the number of fluoroquinolone treatments, sex, body weight or metaphylaxis use, but was associated with the number of days the cattle were in the feedlot ($P = 0.03$). Susceptibility testing revealed that several isolates (68.4%; 175/256) were

above the breakpoint for nalidixic acid (≥ 64 μg/mL) and above the breakpoint (≥ 4 μg/mL) for ciprofloxacin (65.6%; 168/256). Distributions of MICs for individual feedlots, and by *Campylobacter* species, were significantly different for isolates tested against nalidixic acid ($P \leq 0.01$). A similar trend was noted for isolates tested against ciprofloxacin ($P \leq 0.05$). The number of fluoroquinolone treatments, sex, body weight, days on feed and metaphylaxis use were not significantly associated with the differences in MIC distributions among feedlots and within pens. In this study we found no evidence that the number of fluoroquinolone treatments administered to cattle affects fecal prevalence of *Campylobacter* or the sample-level prevalence of resistance to nalidixic acid or ciprofloxacin.

Introduction

In 2013, the Center for Disease Control and Prevention (CDC) reported that drug-resistant *Campylobacter* is considered a serious threat to the United States and requires immediate action to ensure the problem does not continue to grow (CDC, 2013). The CDC estimates that more than 1 million *Campylobacter* infections are reported each year, leading to 13,000 hospitalizations and 120 deaths. Of the cases reported, 310,000 are found to be drug-resistant (CDC, 2014). *Campylobacter* causes bloody diarrhea, fever and abdominal cramps in humans. If the organism is resistant to antimicrobial therapy, symptoms typically last longer and are more severe (CDC, 2014).

Cattle and poultry are considered reservoirs of *Campylobacter,* which is shed in their feces (Besser *et al*., 2005; Humphrey *et al*., 2007). Human illness is frequently associated with the consumption of undercooked meat, unpasteurized milk and contaminated water (CDC, 2014). Human infection with a *Campylobacter* species is often treated with a fluoroquinolone (Allos,

2001). Bovine respiratory disease, a bacterial pneumonia that causes major economic losses in the U.S. feedlot industry, can also be treated with a fluoroquinolone (Bateman *et al*., 1990). Because antimicrobial drug use contributes to the emergence of drug resistant organisms, the U.S. Food and Drug Administration (FDA) recommends that drugs used in both human and animal medicine be used judiciously (FDA, 2012).

The judicious use of an antimicrobial in food-producing cattle is defined as: 1) limiting medically important antimicrobials to uses considered necessary for assuring the health and well-being of animals and 2) only using these drugs under the consultation and supervision of a veterinarian (FDA, 2012). Evaluating the judicious use of antimicrobials in the feedlot industry helps to assure that the public health is protected and animal health needs are being met. Therefore, the objective of this study was to assess the prevalence of *Campylobacter* and its antimicrobial susceptibilities to human quinolones, isolated from pens of pre-harvest, commercial feedlot cattle that were treated with a fluoroquinolone for bovine respiratory disease (BRD). These data enable estimates of *Campylobacter* prevalence and antimicrobial susceptibility in pens of cattle administered various amounts of antimicrobial therapy, and an assessment of whether gender, body weight, the number of days cattle were at the feedlot ("days on feed") and metaphylaxis use (whole pen use of an antimicrobial) are significantly associated with these prevalence estimates.

Materials and Methods

Study Population

Five commercial feedlots in Texas and western Kansas were selected for this study. Feedlots were selected as a convenience sample based on their use of a fluoroquinolone

(Baytril® 100) as first-line therapy for the treatment of BRD and proximity to Kansas State University Pre-Harvest Food Safety Lab. Ten study pens with various levels of BRD morbidity rates were selected by feedlot personnel based on their projected harvest dates. Data regarding antimicrobials administered to cattle within study pens while at the feedlot were collected and summarized.

Between May and July of 2012, 20 freshly voided fecal samples were collected from the pen floor of each study pen (N=10 pens/feedlot), which were approximately 1 to 2 weeks prior to harvest, within each feedlot (N=200 samples/feedlot). Cattle feces were collected using new plastic spoons, placed in individual Whirlpak bags (Nasco, Inc., Fort Atkinson, WI) and transported on ice to the Kansas State University pre-Harvest food safety laboratory for processing and analysis.

Isolation of *Campylobacter*

Campylobacter was isolated from each fecal sample collected using a published isolation method (Burrough *et* al., 2013). Briefly, each fecal sample was diluted 1:10 (1 g feces to 9 ml broth) in Mueller-Hinton broth (Becton Dickinson Co., Sparks, MD) containing selective (polymyxin B, rifampicin, trimethoprim, and cycloheximide; Oxoid, Cambridge, UK) and growth supplements (SS; sodium pyruvate, sodium metabisulfite and ferrous sulfate; Oxoid, Cambridge, UK) and incubated under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen) at 42°C for 48 h. Using a sterile, disposable cotton swab, the broth culture was streaked for isolation onto Mueller Hinton agar (Becton Dickinson Co., Sparks, MD) with selective and growth supplements (MH+SS) and incubated under microaerophilic conditions at 42°C for 48 h. Plates were observed for *Campylobacter* growth and three, well-isolated colonies per sample were streaked for isolation onto MH agar. Plates were incubated at

42°C for 24-30 h under microaerophilic conditions. After incubation, pure culture from each agar plate was transferred to separate cryovials containing MH broth and 30% glycerol (Fisher, Fair Lawn, NJ). Samples were stored at -80°C until genus confirmation and species identification were performed.

Campylobacter Confirmation and Speciation

Suspect *Campylobacter* colonies were removed from the freezer and streaked for isolation on MH+SS agar plates. The plates were incubated at 42°C for 48 h under microaerophilic conditions. Approximately 2 to 3 colonies were transferred to single cell lysis buffer (1 ml Tris-EDTA (1x TE) + 10 µl proteinase K (5 mg/ml)) for DNA extraction (Olah, *et al*., 2006). Multiplex polymerase chain reaction (PCR) was used to identify *Campylobacter* genus and six species: *jejuni*, *coli*, *lari*, *fetus*, *upsaliensis* and *hyointestinalis* subsp. *hyointestinalis* (Yamazaki-Matsune *et al*., 2007). *Campylobacter coli* NCTC 36572, *Campylobacter jejuni* ATCC 33560, *Campylobacter hyointestinalis* subsp. *hyointestinalis* ATCC 35217, *Campylobacter lari* ATCC 35222, *Campylobacter fetus* subsp. *fetus* ATCC 27374, and *Campylobacter upsaliensis* ATCC 49815 were used as positive controls.

Susceptibility Testing

A micro-broth dilution method, described by the Clinical and Laboratory Standards Institute (CLSI, 2008), was used to determine the minimum inhibitory concentrations (MICs) of nalidixic acid and ciprofloxacin for *Campylobacter* isolates tested. One isolate per fecal sample collected was tested.

Stock solutions of ciprofloxacin and nalidixic acid (Sigma-Aldrich, St. Louis, MO) were prepared in sterile, distilled water at a concentration of 1 mg/mL based on the potency of the antibiotic. Nalidixic acid was tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56,

0.78, 0.39 and 0.195 µg/mL and ciprofloxacin was tested at concentrations of 25, 12.5, 6.25, 3.152, 1.56, 0.78, 0.39, 0.195, 0.098 and 0.049 µg/mL.

Inoculum was prepared by streaking pure, *Campylobacter* culture for each sample onto MH+SS agar and incubating at 42°C for 48 hours. A single isolate from each plate was sub-cultured onto MH agar (without supplements) and incubated for 24-30 hours at 42°C. Pure culture was then transferred to 3mL MH II broth using a sterile, disposable swab. A spectrophotometer (OD 600) and MH II broth was used to adjust the innoculum, so that it was equivalent to 0.5 McFarland standard. A 1:100 dilution of the inoculum was then prepared using MH II broth and 2.5% lysed horse blood.

The antimicrobial susceptibilities were performed in 96-well microtiter plates (Becton and Dickinson Co., Sparks, MD) and quality control organism, *Campylobacter jejuni* ATCC# 33560 was used to ensure the validity of the test. Plates were sealed with perforated plate covers and incubated at 37°C under microaerophilic conditions for 48 h. Results were recorded as either growth or no growth (color change) within each well. This process was performed separately for each antibiotic (ciprofloxacin and nalidixic acid) and each *Campylobacter* isolate was tested in quadruplicate. The minimum inhibitory concentration (MIC) was determined by the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism (CLSI, 2008). *Campylobacter* isolates were considered resistant to nalidixic acid and ciprofloxacin if the MIC was $\geq$ 64 µg/mL and $\geq$ 4 µg/mL, respectively (CLSI, 2010 ; NARMS, 2011).

Statistical Analysis

Campylobacter within-pen prevalence and associations with antibiotic use and demographic characteristics of the study pens were evaluated using generalized linear mixed

models. These models were fitted using binomial distribution, maximum likelihood estimation, complimentary-log-log link, Kenward-Roger degrees of freedom and Newton-Raphson and Ridging optimization procedures (Proc GLIMMIX SAS 9.3, SAS Institute, Inc., Cary, NC, USA). Feedlot was included as a random intercept to account for the lack of independence of observations (samples) made within pens within each feedlot.

Campylobacter prevalence was modeled as a binomial outcome by including the number of *Campylobacter* test-positive samples in a pen ("events") divided by the number of samples collected per pen ("trials"). The number of BRD fluoroquinolone treatments administered, body weight at arrival, days on feed, sex (steers/heifers) and antimicrobial metaphylaxis use in the pen (use/no use) were included as independent variables. The variable pertaining to the number of fluoroquinolone treatments was categorized by the number of treatments per 100 cattle due to the variability in pen size. Continuous variables that did not meet the linearity assumption (i.e., number of fluoroquinolone treatments per 100 cattle, body weight and days on feed) were categorized in quartiles.

Correlation analysis was performed prior to building the multivariable model to identify highly correlated variables ($\geq |0.80|$). Our variable of interest (number of fluoroquinolone treatments per 100 cattle) was forced in our multivariable model regardless of its significance. Testing for *a priori* confounders and two-way interactions was performed by keeping those variables in the model that had a > 20% change in the magnitude of the association (confounder) and when the interaction was statistically significant ($P < 0.05$). Following forward selection, if a variable was found to be non-significant ($P > 0.05$), it was removed from the model and prevalence estimates (means and 95% confidence intervals) for the univariable model were reported for that variable. Residual diagnostics were used to assess model assumptions and

overall model fit. Best linearized unbiased predictors (BLUPs) were used to evaluate residuals at the feedlot level, and Pearson and Deviance residuals were used to evaluate residuals at the pen-level.

Because antimicrobial susceptibility testing was performed in quadruplicate for all *Campylobacter* isolates, the mode MIC value was reported for each isolate. When two different MIC values for one isolate were recorded, the MIC value with the highest concentration was reported. If four different MIC values were recorded, the isolate was re-tested and either the mode or the highest MIC value was reported. The overall proportion of *Campylobacter* isolates at each MIC for both nalidixic acid and ciprofloxacin were summarized using descriptive statistics.

In lieu of dichotomizing the susceptibility outcomes in terms of susceptible or resistant isolates, semi-parametric survival analysis was used to analyze the pen-level distribution of the MIC values for isolates recovered from each of the five feedlots (STATA 10, StataCorp LP, College Station, TX). Cox proportional hazard shared frailty regression model was fitted to evaluate the distribution of the MICs for each drug tested (nalidixic acid and ciprofloxacin) and their association with where the isolates were recovered from (Feedlot), species (*C. coli*, *C. hyointestinalis*, or "other" species), number of fluoroquinolone treatments for BRD per 100 cattle, gender, metaphylaxis use, arrival body weight and days on feed. The hazard was defined as isolates that failed to grow at a specific breakpoint (MIC value). Frailty was used to account for clustering within feedlot. Hazard ratios were documented for all variables tested (univariable model) and those variables found significant ($P < 0.05$) were included in the (multivariable) model, along with our exposure variable of interest (# of fluoroquinolone treatments per 100 head of cattle). The proportional hazard assumption was assessed using the log cumulative

hazard plot and Schoenfeld residuals. Martingale residuals were used to evaluate model fit (Goodness of Fit test), as well as to evaluate outlying observations at the pen-level.

Results

Cattle Demographics

Demographic characteristics of cattle within study pens across the five feedlots were previously described in Smith *et al*., 2016. Briefly, the majority (56%; 28/50) of the cattle in study pens were steers with one pen including both steers and heifers. The pen-level mean body weight of cattle on feedlot arrival ranged from 289 kg to 317 kg across the five feedlots and the mean number of days on feed ranged from 96 to 199 days. All feedlots had at least one pen that received a metaphylaxis antimicrobial for the control of BRD and the mean number of fluoroquinolone treatments per 100 cattle ranged from 5 to 23 treatments, with no fluoroquinolone treatments administered to two pens within one feedlot (Feedlot C).

Campylobacter Prevalence

Overall prevalence of *Campylobacter* isolated from cattle feces across all five feedlots was 27.2% (272/1000). Sample-level prevalence of *Campylobacter* varied significantly ($P < 0.01$) across feedlots ranging from 14.5% (29/200) to 40.0% (80/200) (Figure 1). Within-pen prevalence ranged from 0.0% (0/20) to 60.0% (12/20). *Campylobacter coli* was the most prevalent species (55.1%; 150/272) isolated, followed by *C. hyointestinalis* (42.6%; 116/272). Species was not identified for six isolates (2.2%; 6/272), because we only tested for six species (*jejuni , coli , lari, fetus, upsaliensis and hyointestinalis*). *Campylobacter jejuni* was not isolated from any of the cattle feces collected in this study.

Results from the evaluation of the potential associations between the number of fluoroquinolone treatments per 100 cattle, sex, metaphylaxis use, arrival body weight, the number of days on feed, and within-pen prevalence of *Campylobacter* are illustrated in Table 1. The number of days cattle were on feed at the time of sample collection was significantly associated with the within-pen prevalence of *Campylobacter* (Table 1). Cattle that were fed for 102 to 145 days had significantly lower prevalence of *Campylobacter* than those that were fed for 146 to 161 days ($P = 0.03$) or 162 to 192 days ($P = 0.02$).

The number of fluoroquinolone treatments per 100 cattle was not statistically significant ($P = 0.63$). However it was forced into the multivariable model with the significant variable, "days on feed," because it was considered our main variable of interest. After accounting for the number of fluoroquinolone treatments, cattle that were fed for 102 to 145 days had significantly lower prevalence of *Campylobacter* (19.5%; 95% CI = 12.2 to 30.0) than cattle that were fed for 162 to 192 days (33.2%; 95% CI = 22.9 to 45.5; $P = 0.03$). No other statistically significant differences were found among the other (days on feed) quartiles.

When testing whether the number of fluoroquinolone treatments per 100 cattle was associated with any of the *Campylobacter* species, results indicated that the number of fluoroquinolone treatments per pen was not associated with the prevalence of *C. coli* ($P = 0.39$) or *C. hyointestinalis (*$P = 0.85$). Isolates that did not have species identified were not analyzed due to the small sample size (6 isolates).

Campylobacter Susceptibility Results

There were 256 viable *Campylobacter* isolates available for susceptibility (MIC) testing. Of the isolates tested, 31.6% (81/256) were susceptible to nalidixic acid and 34.4% (88/256) were susceptible to ciprofloxacin. Several isolates (68.4%; 175/256) were above the breakpoint

for nalidixic acid (≥ 64 µg/mL) and above the breakpoint (≥ 4 µg/mL) for ciprofloxacin (65.6%; 168/256).

The MIC results for *C. coli* isolates from each feedlot are illustrated in Table 2. MICs ranged from 3.13 µg/mL to > 100µg/mL for nalidixic acid and 0.05 µg/mL to 25.00 µg/mL for ciprofloxacin across all feedlots, with most of the *C. coli* isolates having a MIC of 100.00 µg/mL for nalidixic acid and a MIC of 6.25 µg/mL for ciprofloxacin. The MIC results for *C. hyointestinalis* isolates from each feedlot are illustrated in Table 3. MICs for these isolates ranged from 25.00 to > 100.00 µg/mL for nalidixic acid and 0.20 µg/mL to 25.00 µg/mL for ciprofloxacin, with most of the isolates having a MIC > 100.00 µg/mL for nalidixic acid and a MIC of 25.00 µg/mL for ciprofloxacin.

Univariable survival analysis revealed that the number of fluoroquinolone treatments within each pen, sex, arrival body weight, and days on feed categories were not significantly associated with the MIC results for nalidixic acid and ciprofloxacin for the *Campylobacter* isolated (Tables 4 and 5). However, the feedlot from where the isolates were collected ($P \leq 0.01$), species ($P \leq 0.01$) and whether or not a metaphylaxis antimicrobial was used ($P \leq 0.05$) were significantly associated with MIC results for naladixic acid and ciprofloxacin (univariable models; Table 4). In the multivariable model, after forcing the variable pertaining to the number of fluoroquinolone treatments per 100 cattle into the model, only feedlot ($P \leq 0.01$) and isolated species ($P \leq 0.01$), remained significantly associated with the MIC distribution for the *Campylobacter* isolated (main effects model).

The distribution of the MICs for *Campylobacter* isolates tested against ciprofloxacin was similar to nalidixic acid (Table 5). The MIC distributions were significantly different among feedlots ($P \leq 0.01$) and were dependent upon species ($P \leq 0.01$). No other variables were found

to be associated with the difference in the MIC distribution when each variable was tested separately (univariable model; Table 5). When our variable of interest (# of fluoroquinolone treatments per 100 head of cattle) was forced into the model, the MIC distributions were still significantly different among feedlots ($P = 0.05$) and species ($P = 0.02$). No other variable tested was significantly associated with the outcome (Table 5).

Discussion

This study provides *Campylobacter* prevalence estimates and quinolone susceptibility data for pre-harvest feedlot cattle administered a fluoroquinolone for the treatment of BRD. It also provides evidence that the number of previous fluoroquinolone treatments administered to cattle during the feedlot production phase does not significantly impact the prevalence or the MIC distributions for *Campylobacter*.

Cumulative sample-level fecal prevalence of *Campylobacter* for this study was fairly low with only 272 of the 1000 (27.2%) fecal samples collected across all five feedlots positive for this organism. Though published *Campylobacter* prevalence data for beef cattle are relatively scarce, fecal prevalence estimates from previous studies range from 20-70% (Beach *et al*., 2002; Bae *et al*., 2005; Krueger *et al*., 2008; Abley *et al*., 2012). *Campylobacter* prevalence was highly variable across all feedlots and within pens of cattle, with some pens having no samples test positive for the organism of interest. Prevalence of *Campylobacter* in cattle are believed to be affected by numerous factors such as season, stress, farm management factors and diet (Wesley *et al*., 2000; Sproston *et al*., 2011). Though all samples were collected within the same season (May thru July; sampled in consecutive order “Feedlot A-E”) and within a similar geographic region (Western Kansas and Northwestern Texas), factors such as feedlot management practices may have accounted for the variability in prevalence (Krueger *et al*., 2008; Hannon *et al*., 2009).

Campylobacter coli was the most prevalent species isolated for this study, followed by *Campylobacter hyointestinalis*. According to the literature, *C. coli* and *C. hyointestinalis*, though more commonly seen in poultry and swine feces, have been previously isolated from cattle (Inglis *et al*., 2003; Laatu *et al*., 2005; Sanad *et al*., 2011; Sproston *et al*., 2011; Mattheus *et al*., 2012; Quintana-Hayashi *et al*., 2012; Gaudreau *et al*., 2014). *Campylobacter coli* has been found to be pathogenic to humans, though *C. jejuni* is the species more commonly associated with foodborne illness (Allos, 2001; FDA, 2012; Taylor *et al*., 2013). Approximately 90% of the human Campylobacteriosis cases each year are caused by *C. jejuni*, while only 10% are caused by *C. coli* (FDA, 2016). *Campylobacter jejuni* is typically one of the most prevalent species isolated from both dairy and beef cattle (Wesley *et al*., 2000; Besser *et al*.,2005; Sproston *et al*., 2011). However, *C. jejuni* was not isolated from any of the fecal samples collected during this study.

The number of fluoroquinolone treatments per 100 cattle, metaphylaxis use, body weight or gender were not significantly associated with the within pen-prevalence of *Campylobacter*. However, *Campylobacter* pen-prevalence significantly differed with the number of days cattle were in the feedlot. Specifically, pens of cattle fed for 146-192 days had significantly more *Campylobacter* than cattle fed 102-145 days after arrival at the feedlot. This was similar to what Besser and others found in 2005 where *Campylobacter* prevalence increased significantly throughout the feeding period. The opposite was reported in 2011 where *Campylobacter* prevalence was the highest at feedlot entry (32.1%) and reduced to 11.8% at final sampling (Sproston *et al*., 2011). With various outcomes being reported, we conclude that the number of days cattle are in the feedlot may not be a consistent predictor of *Campylobacter* prevalence at harvest. In the current study, other factors such as the environment and feedlot management

practices may have contributed to the increase in *Campylobacter* prevalence 140+ days after arrival. Numerous *Campylobacter* isolates recovered from fecal samples collected for this study were above the breakpoint for both nalidixic acid (175/256 ≥ 64 μg/mL) and ciprofloxacin (168/256; ≥ 4 μg/mL). Since a large portion of the isolates available for susceptibility testing were *C. coli*, (55.1%; 141/256), these results were expected. Previous studies in cattle have shown that *C. coli*, under fluoroquinolone selection pressure, tend to be resistant to quinolones (Englen *et al*., 2005; Inglis *et al*., 2006; Sanad *et al*., 2011; Gaudreau *et al*., 2014). *Campylobacter coli*, as well as other *Campylobacter* species, are able to resist quinolone antimicrobial therapy by a single point mutation in the quinolone resistance-determining region (QRDR) of DNA gyrase A (Alfredson *et al*., 2007; Luangtongkum *et al*., 2009). When the mutation occurs, the quinolone is unable to bind allowing for decreased susceptibility to the drug (Alfredson *et al*., 2007; Luangtongkum *et al*., 2009).

The distributions of MICs for nalidixic acid and ciprofloxacin were significantly different among the five feedlots. Similar to prevalence, feedlot management practices and environmental factors may have contributed to the variability in the susceptibility results. It is unclear how drug-resistant *Campylobacter* isolates survive in the environment and persist without antimicrobial selection pressure. However, enhanced fitness and transmission of these resistant isolates in the environment may play an important role in the variability in the prevalence of these resistant isolates at different feedlots (Zhang *et al*., 2003; Luo *et al*., 2005; Zhang *et al*., 2006).

Distributions of MICs were significantly different ($P < 0.05$) between *Campylobacter* species for both drugs tested. Numerical differences in the prevalence of *C. coli* and *C. hyointestinalis* were observed among feedlots, which may explain the variation in the MIC

results (Tables 2 and 3). Also, previous studies have shown that *C. coli* tend to have higher resistance prevalence to quinolones when compared to other species of *Campylobacter* (Moore *et al*., 2006; Smith and Fratamico, 2010; Gaudreau *et al*., 2014). The number of fluoroquinolone treatments per 100 cattle, sex, metaphylaxis use, body weight and days on feed were not significantly associated with MIC distributions.

Data from this cross-sectional study can be used to help evaluate the use of fluoroquinolone therapy in feeder cattle and the potential association with the prevalence of pathogenic bacteria and their susceptibility to human quinolones. There are, however, limitations to this study design (Mann, 2003). Without the history of fluoroquinolone use prior to cattle arriving at the feedlot, and without more comprehensive knowledge of the epidemiology of *Campylobacter* both within cattle and within their environment, causal inferences are limited. We can conclude, however, that administering a fluoroquinolone to beef cattle for the treatment of BRD was not significantly associated with the prevalence of *Campylobacter*, and though MIC values for *Campylobacter* isolates were generally high, there was no evidence that the distribution of the MICs was associated with fluoroquinolone treatment administered within study pens.

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Disclosure Statement

The first author (A.Smith) is a graduate student of Kansas State University and an employee of Bayer Animal Health, who produce a commercially licensed fluoroquinolone for the treatment and control of BRD.

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Figure 3-1: Cumulative Sample-Level Fecal Prevalence of *Campylobacter* in Five U.S. Commercial Feedlots (*n* = 200 total samples/feedlot; 20 from each of 10 pens). Error Bars Represent 95% Exact Confidence Intervals For Proportions.

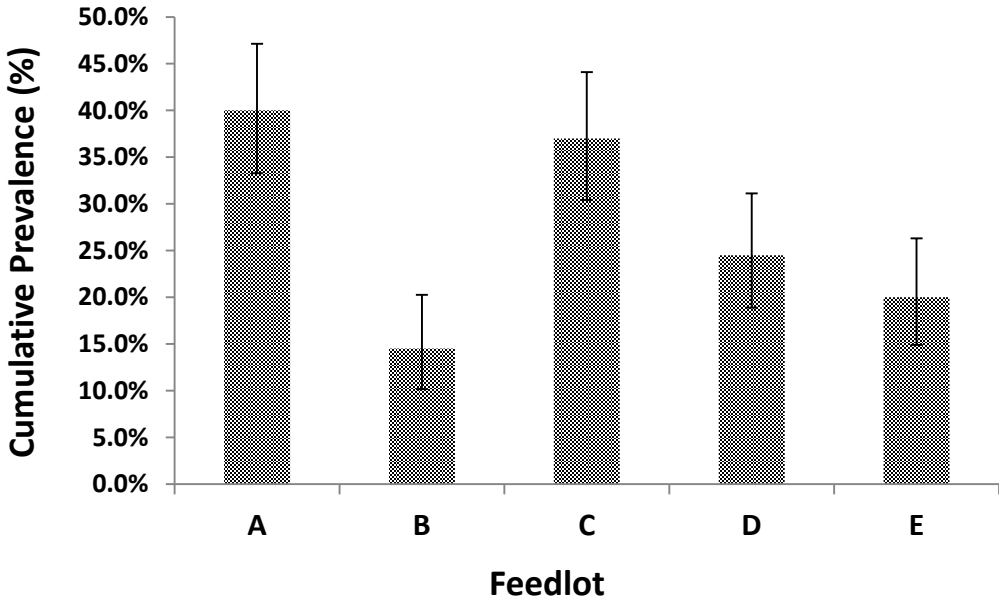

Table 3-1: Results from Univariable Mixed Models[a] of Associations between Pen-Level Risk Factors and Within-Pen Prevalence of *Campylobacter*

Variable, units	Number of Pens	Mean Prevalence (%)	95% Confidence Intervals (%)	*P*-value
Number of fluoroquinolone treatments for BRD per 100 cattle (in quartiles)				0.63
0-2	13	29.8	19.9-42.0	
3-8	12	24.3	15.7-35.6	
9-23	13	28.3	19.0-40.1	
24-66	12	23.0	14.4-35.0	
Sex[b]				0.87
Steer	28	26.0	17.4-36.9	
Heifer	21	26.7	17.6-38.3	
Metaphylaxis use				0.22
No	18	30.5	21.5-41.2	
Yes	32	24.4	17.7-32.7	
Body weight at arrival, kg (in quartiles)				0.34
215-265	12	26.4	16.5-39.4	
266-298	13	30.6	19.9-43.8	
299-319	11	27.1	16.8-40.7	
320-382	14	21.9	13.6-33.4	
Days on feed (in quartiles), days				0.04
102-145	11	20.1[c]	12.5-30.6	
146-161	14	31.2[d]	21.2-43.3	
162-192	12	31.8[d]	21.7-43.9	
193-240	13	21.7[cd]	13.8-32.4	

[a]Models included a random effect to account for lack of independence among pens within feedlots

[b]One pen was a mixed of heifers and steers, and was removed from this analysis

[c,d]Column values with different superscripts differ ($P < 0.05$).

Table 3-2: Minimum Inhibitory Concentration (MIC) results for Nalidixic acid and Ciprofloxacin for *Campylobacter coli* Isolates Recovered from Cattle Feces from the Five Feedlots Sampled

		Nalidixic acid			Ciprofloxacin		
Feedlot	% *C. coli*	Mode MIC (µg/mL)	MIC Range (µg/mL)	% [a]Resistant	Mode MIC (µg/mL)	MIC Range (µg/mL)	% [b]Resistant
A	79.3 (65/82)	100	6.25 to > 100	60.0 (39/65)	6.25	0.195 to 25	73.8 (48/65)
B	88.5 (23/26)	50	3.125 to > 100	26.1 (6/23)	6.25	0.049 to 12.5	78.3 (18/23)
C	7.2 (5/69)	100	25 to > 100	60.0 (3/5)	12.5	6.25 to 12.5	80.0 (4/5)
D	78.6 (33/42)	12.5	6.25 to > 100	24.2 (8/33)	0.195	0.195 to 25	30.3 (10/33)
E	40.5 (15/37)	100	25 to 100	46.7 (7/15)	6.25	0.195 to 12.5	86.7 (13/15)

[a]Nalidixic acid resistance breakpoint is ≥ 64 µg/mL.

[b]Ciprofloxacin resistance breakpoint is ≥ 4 µg/mL.

Table 3-3: Minimum Inhibitory Concentration (MIC) results for Nalidixic acid and Ciprofloxacin for *Campylobacter hyointestinalis* Isolates Recovered from Cattle Feces from the Five Feedlots Sampled

		Nalidixic acid			Ciprofloxacin		
Feedlot	% *C. hyointestinalis*	Mode MIC (µg/mL)	MIC Range (µg/mL)	% [a]Resistant	Mode MIC (µg/mL)	MIC Range (µg/mL)	% [b]Resistant
A	19.5 (16/82)	100	25 to > 100	87.5 (14/16)	25	0.195 to 25	68.8 (11/16)
B	7.69 (2/26)	> 100	N/A	100.0 (2/2)	12.5	N/A	100.0 (2/2)
C	89.9 (62/69)	> 100	N/A	100.0 (62/62)	25	0.195 to 50	66.1 (41/62)
D	21.4 (9/42)	> 100	50 to > 100	88.9 (8/9)	12.5	0.195 to 25	77.8 (7/9)
E	54.1 (20/37)	100	100 to > 100	100.0 (20/20)	0.195	0.195 to 25	45.0 (9/20)

[a]Nalidixic acid resistance breakpoint is ≥ 64 µg/mL.

[b]Ciprofloxacin resistance breakpoint is ≥ 4 µg/mL.

Table 3-4: Results from Univariable and Multivariable Survival Analysis Models[a] Testing Associations between Pen-level Risk Factors and Within-Pen Distributions of Minimum Inhibitory Concentrations (MICs) for Nalidixic acid for *Campylobacter* isolated from Five U.S. Commercial Feedlots

	Univariable Model				Multivariable Model		
Variable, units	Number of Pens	Hazard Ratio	95% Confidence Intervals (%)	*p*-value	Hazard Ratio	95% Confidence Intervals (%)	*p*-value
Feedlot				≤ 0.01			≤ 0.01
A	10	Reference[c]	.		Reference[c]	.	
B	10	1.45[c]	0.85-2.48		1.76[cd]	0.92-3.36	
C	10	0.29[d]	0.17-0.49		0.65[ce]	0.36-1.16	
D	10	1.60[c]	1.00-2.59		2.43[d]	1.34-4.26	
E	10	0.78[c]	0.48-1.28		1.18[c]	0.65-2.15	
Species				≤ 0.01			≤ 0.01
C. coli		Reference[c]	.		Reference[c]	.	
C. hyointestinalis		0.29[d]	0.20-0.41		0.40[d]	0.27-0.61	
"Other"[f]		0.33[cd]	0.10-1.04		0.41[cd]	0.13-1.33	
Number of fluoroquinolone treatments for BRD				0.97			0.45

per 100 cattle (in quartiles)						
0-2	13	Reference	.		Reference	.
3-8	12	1.12	0.58-2.13		0.77	0.47-1.26
9-23	13	0.99	0.53-1.83		0.78	0.51-1.20
24-66	12	1.13	0.57-2.22		0.60	0.31-1.17
Sex[b]				0.39		
Steer	28					
Heifer	21	0.81	0.50-1.31			
Metaphylaxis use				0.05		0.90
No	18	Reference	.		Reference	.
Yes	32	1.58	1.00-2.49		1.03	0.65-1.64
Body weight at arrival, kg (in quartiles)				0.19		
215-265	12	Reference	.			
266-298	13	1.52	0.79-2.93			
299-319	11	2.19	1.08-4.46			
320-382	14	1.48	0.75-2.92			
Days on feed (in quartiles)				0.67		

102-145	11	Reference	.
146-161	14	1.48	0.76-2.85
162-192	12	1.42	0.70-2.89
193-240	13	1.21	0.57-2.57

[a]Univariable models included frailty to account for lack of independence among pens within feedlots

[b]One pen was both heifers and steers and was removed from this analysis

[c,d,e]Row values with different superscripts differ ($p < 0.05$).

[f]Isolates where species was not identified were listed as “other.”

Table 3-5: Results from Univariable and Multivariable Survival Analysis Models[a] Testing Associations between Pen-level Risk Factors and Within-Pen Distributions of Minimum Inhibitory Concentrations (MICs) for Ciprofloxacin for *Campylobacter* isolated from Five U.S. Commercial Feedlots

	Univariable Model				Multivariable Model		
Variable, units	Number of Pens	Hazard Ratio	95% Confidence Intervals (%)	*p*-value	Hazard Ratio	95% Confidence Intervals (%)	*p*-value
Feedlot				≤ 0.01			0.05
A	10	Reference[c]	.		Reference[c]	.	
B	10	1.12[c]	0.72-1.74		1.26[ce]	0.77-2.07	
C	10	0.62[d]	0.44-0.86		0.92[d]	0.62-1.37	
D	10	1.17[c]	0.81-1.71		1.64[e]	1.03-2.61	
E	10	1.17[c]	0.79-1.73		1.43[ce]	0.95-2.17	
Species				≤ 0.01			0.02
C. coli		Reference[c]	.		Reference[c]	.	
C. hyointestinalis		0.58[d]	0.43-0.77		0.63[d]	0.45-0.88	
"Other"[f]		0.52[cd]	0.22-1.23		0.51[cd]	0.21-1.24	
Number of fluoroquinolone treatments for BRD per				0.60			0.15

100 cattle (in quartiles)					
0-2	13	Reference	.	Reference	.
3-8	12	1.06	0.68-1.65	0.95	0.65-1.39
9-23	13	0.95	0.62-1.44	0.87	0.62-1.23
24-66	12	0.75	0.47-1.21	0.60	0.38-0.96
Sex[b]			0.77		
Steer	28	Reference	.		
Heifer	21	0.95	0.68-1.33		
Metaphylaxis use			0.38		
No	18	Reference	.		
Yes	32	1.15	0.84-1.60		
Body weight at arrival, kg (in quartiles)			0.62		
215-265	12	Reference	.		
266-298	13	1.31	0.83-2.06		
299-319	11	1.03	0.63-1.70		
320-382	14	1.18	0.73-1.88		
Days on feed (in quartiles)			0.81		
102-145	11	Reference	.		

146-161	14	1.21	0.77-1.91
162-192	12	1.05	0.65-1.70
193-240	13	1.19	0.72-1.96

aUnivariable models included frailty to account for lack of independence among pens within feedlots

[b]One pen was a both heifers and steers and was removed from this analysis

[c,d, e]Row values with different superscripts differ ($p < 0.05$).

[f]Isolates where species was not identified were listed as “other.”

Chapter 4 - A randomized trial to assess the effect of fluoroquinolone metaphylaxis on the fecal prevalence and quinolone susceptibilities of *Salmonella* and *Campylobacter* in feedlot cattle

Ashley B. Smith[1,2], David G. Renter[1,3], Natalia Cernicchiaro[1, 3], Xiaorong Shi[1] Jason S. Nickell[2], Daniel J. Keil[2] and T.G. Nagaraja[1]

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[1]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506

[2]Bayer Animal Health, Shawnee, Mission, KS 66216

[3] Center for Outcomes Research and Education, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506

Address correspondence to:

David G. Renter, DVM, PhD

College of Veterinary Medicine

309 Coles Hall, 1620 Denison Avenue

Kansas State University,

Manhattan, KS 66506

Phone: 785-532-4801, Fax: 785-532-4851

drenter@vet.k-state.edu

Abstract

The study objective was to determine effects of fluoroquinolone metaphylaxis on fecal prevalence of *Salmonella* and *Campylobacter* and fecal prevalence of quinolone resistant *Salmonella* and *Campylobacter* in feedlot cattle. On Day 0, cattle (n=288) at risk for bovine respiratory disease (BRD) were randomly assigned to either a non-treated, control pen (12 pens), or a fluoroquinolone (enrofloxacin; Baytril® 100) treated pen (12 pens). Rectal fecal samples were collected from cattle on days 0, 7, 14, 21, and 28. Feces were cultured for *Salmonella* enterica and *Campylobacter* spp. using enrichment and selective isolation methods, and confirmed by serology and PCR. Susceptibilities to nalidixic acid and ciprofloxacin were determined using microbroth dilution methods. Data analyses were performed using linear mixed-models. Overall, *Salmonella* and *Campylobacter* were recovered from 10.2% (139/1,364) and 12.4% (170/1,364) of the fecal samples, respectively. *Campylobacter* species included *hyointestinalis*, *jejuni* and *coli*. Neither *Salmonella* nor *Campylobacter* prevalence were significantly impacted by fluoroquinolone treatment ($P = 0.80$, $P = 0.61$, respectively). However, *Salmonella* prevalence differed between study weeks ($P < 0.01$) with prevalence decreasing over time. Prior to treatment, 98.9% (91/92) of *Salmonella* isolates were susceptible to nalidixic acid and ciprofloxacin. All *Salmonella* recovered post-treatment were susceptible to both antimicrobials (n=43). The majority of *Campylobacter* recovered prior to treatment were resistant to nalidixic acid (23/35; 65.7%) and ciprofloxacin (21/35; 60.0%). There were no significant treatment by week interaction ($P = 0.85$) or treatment effects ($P = 0.61$) on the post-treatment prevalence of *Campylobacter* resistance. There was, however, a significant week effect ($P = 0.05$), with *Campylobacter* resistance prevalence decreasing over time. In this 28-day study, we found no evidence that a fluoroquinolone used for metaphylaxis significantly impacts

fecal prevalence of *Salmonella* or *Campylobacter* or the fecal prevalence of nalidixic acid or ciprofloxacin resistance.

Key words: fluoroquinolone; cattle; susceptibility; resistance; prevalence; *Salmonella*, *Campylobacter*

Introduction

Salmonella and *Campylobacter* are two of the most common causes of bacterial foodborne illness in humans (CDC, 1999). These bacteria are commensal gut flora in healthy, beef cattle and are typically shed in their feces (Kunze *et al*., 2008). The United States Foodborne Diseases Active Surveillance Network (FoodNet) indicates that 1 million cases of salmonellosis and 1.3 million cases of campylobacteriosis are reported each year (CDC, 2014; CDC, 2015). Illness is often associated with the consumption of unpasteurized dairy products, raw or undercooked meat, and contaminated produce and water (CDC, 2014; CDC, 2015). Although few human illness cases are directly attributable to beef cattle, cattle may serve as a reservoir for both *Salmonella* and *Campylobacter.*

Human pathogenic organisms, such as *Salmonella* and *Campylobacter*, often carry antimicrobial resistance genes (Luo *et al*., 2003; Chen *et al*., 2007). As antimicrobial resistance continues to become one of the biggest human health threats around the world, the use of antimicrobials in livestock has become highly scrutinized (Jan *et al*., 2012; Page and Gautier, 2012). This is particularly true for medically important antimicrobials such as fluoroquinolones, which are used to treat both human and animal diseases (Jan *et al*., 2012; Page and Gautier, 2012). However, there are minimal data that can be used to estimate the risk to humans associated with the use of fluoroquinolones or other antimicrobials in feedlot cattle.

In the beef industry, the metaphylactic use of antimicrobials is an important tool for reducing bovine respiratory disease (BRD), the most common cause of morbidity and mortality (Bateman *et al*., 1990; Edwards 2010). Metaphylaxis involves treating a group of cattle with a US Food and Drug Administration (FDA)-approved antimicrobial with the intent of controlling the incidence of acute onset BRD in high risk cattle (Ives and Richeson, 2015). To date, there is no evidence that the metaphylactic use of a fluoroquinolone has a negative impact on human health. Prevalence of quinolone resistance in retail ground beef has been very low over the past decade for both *Salmonella* and *Campylobacter* (FDA, 2015). However, the continued judicious use of fluoroquinolones in cattle and monitoring of resistant organisms that may be pathogenic to humans seems warranted. To our knowledge, there are no published data assessing whether metaphylaxis with a fluoroquinolone, such as enrofloxacin, has any impact on fecal shedding of *Salmonella* or *Campylobacter* in feedlot cattle. Therefore, the objective of this study was to assess the effect of using the fluoroquinolone, enrofloxacin, for metaphylaxis on the fecal prevalence of *Salmonella* and *Campylobacter* and the fecal prevalence of quinolone-resistant *Salmonella* and *Campylobacter* in feedlot cattle.

Materials and Methods

Study design

Two-hundred and ninety-eight, eight-month old, cross-bred beef calves were purchased from a livestock auction in Iowa and transported to a Kansas cattle feeder two days prior to study initiation. To be eligible for this study, calves were required to have had 1) no previous history of fluoroquinolone use, 2) no overt clinical signs of illness at arrival, and 3) been considered at high risk for BRD. High risk cattle are typically those with one or more risk factors for BRD, which

may include: auction market sourced, unknown vaccination or preconditioning status, recent weaning, transportation from multiple farm origins, an extended transport time with few to no rest stops, experience a, or exposure to dramatic temperature change of ≥ 30 °F from the origin to the study site, a ≥ 30 °F fluctuation in environmental temperature at a study site within a 24-hour period, exposure to changes, or wet or cold weather conditions (Ives and Richeson, 2015; Bayer Healthcare, LLC, 2012).

Cattle were individually assigned a unique identification number, which appeared on duplicate ear tags and administered a modified-live vaccine (Bovi-Shield Gold®5; Zoetis, Kalamazoo, MI) and a parasiticide (Dectomax®; Zoetis, Kalamazoo, MI) during processing. Cattle were commingled across all truck loads in a large open feedlot pen and managed according to the feedlot's standard management practices, until study initiation.

Prior to study initiation, 24 pens, arranged in a circular-fashion, were randomly assigned to a treatment group. A coin toss determined that the first study pen was assigned to treatment A and the second was assigned to treatment B. Remaining pens were systematically assigned to treatments, so that every other pen was assigned to the opposite treatment. Using the random number function in Excel® 2010 (Microsoft, Redmond, WA), animal ID numbers were then randomly assigned to a pen. The total number of pens, animals per pen, and samples per animal over time for each treatment group were determined via simulation methods in order to detect a relative difference of 50% between treatment groups over time in the pen-level mean resistance prevalence, with type 1 and type 2 errors < 0.05 and 0.2, respectively.

On study day 0, cattle were run through the chute and administered either a single, subcutaneous, 7.5 mg/kg dose of Baytril ®100 (Bayer Animal Health, Shawnee, KS; treatment A) (n=144; 12 pens; 12 calves per pen) or no drug (treatment B) (n=144; 12 pens; 12 calves per

pen), according to their randomized pen assignment; hereafter, groups referred to as treated and control pens, respectively. Body weights were measured prior to treatment to ensure cattle were properly dosed. Once cattle were assigned to a pen and treatment was administered, cattle were placed in their respective pens where they were housed for the duration of the study (30 days).

Cattle health and performance

Routine health observations were conducted daily by the feedlot veterinarian. Cattle that required therapy for non-infectious conditions (e.g., bloat, injury) and did not require an antimicrobial were treated as per the standard site protocol and placed back into their study pen. Cattle that required antimicrobial therapy for any reason, based on the veterinarian's assessment (blinded to treatment group), were removed from the study pen. Once calves were removed from study pens, they were no longer considered part of the study.

Calves were fed a starter ration comprised of wet distiller's grain and grass hay. Monensin (Rumensin®; Elanco Animal Health, Indianapolis, IN) was mixed in the ration, per label, to prevent coccidiosis. No tetracyclines, macrolides, or other antimicrobials were included in the feed. Body weights were measured for all cattle on study day 0 and the last day of the study (study day 30). If cattle were removed from the study, their body weights were measured the day they were removed.

Sample collection

Individual fecal samples (approximately 20 g) were collected from the rectum of all study cattle on study days: 0, 7, 14, 21 and 28 (+/- 2 days). Samples were placed in Whirlpak bags (Nasco, Inc., Fort Atkinson, WI) pre-labeled with the animal ID, study day, and date of sampling. Samples were transported immediately on ice to the Kansas State University pre-harvest food safety laboratory for same-day processing and analysis.

Salmonella isolation

Ten grams of feces were suspended in 90 mL Tetrathionate (TT) broth (Becton, Dickinson Co., Sparks, MD) and incubated at 37°C for 24 h. After incubation, 100 µL of the suspension was transferred to 10 mL Rappaport-Vassiliadis (RV) broth (Becton, Dickinson Co.) and incubated at 42°C for 24 h. Enriched samples were then streaked on Hektoen Enteric (HE) agar plates (Becton, Dickinson Co.) and incubated at 37°C for 24 h. Three colonies with morphology similar to *Salmonella* (blue-green colony with black centers) for each sample were streaked onto blood agar (Remel, Lenexa, KS) and incubated at 37°C for 24 h. A single colony was tested by slide agglutination with polyvalent O antisera for *Salmonella* serogroups B, C1, C2, D1, D2 and E. Isolates that agglutinated with the polyvalent O antisera were considered *Salmonella*. Confirmed isolates were stored in cryo-protection beads (Hardy Diagnostics, Santa Maria, CA) at -80°C.

Campylobacter isolation

Campylobacter was isolated from feces using a method described by Burrough and others (2013). Briefly, fecal samples were suspended at a ratio of 1:10 (1 g feces to 9 mL broth) in Mueller-Hinton (MH) broth (Becton Dickinson Co., Sparks, MD) containing selective (polymyxin B, rifampicin, trimethoprim, and cycloheximide; Oxoid, Campbridge, UK) and growth supplements (SS; sodium pyruvate, sodium metabisulfite and ferrous sulfate; Oxoid) and incubated under microaerophilic conditions (5% Oxygen, 10% Carbon Dioxide and 85% Nitrogen) at 42°C for 48 h. The enrichment culture was streaked for isolation onto MH+SS agar (Becton Dickinson) and incubated under microaerophilic conditions for 48 h at 42°C. Plates were observed for *Campylobacter* growth and three, well-isolated colonies per sample were streaked onto MH agar. Plates were incubated at 37°C for 24-30 h under microaerophilic

conditions. Pure cultures were then transferred from each agar plate to separate cryovials containing MH broth with 30% glycerol (Fisher, Fair Lawn, NJ). Vials were stored at -80°C until genus confirmation and species identification were performed.

Campylobacter confirmation and speciation

Presumptive *Campylobacter* colonies were removed from the freezer (- 80°C) and streaked for isolation onto MH+SS agar plates. Plates were incubated under microaerophilic conditions at 42°C for 48 h. Approximately 2-3 colonies were transferred to single cell lysis buffer (1 ml Tris-EDTA (1x TE) + 10 µl proteinase K (5 mg/ml)) for DNA extraction (Olah, et al., 2006). A multiplex PCR assay (Yamazaki-Matsune et al., 2007) that identifies six species of *Campylobacter* (*jejuni*, *coli*, *lari*, *fetus*, *upsaliensis* and *hyointestinalis* subsp. *hyointestinalis*) was used. *Campylobacter jejuni* ATCC 33560, *Campylobacter coli* NCTC 36572, *Campylobacter lari* ATCC 35222, *Campylobacter fetus* subsp. fetus ATCC 27374, *Campylobacter upsaliensis* ATCC 49815 and *Campylobacter hyointestinalis* subsp. *hyointestinalis* ATCC 35217 were used as positive controls.

Susceptibility testing of *Salmonella* isolates

The Clinical and Laboratory Standards Institute's (CLSI) micro-broth dilution methods were used to determine minimum inhibitory concentrations of nalidixic acid and ciprofloxacin for all *Salmonella* isolates tested (CLSI, 2008). Susceptibility testing was performed on one *Salmonella* isolate per fecal sample. Stock solutions of nalidixic acid and ciprofloxacin (Sigma-Aldrich, St. Louis, MO) were prepared in sterile, distilled water at a concentration of 1 mg/mL based on the potency of the antimicrobial. Isolates were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 µg/mL for nalidixic acid and 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.098, 0.049, 0.024, and 0.012 µg/mL for ciprofloxacin.

The inoculum was prepared by selecting a single colony from a blood agar plate (Remel) and mixing it with 10 mL Mueller Hinton II broth (Becton–Dickinson, Sparks, MD). The inoculum was incubated for 6 h and the cell turbidity was adjusted to 0.5 McFarland turbidity standard. The culture was diluted (1:100) in Mueller-Hinton broth II for an inoculum of 5×10^5 cfu/ml. A 96-well plate was used to perform the antimicrobial susceptibility test for each antimicrobial and plates were incubated for 24 h at 37°C. *Escherichia coli* (ATCC 25922) was used as a positive control. Isolates were tested in quadruplicate and results were recorded as either "growth" or "no growth" for each well.

The minimum inhibitory concentration (MIC) was the lowest concentration of antimicrobial agent that inhibited the growth of the organism. *Salmonella* isolates were considered susceptible to nalidixic acid if the MIC was ≤ 16 μg/mL and ≤ 0.06 μg/mL for ciprofloxacin. Isolates were considered resistant if the MIC was ≥ 32 μg/mL or ≥ 1 μg/mL, for nalidixic acid and ciprofloxacin, respectively (CLSI, 2013). Further testing was performed for *Salmonella* isolates considered resistant using Sensititre® plates that contained 17 antimicrobials and a semi-automated testing system (Sensititre; TREK Diagnostics, Westlake, OH). Isolates determined to be resistant by microbroth dilution were submitted to the National Veterinary Services Laboratory (NVSL; Ames, IA) for serotyping.

Susceptibility testing of *Campylobacter* isolates

One *Campylobacter* isolate per fecal sample was tested using the micro-broth dilution method as described above at concentrations of nalidixic acid of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 μg/mL and concentrations of ciprofloxacin at 25, 12.5, 6.25, 3.152, 1.56, 0.78, 0.39, 0.195, 0.098 and 0.049μg/mL. Inoculum was prepared for each isolate by streaking pure culture onto MH+SS agar and incubating each plate under microaerophilic

conditions for 48 h at 37°C. One isolate from each plate was transferred to MH agar (without supplements) and incubated for 24-30 h at 42°C. Using a sterile, disposable swab, pure culture was transferred to 3 mL MH II broth. The inoculum was adjusted using a spectrophotometer (OD 600) and MH II broth, so that the inoculum was equivalent to 0.5 McFarland standard. The final inoculum was prepared at a 1:100 dilution using MH II broth with 2.5% lysed horse blood.

Antimicrobial susceptibilities were performed using 96-well microtiter plates (Becton and Dickinson, Franklin Lakes, NJ) and a quality control organism, *Campylobacter jejuni* ATCC 33560, which was used as a positive control. Plates were covered using perforated plate covers and incubated under microaerophilic conditions at 37°C for 48 h. Isolates were tested in quadruplicate and considered susceptible if the MIC was ≤ 16 for nalidixic acid or ≤ 1 for ciprofloxacin. *Campylobacter* isolates were considered resistant if the MIC was ≥ 64 μg/mL for nalidixic acid or ≥ 4 μg/mL for ciprofloxacin (NARMS, 2011).

Statistical Analyses

The number of pens used in this study was determined based on an alpha of 0.05, a statistical power of 0.80 and a presumed delta of 50%. Twelve pens per treatment group, with 12 cattle per pen, were found to be sufficient in finding a significant difference in the proportion of antimicrobial resistance between the two treatment groups.

The proportion of fecal samples that tested positive for *Salmonella* or *Campylobacter* within each pen on each sampling day was determined. A sample was considered positive if at least one isolate was obtained from an individual fecal sample. Differences in the pen-level prevalence of *Salmonella* and *Campylobacter* between treatment groups were evaluated using generalized linear mixed models, which were fitted using a binomial distribution, maximum likelihood estimation, complimentary-log-log link, Kenward-Roger degrees of freedom and

Newton-Raphson and Ridging optimization procedures (Proc GLIMMIX SAS 9.3, SAS Institute, Inc., Cary, NC). The outcome variable was the number of *Salmonella* or *Campylobacter* test positive samples in a pen (events)/number of samples collected per pen (trials), for each sampling week. Treatment (metaphylaxis yes/no), week (n=4), treatment by week interaction and a variable representing the baseline prevalence on study day 0 (prior to antimicrobial administration) for each pen were included in the model as independent variables. A random effect, using an autoregressive covariate structure, was used to account for the lack of independence of repeated measures in pens over time.

Descriptive statistics were used to evaluate the MIC data for both nalidixic acid and ciprofloxacin for *Salmonella* and *Campylobacter* isolates recovered during this study. Since all confirmed isolates were tested in quadruplicate, the mode MIC value was reported for each sample. When only two MIC values were available, the MIC value with the highest concentration was used. If MIC values varied for all four plates, the isolate was re-tested and either the mode or the highest MIC value was reported.

Based on the susceptibility results, proportions of *Salmonella* or *Campylobacter* isolates resistant to either nalidixic acid or ciprofloxacin for each pen, for each sampling week, were determined. Differences in pen-level (nalidixic acid or ciprofloxacin) resistance prevalence estimates, for both *Salmonella* and *Campylobacter* were evaluated for the treatment groups using generalized linear mixed models as described above. The outcome variable was the number of resistant isolates per pen/the number of samples collected per pen for each sampling week. Similar to prevalence, treatment (metaphylaxis yes/no), week (n=4), treatment by week interaction and a variable representing the baseline prevalence on study day 0 (prior to drug administration) for each pen were included in the model as independent variables. A goodness of

fit test was used to examine overall model fit and Pearson and Deviance residuals were used to evaluate potential outliers and influential observations at the pen-level.

Average daily gain (ADG; pen-level) was calculated for both treatment groups by subtracting the weight of the cattle within each pen on study day 0 from the their weight on study day 30 and dividing it by the number of days the cattle were in the study. Average daily gain was calculated with and without cattle that died or were euthanized. The proportion of moribund cattle that were removed from the study pens due to BRD, as well as the number of deaths due to BRD, were calculated and summarized. Cattle performance and health data were analyzed with general and generalized linear mixed models, respectively, for normal and binomial distributions (as described above).

Results

Baseline Body Weights and Fecal Shedding of *Salmonella* and *Campylobacter*

On the day of enrollment, mean body weights of cattle were not significantly different between treatment groups (Table 1). Baseline prevalence of *Salmonella* was numerically higher in cattle assigned to a treated pen (35.4%) compared to cattle assigned to a control pen (29.2%), but this difference was not statistically significant (Table 1). The majority of *Salmonella* isolates belonged to serogroup B (87.1%; 81/93), followed by serogroup E (4.3%; 4/93), serogroup A (2.2%; 2/93) and serogroup C1 (2.2%; 2/93). Four isolates did not test positive for any of the six serogroups evaluated. Susceptibility results revealed that 98.9% (91/92) of the *Salmonella* isolates recovered prior to treatment were susceptible to both nalidixic acid and ciprofloxacin. The MIC of the isolate resistant to nalidixic acid was $\geq$ 32 μg/mL and was identified as serotype Agona. The isolate was tested against 17 human antimicrobials (amoxicillin/clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin,

gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulphamethoxazole) and was resistant to all antimicrobials except for ciprofloxacin, gentamicin, and kanamycin.

Baseline prevalence of *Campylobacter* (prior to antimicrobial administration) tended to be higher in cattle randomly assigned to a treated pen compared to cattle assigned to a control pen (Table 1). The majority of the isolates across both treatment groups were *C. coli* (20/35; 57.1%), followed by *C. jejuni* (13/35; 37.1%) and *C. hyointestinalis* (2/35; 5.7%). The recovery of *C. coli* from the feces of enrolled cattle was significantly higher ($P = 0.04$) in cattle assigned to a treated pen (10.4%; 15/144) compared to a control pen (3.5%; 5/144) prior to treatment administration. No difference was found for *C. jejuni* or *C. hyointestinalis* ($P > 0.05$).

Most *Campylobacter* isolates recovered prior to treatment were resistant to nalidixic acid (23/35; 65.7%) and ciprofloxacin (21/35; 60.0%). Of those nalidixic acid-resistant isolates, 73.9% (17/23) were from cattle assigned to a treated pen and 26.1% (6/23) were from cattle assigned to a control pen. For ciprofloxacin resistant isolates, 71.4% (15/21) were from the feces of cattle assigned to treated pens and 28.6% (6/21) were from the feces of cattle assigned to control pens. Mean pen-level (nalidixic acid) resistance prevalence, prior to treatment, was significantly higher for cattle assigned to treated pens (11.8%) when compared to cattle assigned to control pens (4.2%; $P = 0.03$) and also tended to be different for ciprofloxacin ($P = 0.06$) for treated pens (10.4%) versus control pens (4.2%), despite the fact that all of these samples were collected prior to treatment administration.

Post-Antimicrobial Administration - *Salmonella* Prevalence

Overall sample-level prevalence of *Salmonella* across both treatment groups, post-treatment, was 4.3% (46/1076). *Salmonella* prevalence did not differ significantly between the

treated versus the control pens of cattle ($P = 0.80$; Figure 1). The treatment by week interaction was not statistically significant ($P = 0.41$), however, prevalence did vary by study week ($P < 0.01$), with *Salmonella* prevalence decreasing over time for both treatment groups. The majority of the *Salmonella* isolates recovered, post-treatment, belonged to serogroup B (67.4%; 31/46), followed by serogroup E (8.7%: 4/46) and serogroup C1 (4.3%; 2/46). Nine isolates did not test positive for any of the six serogroups tested.

Post-Antimicrobial Administration - *Salmonella* Susceptibility

Of the 46 isolates confirmed to be positive for *Salmonella*, 43 (93.5%) were viable and tested. All isolates tested were susceptible to both nalidixic acid (MIC ≤ 16 μg/mL) and ciprofloxacin (MIC ≤ 0.06 μg/mL). Most of the isolates had a MIC of 6.25 μg/mL (83.7%; 36/43) for nalidixic acid and a MIC of 0.024 μg/mL for ciprofloxacin (74.4%; 32/43). No isolate was above the breakpoint for either antimicrobial tested.

Post-Drug Administration - *Campylobacter* Prevalence

Overall *Campylobacter* sample-level prevalence across all study pens, post-treatment was 12.5% (135/1076). There was no significant difference ($P = 0.65$) in *Campylobacter* prevalence in pens of cattle treated with enrofloxacin compared to the non-treated, control pens (Figure 2). There was no significant treatment by week interaction ($P = 0.62$), nor was there a significant difference by study week ($P = 0.10$).

Of the *Campylobacter* isolates recovered, *C. coli* accounted for 78.5% (106/135) of the isolates and *C. jejuni* accounted for 21.5% (29/135) of the isolates. Model-adjusted pen-level prevalence of *C. coli* was significantly higher in treated pens when compared to the control pens ($P = 0.04$). There was no significant treatment by week interaction ($P = 0.88$), though there was a significant week effect ($P < 0.01$). Mean *C. jejuni* prevalence was not significantly different (P

= 0.70) in the treated pens compared to the control pens of cattle, nor was there a significant treatment by week interaction ($P = 0.80$) or a week effect ($P = 0.07$), post-treatment.

Post-Antimicrobial Administration - *Campylobacter* Susceptibility

All 135 *Campylobacter* isolates obtained post-treatment were viable and subjected to susceptibility testing. Nalidixic acid and ciprofloxacin MICs for these isolates ranged from 1.56 μg/mL to ≥ 100 μg/mL for nalidixic acid and 0.049 μg/mL to ≥ 6.25 μg/mL for ciprofloxacin. Of the isolates tested, 81.5% (110/135) were resistant to both nalidixic acid (MIC ≥ 64 μg/mL) and ciprofloxacin (MIC ≥ 4 μg/mL). One-hundred and four of these isolates were *C. coli* and the remaining 31 isolates were *C. jejuni*. There were significantly more *C. coli* 98.1% (102/104) than *C. jejuni* 28.1% (8/31) isolates resistant to both antimicrobials ($P < 0.01$). All other isolates (18.5%; 25/135) were susceptible to both antimicrobials (i.e., there were no isolates resistant to just one drug).

Pen-level prevalence of resistant *Campylobacter* did not differ significantly ($P = 0.61$) between the treated and control pens for either drug tested (Figure 3), and there was no evidence of a significant treatment by study week interaction ($P = 0.85$). There was, however, a significant week effect as the mean prevalence of resistant *Campylobacter* isolates decreased over time for both treatment groups ($P = 0.05$).

Cattle Health and Performance

Within the four weeks post-treatment, 10 clinically ill cattle were removed from the treated pens and 17 cattle were removed from the control pens. All cattle were removed due to clinical signs of BRD, with the exception of two cattle that were removed from control pens due to lameness. The BRD morbidity was not significantly different among treatment groups (Table 2). Three mortalities occurred during this study (1 from treated, 2 from control pens). Necropsy

results indicated that all deaths were attributable to BRD. There was a statistically significant difference in mean ADG for cattle in the treated and control pens (Table 2). However, when cattle that died were removed from the weight gain analysis, the ADG means were not significantly different (Table 2).

Discussion

This study demonstrated no evidence that enrofloxacin metaphylaxis impacts the prevalence of *Salmonella* and *Campylobacter* in the feces of feedlot cattle, or the prevalence of resistance of these organisms to nalidixic acid or ciprofloxacin. Although not associated with treatment group, the overall prevalence of quinolone resistance in *Campylobacter*, particularly *C. coli*, was quite high (over 80% during the study period). In contrast, the overall prevalence of *Salmonella* resistance to nalidixic acid or ciprofloxacin was very low (0% during the study period). Although the epidemiology and risk factors for antimicrobial resistance in *Campylobacter* and *Salmonella* in feedlot cattle must be further investigated, based on this study, there is no evidence that enrofloxacin metaphylaxis is a significant driver of the prevalence and susceptibility of these organisms.

Considering the study season, location and cattle type, prevalence of *Salmonella* and *Campylobacter* in cattle during enrollment was consistent with other reports (Galland *et al*., 2000; Kunze *et al*., 2008; Sanad *et al*., 2011). *Salmonella* prevalence did not differ significantly when comparing treated to non-treated pens of cattle. These findings were similar to results reported from a previous study where the number of fluoroquinolone treatments administered to cattle at five commercial feedlots was not associated with *Salmonella* prevalence (Smith *et al*., 2016). *Salmonella* prevalence did, however, differ across study weeks with prevalence decreasing over time. It is possible that being exposed to other cattle at the sale barn, the stress of

being transported to the research site, as well as acclimation to a new diet and environment had an impact on overall prevalence of *Salmonella*. Research has shown that the transportation of cattle can be a potential stressor for the increased shedding of *Salmonella* spp. (Barham *et al*., 2002). Type of diet has also been shown to be associated with the culture-positive status of cattle tested for *Salmonella* (Green *et al*., 2010). With prevalence in the current study being 3 to 4 times higher at study initiation than at the end of the study, it seems plausible that initial production or transportation stresses affected the prevalence of *Salmonella* fecal shedding over time.

On the day of enrollment, prevalence of *Salmonella* resistance was low, which was expected given that cattle had no history of previous exposure to a fluoroquinolone (inclusion criteria). The low prevalence of quinolone-resistant *Salmonella* isolated from cattle is consistent with the literature (Beach *et al*., 2002; FDA, 2015). The single *Salmonella* isolate that was resistant to nalidixic acid prior to treatment was also found to be multidrug-resistant (14 human antimicrobials). It is not unusual for *Salmonella* isolates from beef cattle to be either pan-susceptible or resistant to multiple antimicrobials (Dargatz *et al*., 2000; Khaitsa *et al*., 2007; Krueger *et al*., 2014).

All *Salmonella* isolates recovered, post-treatment, were susceptible to both nalidixic acid and ciprofloxacin, with MICs well below the breakpoint for both antimicrobials. These results are consistent with other research, where the isolation of quinolone-resistant *Salmonella* from beef cattle was either low or not detected (Dargatz *et al*., 2000; Beach *et al*., 2002; Kunze *et al*., 2008). Fluoroquinolone metaphylaxis did not significantly affect prevalence of resistant organisms isolated from cattle feces in this study, which is consistent with a previous observational study where previous fluoroquinolone use for BRD treatment within pens of cattle

was not associated with the susceptibility of *Salmonella* to human quinolones (Smith *et al*., 2016).

Data regarding the prevalence of *Campylobacter* isolated from US beef cattle are scarce. The current study showed that the prevalence of *Campylobacter* across study weeks was highly variable for cattle in both the treated and non-treated control pens and that metaphylaxis treatment with a fluoroquinolone was not associated with prevalence. As previously discussed for *Salmonella* sp., it is possible that acclimation to a new diet and environment may have contributed to the variability in *Campylobacter* spp. prevalence (Sproston *et al*., 2011). *Campylobacter jejuni* is reported as the most prevalent *Campylobacter* species isolated from cattle and is the most common cause of human campylobacteriosis in the U.S. (Altekruse *et al*. 1999; Englen *et al*. 2005; FDA, 2014; Gaudreau *et al*. 2014). However, prevalence of *C. jejuni* in this study was fairly low and accounted for less than 25% of the *Campylobacter* isolates recovered. The reason for low prevalence of this *Campylobacter* species in our cattle population is unknown. *Campylobacter coli* was the most prevalent *Campylobacter* species isolated in this study, with significantly more *C. coli* isolates recovered from the treated pens of cattle compared to the control pens. *Campylobacter coli* are typically isolated from swine and poultry and tend to have higher antimicrobial resistance prevalence when compared to *C. jejuni* (Moore *et al*. 2006). A similar trend was noted in the current study with significantly more *C. coli* isolates showing resistance to both nalidixic acid and ciprofloxacin when compared to *C. jejuni*. *Campylobacter* mutants found to be highly resistant to quinolone antimicrobials typically have a single-point mutation in the *gyrA* gene, which is part of the quinolone drug binding site (Luo *et al*. 2003). This single step mutation keeps quinolone antimicrobials from binding, causing clinically relevant levels of resistance to quinolone antimicrobials (Wang *et al*. 1993).

Studies of the effects of fluoroquinolone treatment on the prevalence of fluoroquinolone-resistant *Campylobacter* in swine and poultry have shown that resistance prevalence increases rapidly after treatment and persists for several days in both the animal and the environment (McDermott *et al*. 2002; Usui *et al*. 2014). However, results from the current study showed that the mean *Campylobacter* resistance prevalence in cattle feces within a pen was not significantly impacted by fluoroquinolone use, and declined over time for both treatment groups to less than 10% by the end of the 4 week study period. Assuming this decline reflects what is commonly found in commercial finishing feedlots after the Baytril® 28-day withdrawal time has been met, cattle should have minimal resistance prevalence at harvest. With the National Antimicrobial Resistance Monitoring System (NARMS) no longer reporting *Campylobacter* due to low prevalence of this organism in ground beef, this assumption is plausible (FDA, 2015).

Prevalence of *Campylobacter* resistance at baseline (prior to drug administration) was rather high with > 60% of the isolates resistant to at least one of the antimicrobials tested. These results were unexpected, since cattle had no history of being administered a fluoroquinolone prior to enrollment. However, a similar finding was noted in a longitudinal study examining the prevalence of antimicrobial-resistant *Campylobacter* strains isolated from conventional and antibiotic-free swine (Quintana-Hayashi and Thakur, 2012a). Authors reported a high prevalence of multidrug-resistant (MDR) *Campylobacter* isolates obtained from antibiotic-free swine and their environment, with the majority of those isolates identified as *C. coli*. In a follow-up study, researchers performed multilocus sequence typing (MLST) on the MDR *C. coli* isolates and found that pigs that did not carry MDR isolates at the farm, acquired the resistant strains at the slaughter plant (Quintana-Hayashi and Thakur, 2012b). It is possible that cattle in the current study acquired resistant isolates either at the sale barn or during transit to the study site.

However, the time of acquisition of the resistant organisms could not be determined in the present study as the cattle were not sampled prior to arrival to the study site.

More than half of the cattle were shedding quinolone-resistant *Campylobacter* on the day of enrollment. As there were no data on resistance prevalence for these cattle prior to study initiation, it was not accounted for in the study design, and the resulting randomization process unfortunately resulted in a difference in the prevalence of animals shedding resistant organisms between treated and control pens. While the statistical analysis of treatment effects accounted for the baseline prevalence, the large number of cattle shedding resistant *Campylobacter* prior to enrollment may have influenced the overall *Campylobacter* prevalence or resistance prevalence throughout the study. As noted by Zhang *et al*. (2006), resistant isolates can have a fitness advantage compared to susceptible isolates, even in the absence of antimicrobial selection pressure. This may have influenced the overall resistance prevalence during the four week study period, even though resistance did not differ significantly between treatment groups (Figure 3).

No significant differences were observed when evaluating the pen-level prevalence of quinolone-resistant *Campylobacter* isolates recovered from the feces of cattle administered fluoroquinolone metaphylaxis compared to non-treated cattle. Though MIC values were generally high throughout the study, they were consistently high for isolates recovered from both treatment groups. Adding monensin to the feed of both treatment groups may have influenced the overall resistance prevalence of *Campylobacter* spp. However, ionophores have a different mode of action than fluoroquinolones, and bacteria typically adapt to ionophores rather than mutate or acquire resistance genes (Russell and Houlihan, 2003). With *C. coli* being the most prevalent species isolated, an overall high prevalence of resistance to quinolones could be expected (Moore *et al*. 2006; Gaudreau *et al*. 2014).

Although cattle at high risk for BRD were purposely selected for this study, overall BRD morbidity and mortality throughout the study were relatively low. Only 8.7% (25/288) of the cattle enrolled in the study were removed due to clinical BRD, with no statistical differences detected between treatment groups. Since the impacts of treatment on cattle health was not one of our primary objectives, and thus sample size was not optimized for this outcome, making inferences based on these results could result in Type II error. Data collected on BRD morbidity and mortality were merely recorded as an indicator of clinical disease and a descriptor of the cattle in the study population. The impact of BRD also can be reflected in performance measures such as ADG, as shown by Cusack *et al.* (2007). In the current study, ADG was significantly higher in the treated pens of cattle compared to the control pens when all cattle were included in the analysis, but those results were apparently driven by a disproportional number of mortalities in each group (Table 2). Differences in cattle performance between cattle given metaphylaxis versus untreated controls were expected. Several studies have shown that the type and the timing of BRD treatment can impact cattle performance over time (Bateman *et al*., 1990; Babcock *et al*., 2009 and 2010).

In conclusion, our results indicate that metaphylaxis administration of the fluoroquinolone enrofloxacin to beef cattle for the control of BRD did not affect prevalence and susceptibility of *Salmonella* and *Campylobacter* when compared to non-treated cattle. A relatively large proportion of the cattle enrolled in this study were shedding *Salmonella* and *Campylobacter* prior to study initiation. Furthermore, a high percentage of the *Campylobacter* isolates recovered from the feces of these cattle were resistant to quinolones upon the start of the study, even though cattle were not previously exposed to a fluoroquinolone. Despite cattle harboring quinolone-resistant *Campylobacter* prior to enrollment, metaphylaxis use of a

fluoroquinolone did not increase the prevalence of resistance after administration, and in fact, the prevalence of resistance declined (in both treatment groups) throughout the study period.

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Disclosure Statement

The first author (A. Smith) is a graduate student of Kansas State University and an employee of Bayer Animal Health who produce a commercially licensed fluoroquinolone for the treatment and control of BRD.

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Table 4-1: Model-adjusted mean body weight and pathogen prevalence in feces of cattle at the time of enrollment (pre-treatment) for cattle randomized to either the Baytril® 100 treated pens, or the non-treated, control pens.

Variable, (Unit)	Treated pens	Control pens	*P*-value[b]
Animal (Pens)	144 (12)	144 (12)	-
Arrival Body Weight (kg)			0.98
Mean[a]	271.5	271.4	
95% Confidence Interval	266.2 – 276.8	266.1 – 276.7	
Salmonella Prevalence (%)			0.27
Mean[a]	35.4	29.2	
95% Confidence Interval	27.7 – 44.0	22.0 – 37.6	
Campylobacter Prevalence (%)			0.06
Mean[a]	16.0	8.33	
95% Confidence Interval	10.6 – 23.4	4.6 – 14.5	

[a]Model-adjusted means

Figure 4-1: Model-adjusted mean pen-level *Salmonella* prevalence (and 95% confidence intervals) by week post-treatment, for cattle in Baytril® 100 metaphylaxis treated and non-treated, control groups. There was no significant interaction between time and treatment group, or a significant difference between treatment groups, but prevalence across both groups differed over time.

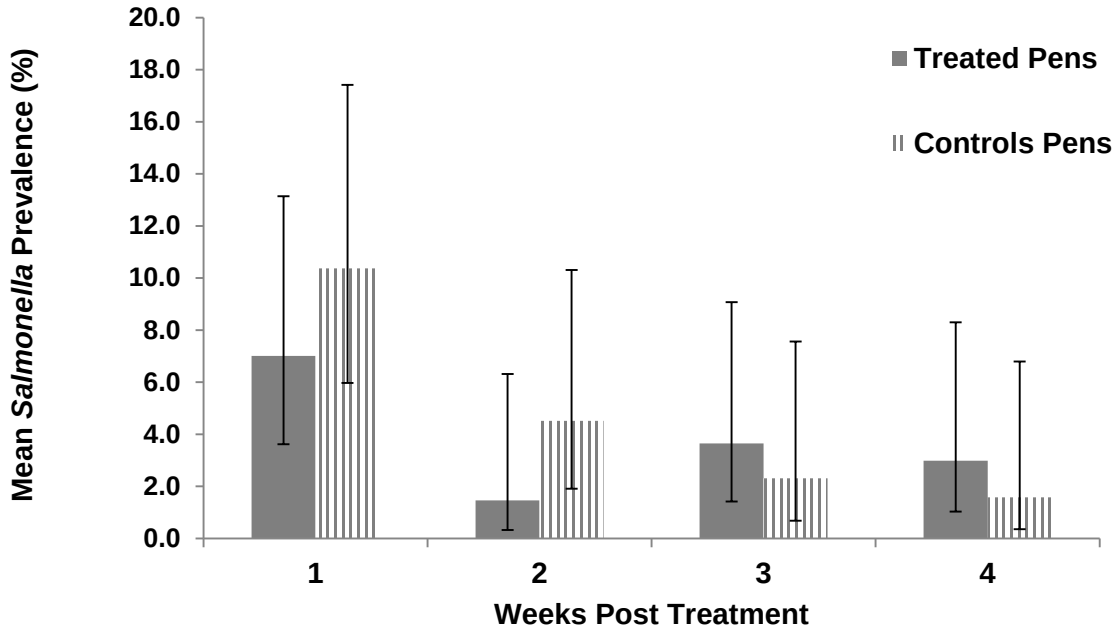

Figure 4-2: Model-adjusted mean pen-level *Campylobacter* prevalence (and 95% confidence intervals) by week post-treatment, cattle in Baytril® 100 metaphylaxis treated and non-treated, control groups. There was no significant interaction between time and treatment group, or significant differences between treatment groups or among weeks.

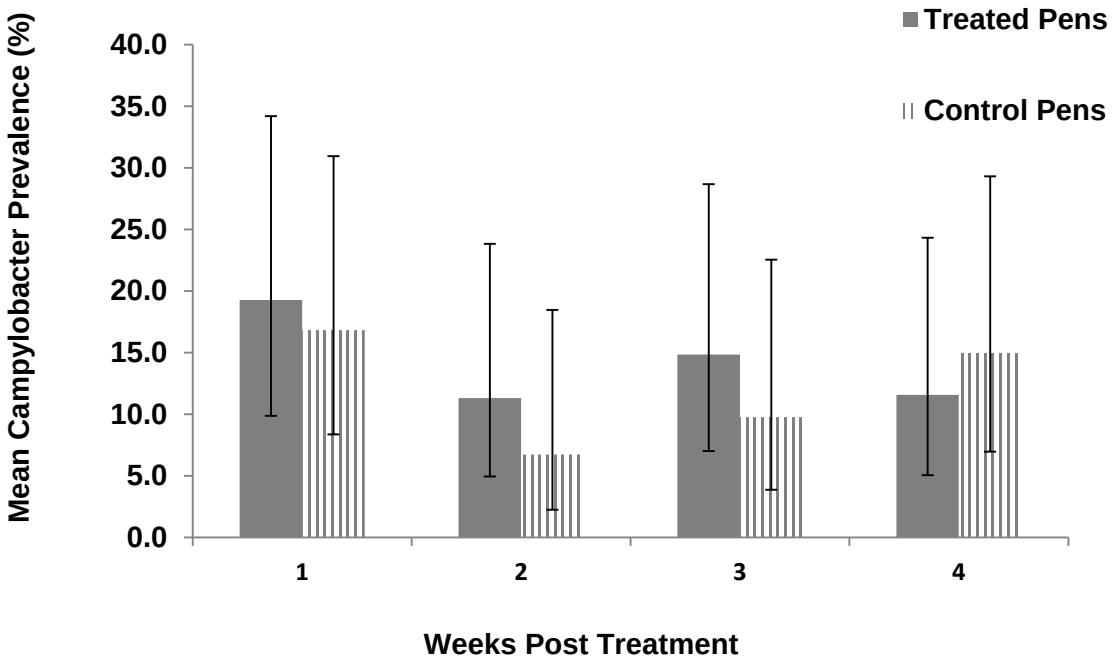

Table 4-2: Bovine respiratory disease (BRD) and average daily weight gain (ADG) as primary health and performance outcomes for cattle randomized to either Baytril® 100 metaphylaxis treated pens or a non-treated, control pens.

Variable, (Unit)	Treated pens	Control pens	*P*-value
Animal (Pens)	144 (12)	144 (12)	-
BRD morbidity (%)			0.31
Mean[a]	6.94	10.4	
95% Confidence Interval	3.6 – 12.8	6.20 – 17.0	
BRD mortality (%)			0.58
Mean[a]	0.69	1.39	
95% Confidence Interval	0.1 - 5.3	0.3 - 5.8	
ADG (Deads in) (kg)			0.05
Mean[a]	1.4	1.2	
95% Confidence Interval	1.3 - 1.5	1.1 - 1.3	
ADG (Deads out) (kg)			0.07
Mean[a]	1.4	1.3	
95% Confidence Interval	1.3 - 1.5	1.2 - 1.4	

[a]Model-adjusted means

Chapter 5 - Conclusion

For over 90 years, antimicrobials have been indispensable tools for decreasing both morbidity and mortality associated with human and animal infectious diseases. They continue to remain effective against most infectious agents, yet prolonged, intense, and sometimes inappropriate use has led to the emergence of multiple antibiotic-resistant, zoonotic bacterial pathogens. Because of this, several U.S. and global agencies, along with the scientific community around the world, are working to assess the effects of using antimicrobials in food animals. The result of this collaboration contributes data needed to assure that the public health is protected and that animal health needs are being met.

Data presented in the previous three chapters will aid in the continued effort to better understand the outcomes associated with the judicious use of antimicrobials in the beef cattle industry. Judicious use of therapeutic antimicrobials is an approach defined by the U.S. Food and Drug Administration (FDA) as a way to "maximize therapeutic efficacy and minimize the selection of resistant microorganisms" (FDA, 2016). Administration of antimicrobials to food animals at low concentrations has been shown to increase weight gain and reduce the spread of stress-associated diseases (Butaye *et. al*, 2003). Though there are few studies that show a true link between the number of antimicrobials administered to livestock and the increased prevalence of resistant pathogenic organisms shed by those animals, this practice has been labeled as a potential driver for the emergence of resistant microorganisms.

In 2013, the FDA released a guidance document that provided recommendations for medically important antimicrobial drug use in veterinary medicine (FDA, 2013). This document states that the FDA promotes the use of antimicrobials as therapy, control and prevention of diseases in animals; however, it also states that the U.S. government will no longer approve the

use of antimicrobials as a means of “increasing weight gain” or “improving feed efficiency.” Veterinary oversight is recommended for all approved drugs, and the microbiological effects on bacteria of human health concern should be evaluated for all new animal drugs, especially drugs that are critically important to human health (FDA, 2013).

As previously stated, fluoroquinolones are broad-spectrum antibiotics that play an important role in treating serious infections in humans. Fluoroquinolones are also used to treat bovine respiratory disease in cattle. Because this drug plays such a critical role in both human and animal medicine, the continued effort to evaluate its use was deemed necessary.

Evaluating the prevalence and quinolone resistance prevalence of the human enteric pathogens, *Salmonella* and *Campylobacter*, in beef cattle administered a fluoroquinolone for the treatment of bovine respiratory disease (BRD), was a way of assessing the impact of judicious use of this critically important drug. Our studies indicated that the use of a fluoroquinolone in feeder cattle was not associated with the pen-level prevalence of *Salmonella* and *Campylobacter*, nor was it associated with nalidixic acid and ciprofloxacin resistance prevalence for either organism. In the observational study, the number of fluoroquinolone treatments administered within each pen for BRD treatment was not significantly associated with the prevalence or resistance prevalence of *Salmonella* and *Campylobacter*, nor was it associated with factors such as metaphylaxis use (with any drug) or gender. In the randomized clinical trial, metaphylactic use of a fluoroquinolone in high risk pens of cattle did not significantly increase prevalence or quinolone resistance prevalence when compared to untreated pens of cattle, nor did it significantly alter the quinolone susceptibilities of *Salmonella* or *Campylobacter* isolated from cattle within each pen.

Prevalence of *Salmonella* isolated from the feces of cattle for both studies was fairly low (38% for Trial #1 and 4.3% for Trial #2), and quinolone-resistance prevalence was negligible, with only two isolates resistant to nalidixic acid. *Campylobacter* prevalence was slightly higher in fecal samples collected for the observational study (27.2%) compared to the randomized clinical trial (12.5%). It was fairly common for *Campylobacter* isolates to be resistant to nalidixic acid and ciprofloxacin for both studies, particularly if the isolate was *C. coli*. Greater than 60% of the *Campylobacter* isolated from the feces of cattle with no known exposure to a fluoroquinolone were resistant to both human quinolones. With cattle being a major reservoir of this zoonotic pathogen, continued monitoring of the development of quinolone-resistant *Campylobacter* in cattle is necessary to reduce the human food safety risk.

As we continue to monitor medically important antimicrobial drug use in food-producing animals, efforts are being made by both veterinarians and human physicians to help mitigate the antimicrobial resistance dilemma. Antimicrobial alternatives such as: antibacterial vaccines, immunomodulators, bacteriophages and the use of edolysins and antimicrobial peptides are all being examined for their ability to reduce pathogenic organisms in humans and animals (Cheng *et al*., 2014). Prebiotics and probiotics are currently being used in livestock to enhance the gastrointestinal microbial environment and to out-compete the pathogenic microorganisms for both space and nutrients (Uyeno *et al*., 2015). Human medical doctors, as well as veterinarians, are culturing pathogens whenever possible to make sure the appropriate therapy is administered, and patients are being advised to complete the full course of therapy when an antimicrobial is prescribed (Madigan, 2006). Increased efforts are being made to preserve the efficacy of our current human antimicrobial therapy and at the same time promote the continued judicious use of antimicrobial drugs in our animal production system.

Future research is still needed, however, to further explore the antimicrobial resistance mechanisms used by pathogenic organisms, to explore the risk factors associated with the transmission of these resistant pathogens and to develop intervention strategies to keep antimicrobial resistance at a minimum. Further investigation will facilitate the development of strategies to maintain and improve antimicrobial use in animal health, while addressing antimicrobial resistance concerns related to human health.

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