TREATMENT STRATEGIES IMPACTING CEFTIOFUR RESISTANCE AMONG ENTERIC BACTERIA IN CATTLE

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

A randomized controlled field trial was designed to evaluate the effects of two treatment strategies on ceftiofur and tetracycline resistances in feedlot cattle. The two strategies consisted of administering ceftiofur crystalline-free acid administration (CCFA) at either one or else all of the steers within a pen, and subsequent feeding/not feeding of therapeutic doses of chlortetracycline. Both strategies were hypothesized to reduce ceftiofur resistance. The effects of treatment strategies were evaluated *via* metagenome-based and culture-based assays. In this 26-day study, 176 steers were allocated to 16 pens of 11 steers each. The two strategies were randomly assigned to the pens in a two-way full-factorial manner resulting in four treatment groups. The $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), *tet*(B), and 16S rRNA gene copies/g feces were quantified using qRT-PCR from fecal community DNA. Antimicrobial susceptibility profiles were determined using microbroth dilution technique from the non-type-specific (NTS) *E. coli* isolates (n=1,050). The NTS *E. coli* DNA was screened for the presence of $bla_{\text{CMY-2}}$, *tet*(A), and *tet*(B) genes. Pens in which all the steers received CCFA treatment showed an increase in $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$ $\log_{10}$ gene copies/g feces and in the proportion of ceftiofur-resistant and $bla_{\text{CMY-2}}$ positive NTS *E. coli*. This was in contrast to the pens where only one animal received CCFA treatment. There was a significant decrease in quantities of tetracycline genes in community DNA in pens where all animals received CCFA treatment. In contrast to metagenome-based assay results, culture-based assays indicated an increase in the proportion of tetracycline resistant NTS *E. coli* upon CCFA treatment. Thereafter, chlortetracycline administration led to rapid expansion both of ceftiofur ($bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$) and tetracycline [*tet*(A) and *tet*(B)] $\log_{10}$ gene copies/g feces. Chlortetracycline treatment delayed the return of the ceftiofur resistance prevalence to baseline among NTS *E. coli* and thus did not lead to the hypothesized decrease in ceftiofur resistance. Our data suggest that chlortetracycline use is contraindicated when attempting to avoid expansion of resistance to critically important 3$^{\text{rd}}$ generation cephalosporins in feedlot cattle. Further studies are required to better establish the animal-level effects of co-housing antimicrobial-treated and non-treated animals together at varying ratios on the levels of antimicrobial resistance.

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Table of Contents

List of Figures

List of Tables

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Chapter 1
Introduction

1.1. Background

Antimicrobial drug resistance is widely regarded to be a major public health threat [1]. The science ministers of the G8 countries in June 2013 jointly declared "…antimicrobial drug resistance as a major health security challenge of the 21st century" [2]. The Centers for Disease Control and Prevention (CDC) in 2011 reported the estimated health care costs, and other societal costs, solely attributed to antimicrobial drug resistance in the United States to be approximately $20 billion per year and $34 million, respectively [3]. In addition, antimicrobial drug resistance is estimated to contribute more than 8 million additional hospital days incurred by patients in the United States [3]. There has been no new class of antimicrobial approved in more than 40 years to treat Gram negative bacillus infections [4]. Therefore, it is essential that we control antimicrobial drug resistance and preserve the efficacy of the existing antimicrobials used both in animal and human medicine. This is particularly important in case of those drugs that have been identified as being critically important for human medicine [5].

Third-generation cephalosporins (e.g., ceftriaxone) are presently classified as critically important drugs for human use. Ceftiofur, an animal-only antibiotic, belongs to the same class as ceftriaxone and is extensively used in veterinary medicine and animal husbandry. An association between ceftiofur usage in animals and ceftriaxone resistance among pathogenic bacteria in humans has been speculated upon in the past [6]. In order to safeguard animal and human health, the Food and Drug Administration (FDA) of the U.S. Department of Health and Human Services, in April 2012 released an order of prohibition restricting certain extra-label uses of

ceftiofur in animal agriculture [7]. There is an urgent need to better understand the complexity of resistance ecology in terms of selection and co-selection of resistance determinants or isolate phenotypes under specific antimicrobial selection pressures; further, there must be well-designed and thoroughly tested animal- and farm-level intervention strategies that can help control this global problem. This work presented in this dissertation is focused on testing the multivariate impacts of two treatment strategies on ceftiofur resistance. Both culture-based and metagenome-based approaches were utilized in an effort to understand these resistance mechanisms both at genotypic and phenotypic levels.

Differential ceftiofur crystalline-free acid (CCFA: Excede®, Zoetis Animal Health, NJ, U.S.A.) treatment, administered to either one or else all the steers within a pen, was the first strategy which was evaluated. Animals within a pen are expected to exchange bacterial strains and resistant determinants (i.e., horizontal transmission) amongst each other *via* fecal-oral transmission (intermediated through the local environment) [8]. Untreated animals in a pen are likely to serve as a ready source of antimicrobial-susceptible bacterial strains for the treated animals in the same pen; these latter animals are far more likely to be harboring resistant bacteria during the post-treatment period. Some scientists believe that resistance genes are associated with a fitness cost to the host bacterium, at least during the period immediately following the introduction of an antimicrobial to the market [9-10]. Readily available susceptible bacteria, from untreated pen mates, may outcompete and help to replace the resistant bacteria and promote re-colonization of the baseline microflora of the treated animal.

The administration of chlortetracycline (CTC: Aureomycin®, Alpharma, Bridgewater, NJ) following treatment with long-acting ceftiofur (CCFA) was the second treatment strategy. This strategy was derived from the results of a previous study which demonstrated that

chlortetracycline treatment selected for a subset of the *E. coli* population that was resistant to tetracycline over the subpopulation that was co-resistant to tetracycline and ceftiofur [11]. Hence, the proportion of ceftiofur-resistant *E. coli* significantly decreased upon chlortetracycline administration. The present study was designed to evaluate this selective mechanism both among *E. coli* (qualitative phenotypic and genotypic analyses) and in the fecal metagenome as a whole (quantitative genotypic analysis only).

1.2. Study objectives

The objective of this randomized and controlled field trial was to investigate the effects of two treatment strategies on both ceftiofur and tetracycline resistance as measured qualitatively at both the phenotypic and genotypic isolate level, and quantitatively at the genotypic metagenomic level. The two treatment strategies were:

1. House either all CCFA (ceftiofur crystalline-free acid)-treated animals, or else one treated amongst all other untreated animals, together within a pen, mimicking metaphylaxis and individual therapeutic regimens, respectively.
2. Feed therapeutic doses of CTC (chlortetracycline) following the earlier treatment with a long-acting ceftiofur formulation (CCFA).

Metagenome-based (Chapter 3) as well as culture-based assays (Chapter 4) were utilized to evaluate the impact of these two strategies on both ceftiofur and tetracycline resistances within the bovine fecal microbiome.

Specific Objectives

1. Perform gene quantification to estimate genotypic changes in enteric microbial populations as measured by variations in target gene copies [$bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), *tet*(B), and 16S rRNA genes] in the fecal community DNA; before, during, and after the influence of these treatments. Absolute target gene copies [$bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), *tet*(B)] as well as these same quantities relative to 16S rRNA gene copies were each assessed separately.
2. Determine phenotypic antimicrobial susceptibility to a panel of 15 different antimicrobials among non-type-specific (NTS) *E. coli* isolates by estimating and interpreting the minimum inhibitory concentrations. In addition, screen the NTS *E. coli* DNA for the presence of *tet*(A), *tet*(B), and $bla_{\text{CMY-2}}$ genes. Further, evaluate the associations between treatment strategies, antimicrobial susceptibility data, and resistance genes (both quantitative and qualitative presence) to better understand the phenomenon observed in this as well as the earlier trial by Platt *et al.* (2008) [11].

We hypothesized that these two treatment strategies would expedite the return of ceftiofur resistance to baseline (pre-treatment) levels among both fecal metagenomic DNA and commensal NTS *E. coli* as measured at the pen-level.

Chapter 2
Literature Review

2.1. Animal agriculture and antimicrobial usage

An animal health report released by the United States Department of Agriculture (USDA) in March 2013 provided an overview of U.S. livestock and summary statistics on major commodities for the year 2011 [12]. According to the report, at the end of that year there were estimated to be approximately 100 million cattle (both beef and dairy) in the United States. As of January 1, 2012, beef cattle constituted 76.4% of the total cattle inventory. Economically, beef cows and calves were the largest commodity with the value of production being $45.2 billion, followed by dairy, poultry, hogs, and sheep [12].

Beef calves, after being weaned at approximately 7 months of age, generally are transported to backgrounder or stocker farms and thereafter to feedlots (or, feedyards) to be 'finished' for slaughter. Cattle are provided with high-energy rations in order to attain an acceptable size and carcass finish for slaughter. Their stay in the feedlot lasts for an average of 6 months, for those placed at around a year of age [13]. Antimicrobials are used in food-producing animals for reasons such as treatment, disease prevention, disease control, and growth promotion/improved feed efficiency. Pneumonia and diarrhea caused by bacterial and viral agents both may cause significant morbidity and mortality. In addition, prevention, control and treatment of conditions like liver abscess and other respiratory diseases is often required during cattle feeding period. Finally, antibiotics and other antimicrobials have been shown to increase weight gain and feed efficiency, even at very low sub-therapeutic doses [13]. A recent report (released March 2013) by USDA on management practices of feedlots presented summary

statistics on antimicrobial usage in feedlots with a capacity of 1,000 head or more [14]. According to the report, 73.4% of feedlots used at least some antimicrobials in feed for their cattle, either for health or production purposes [14]. A previous 1999 survey report by USDA indicated that injectable antimicrobials that were commonly used metaphylactically to prevent shipping fever included tilmicosin (70.3%), oxytetracycline (31.9%), florfenicol (22.1%), penicillins (9.8%), and ceftiofur (8.1%). Note that at that time CCFA (longer-acting ceftiofur) was not yet approved. Chlortetracycline, tylosin, oxytetracycline, and chlortetracycline/sulfamethazine were administered in 51.9%, 20.3%, 19.3%, and 16.8% of the feedlots in either feed or water and for health or growth promotion purposes, respectively. There were more than 50% feedlots that used florfenicol (54%), tilmicosin (51.7%), or tetracyclines (50.8%) for treating bovine respiratory disease complex (BRD) as a part of initial first-line therapy. Feedlots also used cephalosporins (38.1%), fluoroquinolones (32.1%), penicillins (31.1%), and macrolides (17.4%) for initial treatment of respiratory diseases [15].

In 2006, the FDA Center for Veterinary Medicine (FDA-CVM) reported that, since the inception of the agency, they had approved approximately 700 drug products for use in animal agriculture in the United States. These drugs were approved to treat 2006 U.S food-animal population; the animals included: 8.8 billion chickens, 272 million turkeys, 97 million cattle, 59 million pigs, and 7 million sheep along with a number of other minor food-producing animal species. Additionally, more than 700 drugs products have also been approved for use in companion animals. The 2006 U.S companion animal population has been estimated to consist of 60 million dogs, 75 million cats, and 5 million horses [16]. Many of the major antimicrobial drugs that are currently approved and marketed for animal agriculture are listed in Table 2-1 [17].

Antimicrobial Class	Marketed Animals Drugs	Human Medicine
Aminocoumarins*	Novobiocin	-
Aminoglycosides****	Dihydrostreptomycin	-
	Gentamicin	H
	Neomycin	H
	Spectinomycin	H
	Streptomycin	H
Amphenicols***	Florfenicol	-[a]
Cephalosporins†****	Ceftiofur	-[a]
	Cephapirin	-
Diaminopyrimidines*	Ormetoprim	-
Fluoroquinolones****	Danofloxacin	-[a]
	Enrofloxacin	-[a]
Glycolipids*	Bambermycins	-
Ionophores*	Laidlomycin	-
	Lasalocid	-
	Monensin	-
	Narasin	-
	Salinomycin	-
Lincosamides***	Lincomycin	H
	Pirlimycin	-[a]
Macrolides****	Erythromycin	H
	Gamithromycin	-
	Tilmicosin	-[a]
	Tulathromycin	-
	Tylosin	-[a]
Penicillins†****	Amoxicillin	H
	Ampicillin	H
	Cloxacillin	H
	Penicillin	H
Pleuromutilins***	Tiamulin	-
Polypeptides**	Bacitracin	H
Quinoxalines*	Carbadox	-
Streptogramins***	Virginiamycin	-[a]
Sulfas***	Sulfadimethoxine	-[a]
	Sulfamerazine	-[a]

	Sulfamethazine	-[a]
	Sulfaquinoxaline	-[a]
Tetracyclines***	Chlortetracycline	-[a]
	Oxytetracycline	H
	Tetracycline	H

Table 2-1 Antimicrobial classes and products marketed for use in food-producing animals in the United States as of 2013 and the corresponding usage in human medicine
Adapted from [5, 17-18]
****: Critically important antimicrobials
*** : Highly important antimicrobials
** : Important antimicrobials
* : Unclassified
† : 1st and 2nd generation cephalosporins are classified as highly important antimicrobials whereas 3rd and 4th generation antimicrobials are classified as the critically important antimicrobials; similarly anti-staphylococcal penicillins are classified as highly important whereas natural aminopenicillins and antipseudomonal penicillins are classified as critically important antimicrobials by WHO
[a] : Closely related analogues are important in human medicine
H : Marketed human drugs

There are both risks and benefits accruing from the use of antimicrobials in animal agriculture; that is, environmental, animal and human health risks associated with the development of antimicrobial resistance and other effects relating to residues and metabolites, as well as economic and welfare benefits. Some benefits of antimicrobials include reduced morbidity and mortality, improved feed efficiency resulting in an increase in daily weight gain and reduction in feed inputs per unit animal produced, increased production efficiency resulting in reducing the number of animals required to produce given amount of animal product such as beef; reduction in the resources required to produce feed such as land (additional 2 million acres required if the use of antimicrobials as growth promoters is withdrawn), fertilizers, insecticides, and gasoline; reduction in the amount of manure per unit animal product; and reduced consumer cost per unit animal product (increase by $5-10 per capita per annum required if the use of

antimicrobials as growth promoter is withdrawn) [19-22]. According to several authors, the benefit-risk ratio should be taken into account to determine the overall impact of antimicrobials on the health of animals/humans, and the environment.

In order to track antimicrobial usage trends in food-producing animals and to examine the relationship of these trends to antimicrobial resistance, the FDA introduced Section 105 of the Animal Drug User Fee amendments in the year 2008 [23]. This legislation mandated that sponsors of approved antimicrobial drugs report their total antimicrobial product sale information to the FDA. This does not summarize the antimicrobial amount purchased by the end user or utilized in animals; however, it may be used as an estimate or surrogate of the same. The legislation also directed the FDA to issue annual summaries of the information reported from sponsors to the public; albeit, while protecting proprietary commercial sale information for those products with a very limited number of sponsors. Annual totals of antimicrobial sales, and distribution summaries by antimicrobial drug class, for the years 2009, 2010, and 2011 are presented in Table 2-2 [17, 24-25]. Approximately 13.1 million, 13.2 million, and 13.5 million kilograms of antibacterial drugs were sold for the use in food-producing animals in the years 2009, 2010, and 2011, respectively.

Sales data for human medicine for 2009, 2010, and 2011 as released by the FDA are also summarized in Table 2-2 [26-27]. These data provide an estimate of antimicrobials sold from the manufacturers to various channels of distribution (outlets). They are not a direct estimate of antimicrobials sold or administered to patients directly. These data may be used as surrogates of human antibacterial drug use in the U.S. market; albeit, with great caution. With some minor variability, sales were flat and at approximately 3.3 million kilograms of antibacterial drugs sold in the years 2009, 2010, and 2011.

The data clearly suggests that the total amount of antimicrobials distributed for use in food-producing animals is approximately four times higher than that used in human medicine. However, FDA cautioned the direct comparisons between the quantities of animal and human sales data [28]. Some variables to be considered before such comparisons are made include the: physical characteristics like weight of humans and animals, differences in antimicrobial dosage and duration of requirements between animals and humans, purpose of antimicrobial use in humans (disease prevention or treatment) versus animals (treatment, control, prevention, and growth promotion), and the number of humans versus animals [28].

Antimicrobial Class	**Animals (kgs)**			**Humans (kgs)**		
	2011	2010	2009	2011	2010	2009
Aminoglycosides	214,895	200,794	339,678	6,485	6,991	9,381
Cephalosporins	26,611	24,588	41,328	496,910	502,561	499,616
Ionophores	4,123,259	3,821,138	3,740,627	NA	NA	NA
Lincosamides	190,101	154,653	115,837	71,455	69,235	69,737
Macrolides	582,836	553,229	861,985	164,028	164,309	176,278
Penicillins	880,163	870,948	610,514	1,460,421	1,439,930	1,459,219
Sulfas	371,020	506,218	517,873	481,664	479,484	471,442
Tetracyclines	5,642,573	5,592,123	4,611,892	113,832	129,183	131,137
Others	1,510,572	1517447	2,227,366	494,381	487,213	500,096
Total	13,542,030	13,241,138	13,067,100	3,289,176	3,278,906	3,316,906

Table 2-2 Total mass of antimicrobials (kgs) sold and distributed for food-producing animals and humans through retail and non-retail channels in the United States (2009-2011)

2.2. Cephalosporins and bacterial resistance mechanisms

Cephalosporins belong to the β-lactam class of antimicrobials. Cephalosporins were introduced into human medicine in 1964. They have been extensively used in human medicine. They are the third most common category of antimicrobials (after penicillins and macrolides) prescribed in the United States in outpatient settings for humans. In 2010, cephalosporins made up 14% of the total out-patient prescriptions; this accounted for approximately 36.2 million prescriptions per year in the United States [29]. Human sales data released by the FDA suggest that approximately 0.5 million kgs of cephalosporins are sold or distributed each year in the United States (Table 2-2). The 3rd generation cephalosporins are categorized as critically important antimicrobials for human use [5]. As such, they are used to treat critically ill patients with life threatening diseases such as severe and invasive *Salmonella* and *Shigella* infections, particularly in children and pregnant women. This is significant because fluoroquinolones interfere with cartilage formation in children/fetuses and thus should not be used in children and pregnant women [7, 30].

The mechanism of action of cephalosporin drugs is to interfere with the final stages of bacterial cell wall synthesis, thereby increasing the permeability and thus leading to hydrolysis of the bacterial cell. These antimicrobials bind to enzymes called penicillin-binding proteins (PBP; a transpeptidase) and form an irreversible bond with the enzyme, thereby inactivating it. This enzyme plays a major role in building the peptidoglycan, which is a component of the cell wall [31].

Ceftiofur and cephapirin are two cephalosporin drugs that are presently approved for use in animal agriculture in the Unites States [7]. Ceftiofur is a third generation cephalosporin. It was licensed for therapeutic use in animal agriculture in 1988 [32]. It was first licensed as a

short-acting formulation requiring reconstitution (Naxcel®) followed by a slightly longer duration and more convenient formulation in 1996 (Excenel®), and then in 2003 as an even longer acting ceftiofur crystalline-free (CCFA) formulation called Excede® (Zoetis Animal Health, NJ, U.S.A.). Excede® is now marketed for use in beef cattle, dairy cattle, pigs, and horses. Presently, ceftiofur is used as an injectable product to control or treat respiratory diseases and to treat foot rot (bovine interdigital necrobacillosis) in beef cattle [7].

Generally, there are 3 major mechanisms by which bacteria can develop and express resistance: (1) efflux of the antimicrobial agent or alterations in the bacterial cell permeability, (2) enzymatic or other inactivation of the antimicrobial agent, and (3) decreasing access of the agent to the antimicrobial target enzymes (PBP's) [33-34]. Enzymes called β-lactamases are utilized by Gram negative bacteria to inactivate cephalosporin drugs by disrupting the β-lactam ring and thereby inactivating the drug. Alteration in PBP is the main mechanism of β-lactam resistance in Gram positive bacteria, examples of which are the PBP5-mediated resistant *Enterococcus* species and the PBP2a-mediated methicillin-resistant *Staphylococcus aureus* [34].

Ambler's molecular classification has been used to categorize the β-lactamases into 4 classes (classes A, B, C, and D). This is a classification based on sequence similarities. Classes A, C, and D group enzymes are serine enzymes while the class B is comprised of metallo β-lactamases [34-35]. Third generation cephalosporin resistance can occur *via* AmpC β-lactamases (class C), extended-spectrum beta-lactamases (ESBLs) (class A; e. g. CTX-M family, and SHV or TEM derivatives), OXA ESBLs (class D), or carbapenem-hydrolyzing β-lactamases (class A and B) [36]. The two most important classes of β-lactamase enzymes for cephalosporin resistance are the AmpC cephalosporinases and the ESBLs.

The plasmid mediated β-lactamase cephamycinase-2 (*bla*$_{CMY-2}$) enzyme belongs to the AmpC cephalosporinase group of enzymes. Discovery of this Amp C plasmid-encoded *bla*$_{CMY-2}$ gene occurred in Greece in 1990 from a *Klebsiella pneumoniae* isolate. Resistance among *Salmonella* spp. from cattle attributed to *bla*$_{CMY-2}$ in the United States was first documented in 1998 [37]. The plasmids (as opposed to chromosomes) encoding the *bla*$_{CMY-2}$ gene are predominantly associated with ceftiofur resistance in *Salmonella* isolates, both from humans and animals, in the United States [31, 34, 38]. In the United States, the plasmid harboring *bla*$_{CMY-2}$ gene almost always appears to harbor other antimicrobial resistance genes [39-41]. There have been many studies that have identified associations among *bla*$_{CMY-2}$ gene and several other tetracycline genes [42-45]. The gene has also been associated with integrons/transposons, therefore readily aiding in the horizontal dissemination of the gene among various enteric bacteria [34].

A chromosomally located ampC β-lactamase gene has been discovered in *E. coli*, but has so far not been detected in *Salmonella* [34]. The *ampC* gene is suggested to be a structural gene for the enzyme; *ampC* positive strains possess very sparse β-lactamase activity [46-47]. The role of this gene in enhancing β-lactamase activity requires further investigation. Point mutations in the ampC operator or promoter region are found to render resistance towards cephalosporin drugs by over expressing the ampC β-lactamase gene [33].

Extended-spectrum β-lactamases (ESBL) have not been detected among the *Salmonella* isolates collected from food-producing animals by NARMS as of the 2011 report [33]. However, they have been widely found in Europe and the rest of the world. The CTX-M enzymes are one of the families of ESBLs that are encoded on plasmids. They have a preferential ability to hydrolyze cefotaxime and thus the acronym [36]. Overall, the enzyme

substrates include penicillins, cephalosporins including oxyiminocephalosprins, and monobactams [34]. Worldwide, the CTX-M family of enzymes are the most widespread ESBLs among both animal isolates [34] as well as human isolates [48-49]. The CTX-M type enzymes have been reported in United States in humans [50-51]. However, in food-producing animals the reports of genes coding for CTX-M type enzymes are rare and require careful sample enrichment to identify; that is, they do not appear in non-selective media or in surveillance isolates from clinical or non-clinical sources [52-53]. The substrate specificity of ESBLs is relatively broad and therefore they can hydrolyze a variety of β-lactam antimicrobials in contrast to AmpC cephalosporinases [34]. The plasmids harboring $bla_{\text{CTX-M}}$ genes are also associated with multidrug resistance; they are commonly found to be associated with bla_{TEM} genes as well as aminoglycoside, chloramphenicol, sulfonamide, tetracycline, and trimethoprim resistance genes [34, 54-56].

2.3. Tetracyclines and bacterial resistance mechanisms

Tetracyclines were first approved by the FDA in 1948 for therapeutic use in humans [57]. Presently, in the United States, tetracyclines constitute 8% of total prescriptions, which in turn represent approximately 20.7 million prescriptions per year in human medicine [29]. Human sales data released by the FDA suggest that approximately 0.1 million kgs of tetracyclines are sold or distributed each year in the United States (Table 2-2). Tetracyclines have been classified as highly important antimicrobials for human use [5]. These are broad spectrum bacteriostatic drugs, that are active against a wide array of Gram negative and Gram positive pathogens, protozoan parasites, and other atypical organisms (*Chlamydia*, *Mycoplasma*, and *Rickettsia*) [58]. The tetracycline drugs, at therapeutic concentrations, bind to the 30s subunit of the ribosome,

thereby preventing the aminoacyl-tRNA from attaching to the ribosomal acceptor (A) site, resulting in inhibition of bacterial protein synthesis [58-59].

Tetracycline, chlortetracycline, and oxytetracycline are the core tetracycline drugs that are marketed for use in food-producing animals. In food animals, these drugs are utilized to combat bacterial enteritis, bacterial respiratory infections, liver abscess, and also vector borne infections (e.g. rickettsiosis and borreliosis). In addition, they are used as growth promoter agents in cattle, poultry, and swine [60]. The total quantity of tetracyclines sold or distributed for animal agriculture purposes amounted to approximately 5.6 million kilograms in the year 2011 (Table 2-2).

The mechanisms by which the bacteria can develop resistance against tetracycline antimicrobials are (1) efflux of the tetracycline drug, thereby reducing the drug concentration in the bacterial cytosol, (2) enzymatic alteration of tetracycline drugs, and (3) ribosomal protection mechanisms that affect the binding of tetracyclines to the bacterial ribosome. Data from the 2010 U.S. National Antibiotic Resistance Monitoring System (NARMS) indicated that 11% and 33.6% of the non-Typhi *Salmonella* isolates arising from human clinical cases and food-producing animals at slaughter, respectively, demonstrated decreased susceptibility towards tetracyclines. Among human isolates, these were the lowest resistance levels recorded since 1999; in contrast, these were among the highest resistance levels recorded for food-producing animals (Figure 2.1). As of 2001, there were approximately 29 tetracycline resistance genes (*tet*) and 3 oxytetracycline genes (*otr*) that had been characterized. Out of these 32 genes, 18 *tet* genes and one *otr* gene code for an efflux pump; while seven *tet* genes and one *otr* gene code for proteins that aid in ribosomal protection; one *tet* gene [tet(X)] code for enzymatic inactivation of drug molecule; and mechanism of resistance for one *tet* and one *otr* genes are not established yet

[58]. Among food-producing animals, *tet*(A), *tet*(B), and *tet*(C) are the most abundant tetracycline resistance genes detected [61-63]. All three genes confer resistance by coding for membrane associated proteins that transport the tetracycline molecules outside of the bacterial cell [58]. Tetracycline resistance is very often associated with co-resistance towards other antimicrobials [63]. The tetracycline resistance genes *tet*(A) and *tet*(B) are typically found to be negatively correlated with each other's presence, while *tet*(C) can be found with either of the others [62-64].

2.4. Transfer of antimicrobial resistance from animals to humans

Historically, transferable mechanisms of resistance to antimicrobial agents were demonstrated by the Japanese as early as 1959 [65-67]. Consequently, the worldwide scientific community started to observe more carefully the non-therapeutic usage of antimicrobial drugs in food animals [68]. The Joint Committee on the Use of Antimicrobials in Animal Husbandry and Veterinary Medicine (established in 1968) presented the 'Swann Report' to the British Parliament in November 1969 [69]. In this report, antimicrobial use in food-producing animals was reported to be the cause of a drastic increase in resistant bacteria in animals over the preceding decades. Such resistance was found to be transferable to human bacteria and therefore could pose a public health risk. Ongoing debate concerning the continued use of antimicrobials in animal agriculture, especially regarding threats to human health, was initiated by this report in UK [69]. This debate continues to the present. The acquisition of antimicrobial-resistant bacteria by humans has been thought to occur *via* a variety of routes [20, 70]. Some suggested routes of transmission are: meat products contaminated at slaughter, animal and human fecal waste contamination of the environment, and direct animal-human contact [20, 70]. Several studies have demonstrated the transfer of antimicrobial resistant bacteria through direct contact

with animals or their manure [71-72], indirect contact *via* food contamination by animal-derived bacteria (e.g., *Salmonella* and *Campylobacter*) [73], or from person-to-person contact following primary exposure of farm workers to animals [74].

There is increasing concern about the use of antimicrobials in animal production systems as this may increase antimicrobial resistance to drugs used in human medicine. This led the World Health Organization (WHO) to categorize and prioritize the antimicrobials into 'critically important', 'highly important', and 'important' based on the importance of the antimicrobial drug for treating human diseases (Table 2-1) [5]. This provides a reference for the prudent use of antimicrobials in humans and animals that are critically important for human medicine.

Several reviews of the scientific literature regarding the evidence of use of antibiotics in food animals leading to increase in resistance among humans have been published [20, 70, 75]. A substantial amount of the data demonstrating such a linkage comes from: 1) case studies where the reported infection is traced backwards to a farm or higher risk groups such as farm workers, 2) experimental studies demonstrating the transfer of resistant determinants among gut flora, and 3) microbiological and molecular ecological studies of bacterial isolates from humans, food animals, and retail meat. Resistant pathogen transfer from animals to humans has attracted the most attention for studies investigating antimicrobial-resistant bacterial ecology [74, 76-77]; however, commensal bacteria make up the vast majority of the gut flora and function as a reservoir of resistance genes and thus can transfer resistance to the pathogenic bacteria (reservoir hypothesis) [78]. Zoonotic as well as commensal bacteria are frequent carriers of transferable resistance genes and can transfer and colonize successfully from the GI tract of the animals to the human gut [79-83]. Also, studies with humans have shown that ingested animal bacteria have transmitted their resistance genes to the normal flora and pathogens of the human GI tract [84-

85]. Therefore, one opinion is to use a wide variety, or else a more predominant bacterial species like *Bacteroides* and Gram-positive colonic anaerobes to study the impact of antimicrobial use in animals and humans [78, 86]. Another opinion is to study the ecology of resistance genes rather than resistant bacterial strains as the resistance genes are thought to readily move among various strains [78].

Results from a USDA nationwide survey in 1994 indicated that 15% of the beef carcasses were contaminated with one or more species of disease-causing bacteria [87]. Another survey indicated that 30% of chicken products were contaminated with viable *Salmonella* and 60-80% of the chickens were contaminated with *Campylobacter*; of which, many strains were resistant to common antibiotics [88]. However, it remains exceedingly difficult to track and document the exact link between animal antimicrobial use and subsequent transference of resistance to the humans [20, 89-91]. There are many opportunities from the farm to the table (e.g., production sites, processing, packaging plants, and transport depots) for the bacteria carrying resistance determinants to enter the food chain. In addition, there are other factors that likely play a role; for example, consider the actions of individuals operating before and after the food harvest – improper storage, poor home sanitary practices, or improper cooking. Therefore, a low likelihood of transfer of resistant bacteria from animal to human is suggested by some [90]. Dietary and occupational human history are not the sole variables that determine the antimicrobial resistance transfer; other variables like international transport of animals, humans, feedstuffs, and food products all play an important role in dissemination of resistant strains or determinants. Therefore, it is extremely difficult to have a valid exposure assessment in this case. It is important to quantify the actual risk of antimicrobial resistance transfer; so far, this risk seems low. However, the continued and cumulative effects on the animal populations of

antimicrobial use means that even low risk, but frequent events, and an increasing hazard can pose problems over time [89].

Ecological studies demonstrating the correlation between resistant strains obtained from animal and human infections are often used as evidence of causation for transfer of resistant organisms from animals to humans [92]. While this may well be true, the directionality of transmission should also be carefully considered before drawing causal inferences based on ecological correlation studies. It is possible that humans select for resistant organisms and these are further disseminated to animals [93]. The antimicrobial resistance issue involves entire ecosystems where resistant organisms or resistant determinants keep flowing constantly among different environments [91]. A better understanding of background levels of antimicrobial resistant organisms and their genes is needed to conclude the directionality of flow of resistance from animals to humans or vice versa and also the impact of use of antimicrobials in animals on human health [91].

2.5. Economic impact due to antimicrobial resistance

The Centers for Disease Control and Prevention (CDC) in the year 2011 reported the national annual estimates for number of illnesses, hospitalizations, and deaths among humans caused by 31 major known pathogens [94]. The estimates were largely derived from laboratory based surveillance data from 2000-2008 reported through 5 surveillance programs: the Foodborne Diseases Active Surveillance (Food-Net), the National Notifiable Diseases Surveillance Systems (NNDSS), the Cholera and Other *Vibrio* Illness Surveillance (COVIS), the National Tuberculosis Surveillance System (NTSS), and the Foodborne Disease Outbreak Surveillance System (FDOSS). All of the estimates were based on the United States population in 2006 (299 million people).

It was estimated that these 31 pathogens caused 37.2 million illnesses, 228,744 hospitalizations, and 2,612 deaths annually in the United States. Of these, 9.4 million illnesses, 55,961 hospitalizations, and 1351 deaths annually were due to food-borne sources [94]. The second major cause of foodborne illnesses after noroviruses (58%) was found to be non-Typhoidal *Salmonella* species (11%; 1.03 million cases). Non-Typhoidal *Salmonella* were found to be the foremost leading cause of hospitalizations due to food borne pathogens (35%) and also deaths (28%). Similarly, *Campylobacter* species were among the leading causes of foodborne illnesses (9%; 0.85 million cases) and hospitalizations (15%) [94]. Both non-Typhoidal *Salmonella* and *Campylobacter* species are considered zoonotic pathogens.

Antimicrobial usage in food-producing animals has been debated over the last several decades since food of animal origin may act as a vehicle to carry resistant food borne pathogens and genetic resistance material. There can be a major public health impact if significant percentages of these zoonotic pathogens develop resistance to the antimicrobials that are currently being used in human medicine [95].

The CDC in 2013 reviewed the data obtained from outbreak-associated illnesses from 1998-2008 in order to attribute food borne illnesses, hospitalizations, and deaths annually to 17 food commodities in the United States. Among bacterial infections, beef contributed to 13.4% (482,199 cases) of illnesses, 7.4% (2640 cases) of hospitalizations, and 5.9% (51 cases) of deaths [96].

The CDC also provided cost estimates of food borne illnesses in humans caused by the combined 31 identified pathogens and a broad category of unspecified agents [97]. The annual economic burden estimates were modeled based on the estimate provided by CDC [98]. The average cost per case of a food borne illness was $1,626. The annual estimated cost due to

health losses from food borne illnesses was as high as $77.7 billion [98]. The illnesses caused by resistant pathogens can fail to respond to antimicrobial treatment (where indicated) and may prolong the illness, lead to an unfavorable prognosis, and increase in-hospital treatment days. This increases the financial burden to health care systems and society [99]. Older annual cost estimates of illnesses caused by resistant bacteria in 1998 by the Institute of Medicine were approximately $4 to $5 billion [100]. In 2003, the CDC estimated the annual 'dead-weight' loss due to resistance (costs incurred by the antibiotic resistance in excess of the benefits obtained by using antibiotics more than the maximum welfare levels) associated with outpatient amoxicillin prescriptions to be $225 million [101]. A single hospital study in Chicago in 2008 estimated the cost associated with antimicrobial-resistant infections. The costs and mortality attributable to occurrence of antimicrobial-resistant infections were approximately $21,000 per infected patient and 6.5% of deaths, respectively [102].

2.6. Antimicrobial resistance surveillance in food animals

The National Antimicrobial Resistance Monitoring System (NARMS; formerly known as National Antimicrobial Susceptibility Monitoring Program) was established in 1996. It is a collaborative effort of the U. S. Food and Drug Administration (FDA), the U. S. Department of Agriculture (USDA), and the U.S. Centers for Disease Control and Prevention (CDC). This national public health surveillance system prospectively monitors the changes in the resistance or susceptibility patterns among select enteric zoonotic pathogens and commensal organisms from human and animal diagnostic samples, healthy farm animals, and retail meat at slaughter and processing [103]. Resistance to a wide range of antimicrobials is tested on the basis of their importance in human and animal medicine. Initially in 1996, non-Typhoidal *Salmonella* was

chosen as the sentinel bacteria to study resistance patterns among enteric bacteria. In 1998, *Campylobacter* was introduced to the animal arm of NARMS. Later in 2000, *E. coli* and *Enterococcus* were also added to the list of organisms to be monitored [13]. The testing is still restricted to *Salmonella* spp. for the cattle samples.

The descriptive data generated by NARMS helps to identify the trends of existing, detection of emerging, antimicrobial resistance in animals and humans. This aids in providing timely information to veterinarians, human doctors, and regulatory bodies like the FDA which has proven instrumental in promoting interventions, research, and legislative decisions that may reduce the antimicrobial resistance among enteric food borne bacteria [104].

There are important factors to be considered before interpreting the NARMS results. Factors include inherent sampling bias and test methodologies [13, 39]. An external subcommittee of the Science Board to the FDA reviewed the data obtained through the NARMS program in 2007 [104]. They expressed concerns about sampling bias in both animal and human arms. The animal arm utilized *Salmonella* isolates that came from clinically ill animals, healthy animals, and animals at slaughter to study resistance trends. The human arm of NARMS relies solely on public health laboratory-based surveillance. Only clinical isolates are tested for susceptibility profiles. There is a serious concern about extrapolating these results to the general animal and human population of the United States. Prior treatment bias could severely affect the external validity of the results at the national level [39]. In response to making the samples more representative of the U.S. animal population, NARMS discontinued the analysis of diagnostic *Salmonella* isolates from sick animals. The human clinical isolates are still analyzed. However, the committee suggested also including isolates from healthy humans in the sampling [39]. The present methodology to test isolates is limited to phenotypic culture-based approaches. The

committee suggested that NARMS ought to expand their methodology to incorporate molecular methods for gene detection from samples irrespective of bacterial species or whether the gene is present in a pathogen or a commensal organism [104].

The sole reliance on culture-based approaches has been criticized since it may lead to selection of only a subpopulation, processing particular phenotypic and genotypic characteristics, and therefore does not represent the entire bacterial population and especially the un-culturable bacteria [13, 105]; further, antimicrobial genes may not be expressed or may be lost under the selection pressure of the media [106]. In contrast to the culture-based approaches, a metagenome-based approach enables the researcher to screen all the bacterial species from the samples, since community DNA represents the entire gene pool. However, information about the presence of a particular gene in specific bacterial species cannot be identified [105-106] from such analyses.

Data from the 2010 U.S. National Antibiotic Resistance Monitoring System (NARMS) indicate that 2.8% of the non-Typhi *Salmonella* isolates arising from human clinical cases were resistant to both ceftiofur and ceftriaxone. At the same time, 21.5% of the non-Typhi *Salmonella* isolates, obtained from food-producing animals at slaughter, were resistant to both ceftiofur and ceftriaxone. This is in sharp contrast to the 14.5% of the non-Typhi *Salmonella* animal isolates that were resistant in 2009. The prevalence of ceftriaxone resistance among human isolates has been observed to be relatively stable since 2004; however, in 2010 ceftriaxone resistance among non-Typhi *Salmonella* cattle isolates (22%) was observed to be at its highest since NARMS testing began in 1997 (Figure 2.1) [107]. Caution should be employed while comparing the data from before and after 2006, especially from cattle sources, since clinical samples were not considered for susceptibility testing after the year 2006. However, the trend clearly shows an

ongoing increase in ceftiofur and ceftriaxone resistance (a critically important 3rd generation cephalosporins) among the cattle isolates.

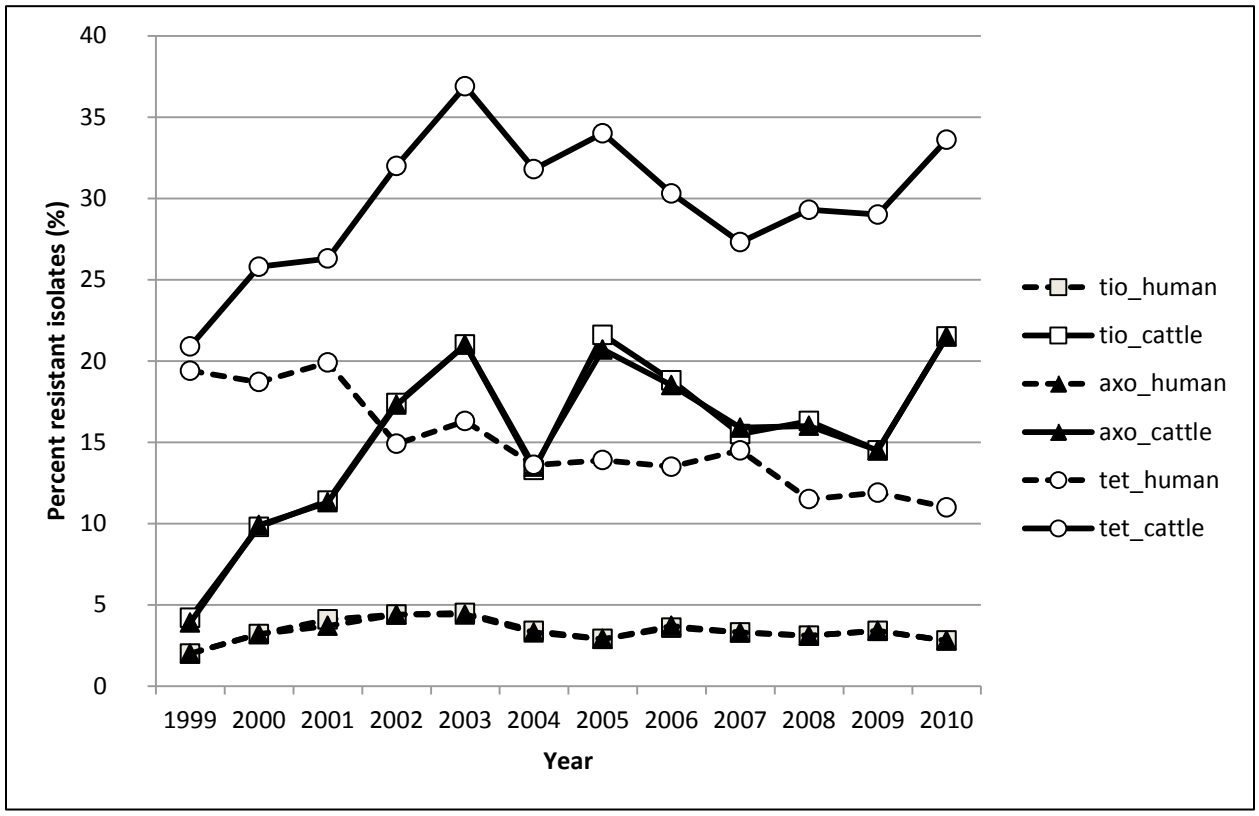


Figure 2.1 Percentage of *Salmonella* isolates resistant to ceftiofur, ceftriaxone, and tetracycline among cattle and humans isolates analyzed by the National Antimicrobial Resistance Monitoring System (NARMS) from 1999-2010.
Legend key: tio_human represents percentage of human isolates resistant to ceftiofur, tio_cattle represents percentage of cattle isolates resistant to ceftiofur, axo_human represents percentage of human isolates resistant to ceftriaxone, axo_cattle represents percentage of cattle isolates resistant to ceftiofur, tet_human represents percentage of human isolates resistant to tetracycline, and tet_cattle represents percentage of cattle isolates resistant to tetracycline.

2.7. Regulatory bodies and their actions to control antimicrobial resistance

Over the years, based on the scientific literature and the surveillance data gathered from NARMS (since 1997), the FDA has released several guidance documents to help control antimicrobial resistance in human medicine that may be caused by the use of antimicrobials in

animal agriculture. While not mandated, a guidance document consists of the FDA's current view on a subject and it recommendations to the animal pharmaceutical industry.

Subsequent to the Swann report in U.K. in 1969, indicating the possibility of transfer of antimicrobial resistance from animals to humans; the FDA in 1970 established a task force of scientists to perform a comprehensive review of the use of antimicrobials in animal feed. The task force report acknowledged that antimicrobial use, especially in sub-therapeutic doses, and the development of resistant bacteria in animals were associated. On the basis of the task force report, the Code of Regulations (21 CFR 558.15) outlined the requirements for the sponsors to submit data demonstrating that the antimicrobial product intended for sub-therapeutic purposes did not lead to bacterial resistance [108].

In 1977, the FDA proposed to prohibit the in-feed, sub-therapeutic uses of tetracycline and penicillin due to concerns about the development of resistance. These two drugs were widely used in human medicine at that time. However, due to lack of sufficient epidemiological evidence, this action was never taken [108].

In 1987, the National Cattlemen's Beef Association producer guidelines for 'Judicious use of Antimicrobials' were released. These outlined the appropriate use of the available antimicrobials in animal agriculture [109].

In 1997, the Center for Veterinary Medicine (FDA) for the first time prohibited the extra-label use of fluoroquinolones and glycopeptides in the animal agriculture industry on the basis of the development of bacterial resistance as a sole cause for this legislation [110]. This order was released under the Animal Medicinal Drug Use Clarification Act (AMDUCA) which has been active since 1994. The use of antimicrobial drugs like chloramphenicol, clenbuterol, diethylstilbesterol, dimetridazole, furazolidone, nitrofurazone, and sulfonamide drugs in lactating

dairy cattle (except approved uses) were prohibited earlier in animal agriculture. However, these prohibitions were based on hazardous effects on human health because of the residues in animal products. Drug residues could lead to direct exposure to the pharmaceutical compounds or their metabolites and pose public health risk [39].

In October 2003, the Center for Veterinary Medicine (FDA) released Guidance for Industry (GFI #152) as a regulatory tool consisting of guidelines to be considered in order to help prevent antimicrobial resistance in humans that may be a result of antimicrobial use in animals. The FDA recommended all new drugs intended to be used in food-producing animals undergo a qualitative risk assessment as a part of the pre-approval safety evaluation process, while applying for new drug approval. The risk analysis process was based on the Office International des Epizooties (OIE) approach. The three steps for the qualitative risk assessment of an animal drug involved release assessment, exposure assessment, and consequence assessment. Release assessment was based on the probability that the target animal will develop resistant bacteria as a result of the drug use; exposure assessment involved the probability that humans will be exposed to those resistant bacteria through food; and consequence assessment was the probability that humans will face an adverse consequence because of the prior exposure to resistant bacteria. The overall risk estimate was classified into high, medium, and low. The document also provided the list of antimicrobial drugs as classified into important, highly, or critically important categories. This list was utilized in the consequence assessment component of the risk assessment [111].

In 2006, the Center for Veterinary Medicine (FDA) released Guidance for Industry (GFI#159) and then further revised it in March 2012. This guidance highlights the effects of veterinary drug residues in food on human intestinal microflora. The document provides

recommendations on (1) steps to determine if there is a need to establish a microbiological average daily intake (ADI) for particular antimicrobials used in animals; (2) test systems and methodologies for determining the concentration and levels of antimicrobials for microbiological endpoints; and (3) a procedure to derive microbiological ADI of antimicrobials in animals. Recommended microbiological endpoints to be considered are colonization barrier disruption leading to colonization of exogenous, possibly pathogenic bacteria and expansion of resistant bacteria in human gut. The guidance recommended *in vivo* and *in vitro* tests to determine the threshold antimicrobial doses to be used in animals so that the residues do not adversely affect the ecology of human gut microflora using a harmonized approach [112].

In July 2008, the Center for Veterinary Medicine (FDA) issued a order of prohibition restricting the extra-label use of cephalosporin drugs in animal agriculture [113]. However, the rule could not be implemented immediately because of the large negative response from the animal health community. The final prohibitory legislation for cephalosporins in animal agriculture came into place in April 2012 [7]. The order bans certain extra-label uses of cephalosporins such as biobullets in cattle, injection or dipping of poultry eggs, and self-compounded preparations. In essence, the use of cephalosporin drugs other than at approved dose levels, duration, frequency, and route of administration were prohibited. This order was also released under the Animal Medicinal Drug Use Clarification Act (AMDUCA) that came into action in 1994.

In April 2012, the Center for Veterinary Medicine (FDA) released Guidance for Industry (GFI#209). This document recommended judicious use of the current medically important human drugs used in animal agriculture. Uses of such drugs for growth promotion and feed efficiency were deemed to be injudicious. The document provides the framework for voluntary

steps to be taken by the animal agricultural community in order to safeguard the efficacy of medically important drugs for human medicine. The two principles outlined are (1) these medically important drugs should only be used in food-producing animals when it is deemed necessary for their health; and (2) the application of these drugs should be limited to purposes that mandate veterinary supervision or prescriptions [108].

The Preservation of Antibiotics for Medical treatment Act (PAMTA) is a proposed amendment to the Federal Food, Drug, and Cosmetic act that came into action in 1938. PAMTA was reintroduced into congress in March 2013 after its previous introductions in March 2009 and 2011. The aim of this legislation is to protect the effectiveness of antimicrobials used in human and animal medicine by banning the non-therapeutic uses of medically important drugs in food-animal production. The 7 classes of antimicrobials included in the bill are penicillins, tetracyclines, macrolides, lincosamides, streptogramins, aminoglycosides, and sulfonamides. If the bill is enacted, the non-therapeutic use of these 7 classes of antimicrobials will be banned unless drug companies can prove that human health is not impacted by their use. It would also require the FDA to re-examine the approvals of antimicrobials used in animal agriculture [114].

2.8. Studies on ceftiofur and tetracycline resistance among enteric bacteria in cattle

The effects of feeding chlortetracycline on antibiotic susceptibility among *E. coli* and *Enterococcus* was studied in 20 feedlot cattle in Texas [11]. Minimum inhibitory concentrations (MICs) against selected antimicrobials were determined by using a microbroth dilution technique. The study results revealed that chlortetracycline treatment leads to a transient increase in the proportion of *E. coli* and *Enterococcus* resistant to tetracycline. However, the same authors observed a drastic reduction in ceftiofur resistance among *E. coli* isolates while

chlortetracycline was being fed to the cattle. Chlortetracycline differentially favored the *E. coli* isolates that were singly resistant to tetracycline over the ceftiofur and tetracycline co-resistant isolates. These results suggested that chlortetracycline might minimize the proliferation and accumulation of ceftiofur resistant bacteria in production-animal agriculture settings. The bacteria load was not quantified in the study. Therefore, the increased proportion of tetracycline resistant isolates could be either due to a transitory increase in the resistant population due to antibiotic selective pressure or else to a decrease in the susceptible population that simply made the probability of detection of resistant isolates easier.

The results from this study formed the basis of one of the hypothesis of the research project described in this thesis. A total of 525 *E. coli* isolates were tested for susceptibility profiles. There were 397 isolates that exhibited resistance to atleast one antimicrobial. Out of those 397 isolates, 213 isolates were derived from the tetracycline treated group and 184 isolates were derived from the control group. The proportions of isolates resistant to one or more antimicrobials in the treated and control groups were 81% and 70%, respectively. The *E. coli* isolates from the control group were significantly less likely to possess resistance towards one or more antimicrobials as compared to the treated group ($p=0.04$). However, the results from *Enterococcus* isolates revealed no significant differences in resistance proportions (approximately 90%) among both control the treated groups. These results indicate that Gram negative and Gram positive bacteria may behave differently in terms of propagating antimicrobial resistances under similar antimicrobial selective pressure. Therefore, drawing conclusion on the effects of antimicrobials on all bacterial populations based on a single or sentinel bacterial species may be misleading. Other bacterial populations (culturable or non-culturable) may act a reservoir or may behave differently under similar selective pressures.

The effect of therapeutic doses of ceftiofur on *E. coli* diversity and changes in prevalence of $bla_{\text{CMY-2}}$ genes among *E. coli* was studied in a single dairy farm in Illinois [115]. An observational study was conducted on 10 dairy cows, out of which 5 cows had been treated with ceftiofur for *Leptospira borgpetersenii* infection and 5 were matched untreated cows. Total *E. coli* were enumerated as log colony forming units per gram sample. A total of 468 *E. coli* isolates (203 and 265 isolates from treated animals and control group, respectively) were screened for antibiotic susceptibility testing against 16 antimicrobials using a microbroth dilution technique. The $bla_{\text{CMY-2}}$ gene was qualitatively detected using PCR assay from DNA derived from *E. coli* isolates as well as from the fecal community DNA. The effect of ceftiofur treatment on genetic diversity among *E. coli* isolates was studied through repetitive element PCR (Rep-PCR). The total *E. coli* counts in the treated group were significantly lower as compared to the untreated group on 2 days during 5-day treatment period and 2 days post-treatment. There was no significant increase in the quantity of resistant bacteria as measured by an antimicrobial resistance index, except for 3 days during treatment. Susceptible bacterial population overtook the resistant fraction once the antibiotic selection pressure was removed.

Overall, 6% of the 203 *E. coli* isolates in treated animals and none of the 265 isolates from untreated animals harbored the $bla_{\text{CMY-2}}$ gene. There was no horizontal cow-to-cow transmission from treated to the non-treated cows noticed during this phase even though the cows were allowed to co-mingle. The probability of transmission of resistant bacteria was expected to be high since there was a significant decrease in the susceptible bacterial population in the treated group. According to authors, the lack of detection of resistant strains could be because of small sample size, less sensitive test methodologies, or else ceftiofur-sensitive

bacteria in the non-treated animals were more fit than resistant strains and therefore the effect of exposure to resistant bacteria was not appreciable. The probability of detecting transmission between treated and non-treated animals could have been increased by evaluating more isolates per animal, including using selective media for ceftiofur-resistant isolates to increase the assay sensitivity. A cluster analysis of the antibiotic susceptibility profiles of *E. coli* isolates revealed that there were only two multidrug resistance clusters. All *E. coli* isolates in these two clusters came from treated cows. Importantly, the Rep-PCR results indicated that genetic diversity in *E. coli* population was indistinguishable between the treated and the control group, before treatment, during and after the treatment during the washout phase. Therefore, the authors concluded that ceftiofur treatment did not cause either the emergence or amplification of ceftiofur resistant *E. coli* population. However, considering the sample size there could be a possibility that in between group variation was not captured. Also, caution should be taken to extrapolate these results to other bacterial genera that could behave differently upon ceftiofur treatment.

Additionally, the emergence and amplification of resistance is a function of repeated usage over a long period of time. Given the extensive usage of ceftiofur over decades in animal agriculture, it could significantly contribute to the emergence and amplification of ceftiofur resistant bacteria over time. This may be evident by the NARMS data that indicate the increase in the baseline ceftiofur resistance among *Salmonella* isolates in cattle from 0% in 1998 to 14.5% in 2009 to 21.5% in 2010 (Figure 2.1). The $bla_{\text{CMY-2}}$ gene was detected in the community DNA from both treated as well as untreated cattle throughout the sampling days. The authors concluded that resistant microbes or resistant determinants were present in the herd even prior to antibiotic treatment as revealed by the community DNA results. Antibiotic treatment enhanced

the probability of detecting resistant isolates, primarily by decreasing the susceptible *E. coli* population. Also, the authors rightly suggested that studying the effects of antibiotics on resistant culturable isolates alone is not sufficient to determine the true impact of antibiotic on entire gut microbiota. The true strength and relationship of antibiotic use and antibiotic resistance among gut microbiota cannot be measured by evaluating the effect on one bacterial population like *E. coli*. Quantifying particular target resistance genes in community DNA, derived from the entire gut microbiota, will be more useful to determine the true impact of antimicrobials on gut flora.

A follow up study to Singer *et al.* (2008) [115] was conducted where absolute quantities of $bla_{\text{CMY-2}}$ gene was measured by real time quantitative PCR from bacterial community DNA obtained from the previous study [8]. The mean $bla_{\text{CMY-2}}$ gene copies in community DNA were significantly higher during the treatment phase in the ceftiofur-treated group as compared to the untreated animals. There was also a small increase in the $bla_{\text{CMY-2}}$ gene copy numbers among the non-treated animals. The authors attributed this effect to co-mingling of treated and non-treated animals together. The increase in the $bla_{\text{CMY-2}}$ gene copies among the non-treated animals could be indicative of the horizontal transmission of bacteria among treated and non-treated co-mingled cattle. The results from this study suggested that the burden of resistance in terms of target resistance gene copies increased temporarily during the treatment phase. Importantly, these findings were not evident with culture-based assay used in their previous study where the decrease in susceptible *E. coli* population was attributed to the ability to detect resistant bacteria rather than an actual increase in the population of the resistant population upon antibiotic treatment. Overall, $bla_{\text{CMY-2}}$ gene quantities ranged from a single copy to 41 copies per qPCR

reaction (per 5µl of sample community DNA). There were 84% observations that were considered censored with either no Cq (cycle threshold) or genes less than 10 copies per qPCR reaction. A hierarchical model was used to impute these censored observations. The model was based on the number of censored observations among the sample triplicates used in the PCR assay. Sixty one percent of the 129 fecal samples screened had at least one well among the triplicates which tested positive for the $bla_{\text{CMY-2}}$ gene. Among the 10 samples collected prior to ceftiofur treatment, there were 5 and 4 fecal samples that tested positive for $bla_{\text{CMY-2}}$ gene from both treated and untreated groups, respectively. The authors concluded that ceftiofur administration resulted in short term selection of $bla_{\text{CMY-2}}$ gene. They did not quantify the housekeeping gene for entire bacterial community like 16S rRNA gene. Therefore, it is not possible to determine the impact of ceftiofur treatment on the susceptible population of bacteria, whether culturable or unculturable.

The effect of three different doses or durations of ceftiofur (ceftiofur crystalline-free acid: CCFA) treatment was evaluated and compared on 61 steers in an experimental feedlot in Texas [116]. CCFA treated and non-treated steers were housed together in pens in the ratio of 1:1, where each pen housed 10 steers, except for one pen that had housed 11 steers. This was done to mimic a real-life situation of feedlots in which treated and non-treated animals are allowed to co-mingle. A microbroth dilution technique was utilized to determine antimicrobial susceptibility towards 15 antimicrobials. The three regimens of ceftiofur tested were single two-third dose regimen (4.4mg/kg administered once on day 0), single therapeutic dose regimen (6.6mg/kg administered once on day 0), and three consecutive therapeutic doses regimen (6.6mg/kg administered thrice on days 0, 6, 13). The *E. coli* concentration was measured in terms of total

colony counts of non-type specific *E. coli*. Approximately 68% of the 1,441 *E. coli* isolates possessed resistance to at least one out of the 15 antimicrobials tested. Ceftiofur resistant isolates were found resistant to approximately 7 other antimicrobials. Co-selection of resistance towards other antimicrobials was observed upon ceftiofur administration. A perfect agreement was observed between ceftiofur resistance and the ACSSuT phenotype. Also, there was a perfect agreement between ceftiofur resistance and reduced susceptibility towards ceftriaxone. Ceftiofur administration led to transient increases in the population of ceftiofur-resistant isolates along with the reduction in total non-type-specific *E. coli* population. The resistance levels returned to baseline values around 14 days post-treatment. There was no statistical difference observed in the proportion of ceftiofur resistant isolates between the cohorts that received single dose of 4.4mg/kg and 6.6mg/kg ceftiofur treatment. Therefore, a causal ceftiofur dose effect (varying levels of antimicrobial selection pressure; e.g., sub-therapeutic verses therapeutic doses) on ceftiofur resistance was not evident from study results.

The scientific literature and lay media tend to implicate antimicrobial resistance more to the sub-therapeutic administration of antimicrobials in food production animals. However, duration of administration could be a confounding factor to such inferences. Sub-therapeutic doses are usually given for longer duration of time for purposes described previously in this section. The effect of co-mingling of treated and non-treated cattle was also not evident in this study. The horizontal transmission of ceftiofur resistant bacteria among treated and non-treated cattle were not observed during the treatment phase. The authors attributed this observation to the possibility of susceptible bacteria (harbored by non-treated animals) being more fit than resistant bacteria and therefore non-treated animals were not affected by exposure to resistant bacteria released into the environment by treated animals. On the basis of this assumption, they

concluded that ceftiofur treatment may be a prerequisite to develop and disseminate ceftiofur resistance in *E. coli*. This may not be entirely true. Although fitness costs may be a valid explanation, assay sensitivity should also be considered. The results were based entirely on phenotypic culture-based assay. Culture-based assays may result in expulsion of resistance genes or the ingredients used in the agar may select for a subpopulation that may not possess those phenotypic characteristics. The transmission probability as described earlier could have been increased by increasing the number of isolates tested per animal or by using selective media to select for ceftiofur resistant isolates [115].

A follow up study to [116] was conducted where absolute quantities of $bla_{\text{CMY-2}}$ gene and 16S rRNA gene per gram of feces were measured by real time quantitative PCR assay [106]. The quantification was performed on bacterial community DNA extracted from cattle feces. The standardized (as ratio to $\log_{10}$ 16S rRNA gene) $\log_{10}$ $bla_{\text{CMY-2}}$ gene quantities for treatment groups receiving three doses of 6.6mg/kg and a single dose of one 4.4mg/kg both were significantly lower than the control group. The decrease in standardized quantities on a $\log_{10}$ scale (difference between the target gene copies and the house keeping gene) indicated either expansion of $bla_{\text{CMY-2}}$ gene quantities, or concurrent reductions in the total bacterial population, or both among the total microbial fecal flora. This led to lower standardized $bla_{\text{CMY-2}}$ gene quantities in the two regimens described above. The nonstandardized $bla_{\text{CMY-2}}$ gene quantities (absolute quantities) for these two treatment groups were significantly higher than the control group. The results suggested that different ceftiofur regimens in feedlot cattle may provide varied selection pressure. This study also highlights the importance of reporting both absolute and standardized quantities that take into account the effect on the background population.

Although not explicitly mentioned, there was increase in the standardized *bla*$_{CMY-2}$ gene copies over study days in the control group. The least square means (lsmeans) $\log_{10}$ copies per gram ± standard error (SE) for standardized gene quantities of control group was 3.40 ± 0.34 and the baseline values for standardized *bla*$_{CMY-2}$ gene quantities were approximately 2.6 $\log_{10}$ copies per gram. This may indicate the effect of pen mates that underwent ceftiofur treatment on the untreated animals (co-mingling effect). The impact of ceftiofur treatment on specific bacterial species could not be determined through this community DNA based assay.

An observational study was conducted on 18 dairy herds in Ohio [117]. *E. coli* isolates from 1,266 fecal samples were screened to determine the association between the ceftiofur use and reduced ceftriaxone susceptibility. *E. coli* with reduced susceptibility towards ceftriaxone were isolated from 12 out of these 18 herds. Ten out of the 12 herds reported ceftiofur use. A mean of 70 cows per herd was sampled. The herds reporting ceftiofur use had 40% mean herd prevalence for reduced ceftriaxone susceptibility *E. coli* as compared to 9% in herds that did not report ceftiofur use. The *bla*$_{CMY-2}$ gene was identified in 355 of the isolates (83%) with reduced susceptibility to ceftriaxone. Eighty one of these *bla*$_{CMY-2}$ positive isolates representing each of the herds were tested for resistance towards 16 antimicrobials by a broth micro-dilution technique. All ceftiofur-resistant isolates were classified as resistant to 8 antimicrobials. Herds reporting ceftiofur use were 25 times more likely to have cows from which reduced ceftriaxone-susceptibility *E. coli* were isolated versus those that never used ceftiofur (p=0.01). This was not true at the individual cow level within those herds that used ceftiofur (adjusted odds ratio=1.01; p=0.83).

There are several factors that could have influenced the study results. The length of time between the recorded ceftiofur treatment and fecal sample collection was not controlled. Detailed antimicrobial usage data was recorded only for subset of dairy herds in the past 6 months prior to sample collection. Previous studies indicate that the probability of detecting ceftiofur resistant bacteria increases in the post ceftiofur treatment period. However, this increase is found to be transitory and the resistance levels drop to baseline levels shortly after the selection pressure is removed [115-116]. The probability of finding a significant relationship between the ceftiofur treatment and resistant bacteria post antimicrobial washout phase seems low [116]. Also, this was a cross-sectional prevalence study so cause-effect relationship is hard to determine. A convenient sampling of herd was performed. This herd sample was studied despite having broadly similar management practices as other herds, but was not a true random representation of all dairy farms in Ohio or the United States. The study was conducted in dairy cattle where only the short acting formulation was used. Therefore, these results should not be extrapolated to beef cattle production systems where long acting formulations are more often being used. One of the most important observations of the study was the significant herd level association of ceftiofur use and subsequent development of reduced susceptibility towards ceftriaxone. This points to the treatment dependent ceftriaxone resistance baseline shift over time [116].

The effects of antimicrobial growth promoters on persistence and diversity of resistant *E. coli* in feces at both phenotypic and genotypic levels were studied in 90 steers [118]. A control group (no antibiotic exposure) and two cattle groups administered tetracycline based antimicrobial growth promoters (chlortetracycline alone or chlortetracycline and sulfamethazine)

were involved in the study. Fecal deposits were prepared subsequent to the group treatments which were administered to steers form arrival day of feedlot to 197 days. Fecal deposits were sampled 12 times over 175 days and examined for proportions of ampicillin and tetracycline-resistant *E. coli*. *E. coli* were isolated using plain MacConkey agar or else MacConkey agar supplemented with ampicillin or tetracycline at breakpoint concentrations according to CLSI. The resistant *E. coli* were screened for the genotypic diversity through pulse field gel electrophoresis and were subjected to *PCR* assay for detection of various genes coding for tetracycline, ampicillin, and sulfonamide resistances. The generic *E. coli* CFU counts were found to be similar among the three study groups. Numbers of tetracycline-resistant *E. coli* were higher in the groups fed chlortetracycline as compared to the control group. High numbers of tetracycline resistant bacteria (more than 5 $\log_{10}$ CFU/gram of dry matter in both treated groups) were found even after the fecal deposits were exposed to environment for 175 days, post-treatment. High numbers of tetracycline-resistant bacteria were observed even on day 0 in the control group. Results of this study suggest that tetracycline resistant bacteria can survive for long durations in environment. Also, the baseline tetracycline resistance is relatively high (Figure 2.1), likely due to tetracycline usage over many decades. This suggests that harboring tetracycline resistant determinants imparts minimal fitness cost to the bacteria even in the absence of selective pressures.

The cattle themselves may have acquired these resistant bacteria from environmental sources. The number of *E. coli,* except ampicillin-resistant *E. coli*, increased in the fecal deposits up to day 56 ($p<0.001$). The authors attributed a decrease in ampicillin resistant *E. coli* population to selective pressure of growth promoters in the ruminant gut that selected for the population that could not survive as well as the resistant population that did not have antibiotic

exposure. These results suggest persistence of resistant bacteria in fecal deposits for extended durations. It makes these deposits a significant source of these bacteria that may spread in environment *via* various channels like water and thereafter pose a public health risk. Factors listed that influenced bacterial persistence included: moisture content, external temperature, ability of oxygen to penetrate fecal deposits, chlortetracycline degradation time, and nutrient availability. Both antimicrobial growth promoter and the sampling time influenced the *E. coli* genotypes as determined by PFGE.

This study demonstrated the phenomenon of differential selection of resistance genes depending on the usage of antimicrobial growth promoter. The majority of the ampicillin resistant *E. coli* that harbored a *tet*(A) gene came from the chlortetracycline group and the ones that harbored *tet*(B) came from the chlortetracycline and sulfamethazine group. These results indicate the possible association of *tet*(A) gene with chlortetracyline when administered alone. The most prevalent tetracycline resistant determinant reported among tetracycline resistant isolates was *tet*(B). The between animal variation could not be reported as the samples per treatment were pooled.

The effect of ceftiofur on the selection and dissemination of the $bla_{\text{CMY-2}}$ gene was studied in cattle, both through *in vivo* conjugation experiments and a multiple herd based field study [119]. The two donor (*E. coli*) and six recipient (four *E. coli* and two *Salmonella*) strains were utilized in the *in vivo* experiment after determining their capability to exchange plasmids through prior *in vitro* experiments. Glycerol stocks for both donor and recipients were prepared for oral inoculation. The *in vivo* experiment involved 10 calves aged 3-4 months. They were randomly allocated to treatment and control groups. The study was divided into 3 phases:

pretreatment phase (Day -7 to -1), treatment phase (Day 0 to 8), and post treatment phase (Days 9 to 14). A single dose of ceftiofur treatment was given at day -7 to increase the probability of detecting ceftiofur resistant populations that could behave as donors during the experiment. Ceftiofur was administered at dose rate of 2.2 mg/kg body weight from day 2 through 6. Oral inoculums consisting of recipient strains and donor strains were given alternately from day 0 through day 13. Selective and non-selective agars were utilized to enumerate the total *E. coli* load as well as donor and transconjugant ceftiofur resistant colonies. The transconjugants were confirmed using molecular methods. No donor, recipients, or transconjugants were detected, during the pretreatment phase. The donor and recipients were detected and constituted substantial proportions of the total *E. coli* numbers in both groups during the entire course of treatment and post treatment phases. The *E. coli* populations were found to decrease during the ceftiofur treatment in the treated group. *Salmonella* isolates were detected in all calves on some experimental days but the recipient strains formed a small proportion of total lactose fermenting CFU's. Three calves in each group possessed transconjugants in their fecal samples showing the horizontal plasmid transfer in the animal gut. However, the ceftiofur treatment was not found to increase the probability of detection of transconjugants as hypothesized. There were just 5 calves in each group. The sample size does not seem enough to capture the variability in between the two groups and demonstrate statistic differences in between the two groups. The standard errors were large. The inferences were based on single trial on 10 animals. Most importantly, the ceftiofur treatment clearly reduced the *E. coli* populations during the ceftiofur treatment phase. This was the phase where authors expected to find increased proportion of transconjugants. Ceftiofur killed the sensitive bacteria that were supposed to act as the recipients. Therefore, the reduced presence of recipient *E. coli* likely adversely affected the

transconjugation process as seen from these results. The value of relative proportions of donor and recipient strains (to the total fecal coliform) drop to zero on a $\log_{10}$ scale on several occasions in both groups during the course of the study. This indicated the lack of either donor or recipient strain expected to participate in transconjugation process, at those time points. Moreover, the administration of donor and recipient strains were alternated over days both during and after ceftiofur-treatment phase. This was done to reduce the probability of conjugation process to begin in the mouths of the calves. The alternate administration of strains might have affected the transconjugation process. The successful conjugation process requires sufficient quantities of both donor and recipients strains. It was found that the fecal flora was predominated by the strains (donor or recipients) that were administered on the previous day. Therefore, the donor and the recipient may not be adequate proportion at the same time point in the animal gut. Study results may also be affected by the donor and recipient strains used. The donor strains were obtained from calves and the recipient strains were obtained from cows. The experiment was performed in calves and therefore the cow adapted recipient strains may not be able to adapt well to calf gut environment.

The herd level component of the study was conducted on 42 dairy herds in the state of Washington. Twenty eight pooled fecal samples from each herd were collected from 2006 to 2007. Each pooled samples was a composite of 10 individual fecal samples. Herd records were checked for past cases of ceftiofur resistant clinical *Salmonella* from 2003 to 2007. Purchase records of ceftiofur formulations as well as ceftiofur use within the herd were recorded. The herd level pooled fecal samples were subjected to *E. coli* enumeration and detection of *Salmonella* isolates. Antimicrobial susceptibility testing was performed using the agar diffusion technique in *E. coli* and the microbroth dilution technique in *Salmonella*. The $bla_{\text{CMY-2}}$ gene was

detected among the isolates. The herd level study also did not identify any association of the magnitude of ceftiofur usage in the herd and frequency of ceftiofur resistant *E. coli*. This led the authors to conclude that ceftiofur use does not play an important role in the persistence and dissemination of ceftiofur resistant commensal *E. coli*. However, as shown in Tragesser *et al.* (2006) [117], the within herd effects are much more difficult to identify than between herd (use/no use) effects. The usage of other antimicrobial drugs used during the dairy farm operations may have confounded the results. The short acting formulation of ceftiofur is often used in dairy farms; therefore, the results cannot readily be extrapolated to beef cattle systems where longer acting formulations usually are used.

A longitudinal study was conducted at the U.S. Meat Animal Research Center, Clay Center, Nebraska feedlot on 763 cattle [120]. Ceftiofur was the drug of choice for therapeutic purposes in this feedlot. Animals were monitored longitudinally for 10 months and fecal samples were collected during 6 sampling periods. Antimicrobial growth promoters were not administered to the animals. The authors studied the fecal and hide prevalence's of extended spectrum cephalosporin resistant *E. coli* ($ESC^{R}EC$) among the cattle, during their stay. This included the cattle that received therapeutic ceftiofur treatment. A total of 312 isolates was found to be $ESC^{R}EC$ and these were characterized using pulsed field gel electrophoresis, antibiotic susceptibility testing, plasmid size analysis, qualitative detection of bla_{CMY-2} gene, and plasmid replicon typing for 18 plasmid incompatibility groups. The authors reported that therapeutic ceftiofur use did not significantly increase the $ESC^{R}EC$ prevalence at the herd level. However, a significant increase was observed in ceftiofur-resistant *E. coli* shedding following the ceftiofur use. The authors only presented the absolute $ESC^{R}EC$ CFU counts over the study

period. They did measure the total lactose-fermenting coliforms in both fecal and hide samples and indicated that the proportion of the $ESC^{R}EC$ on the feces and hide samples were significantly lowered as compared to the total lactose-fermenting coliforms. However, the relative counts over the study period were not presented. Therefore inferences regarding the effect of ceftiofur administration on the background susceptible population cannot be made and this may have biased the results.

The baseline fecal $ESC^{R}EC$ prevalence upon cattle arrival was reported to be 3.9%. Subsequently, the herd prevalence was found to be in the range of 1.7 to 11.2%; these prevalences were not statistically higher than the $ESC^{R}EC$ baseline prevalence on arrival. The hide prevalence of $ESC^{R}EC$ was 15% on arrival. This prevalence also did not significantly differ during most of the study. All 312 $ESC^{R}EC$ isolates harbored the bla_{CMY-2} gene. The study results indicate a clear association between ceftiofur resistance and the bla_{CMY-2} gene. The persistence of bla_{CMY-2} gene was contributed to clonal expansion as opposed to the horizontal transfer of the gene. This was evident by the low diversity of PFGE results that revealed only 26 unique patterns, 12 of which were identified from the samples collected during multiple sampling periods. All 312 $ESC^{R}EC$ isolates were found to be resistant to at least 3 other antimicrobials. The most common phenotype observed in 99.7% of $ESC^{R}EC$ isolates included resistance to nine other antimicrobials. The plasmid replicon typing results revealed that IncA/C replicon was the most prevalent replicon (69.2%) followed by IncY replicon, among the 18 plasmid replicons they tested. The authors concluded that ceftiofur use on this farm may not be the most significant source of ceftiofur resistant organisms that would be present in the final meat products that pose a risk to human health.

2.9. Summary of literature review

There has been an increase in antimicrobial usage over the years, used to promote and to sustain animal agriculture in the United States. The usage of critically important drugs like ceftiofur in animal agriculture has resulted in a public health concern due to the possible transfer of resistant strains or gene determinants to humans. The percentages of ceftiofur and tetracycline resistant cattle isolates in NARMS have been found to increase over the past 15 years. The important determinants for ceftiofur resistance are the genes coding for β-lactamases like $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$. In the United States, $bla_{\text{CMY-2}}$ is mostly associated with ceftiofur resistance in animals. The $bla_{\text{CTX-M}}$ is more prevalent in Europe and is rarely reported in the United States. The most common tetracycline resistance determinants in cattle are *tet*(A), *tet*(B), and *tet*(C). Most studies investigating the effect of ceftiofur and tetracycline conclude that the antimicrobial treatment only aids in transitory increase in the resistance levels and the levels returns to the baseline once the effect the selective pressure effect is removed. However, the temporal and aggregated scale upon which these baselines are observed makes it difficult to observe and trace their steady increase over the decades.

There have been several observational studies evaluating the association of ceftiofur or tetracycline use in farms and the corresponding levels of the resistance and associated multidrug resistances. Most studies base their finding on phenotypic and genotypic culture-based assays focusing on evaluating antimicrobial effects on a single or few sentinel organisms. More recently, metagenome-based approaches are being used; these utilize community DNA to evaluate the effect of antimicrobials on the entire microbial community rather than just a few bacterial species.

Regulatory bodies like FDA have passed new guidelines and rules for restricting the usage of antimicrobials, especially the critically important antimicrobials, in food-producing animals to ensure human health. It is critical to better understand those factors that could influence resistance dynamics in terms of development, propagation, and persistence of antimicrobial resistance determinants among enteric bacteria. This understanding is imperative for being able to design and test treatment strategies at the farm levels in order to control this global problem. This study is aimed at evaluating the effects of two treatment strategies on ceftiofur and tetracycline resistance using both culture-based and metagenome-based approaches. Understanding the associations between the treatment and the resistant determinants and also the association among the various determinants is important for designing interventions strategies to control the problem of antimicrobial resistance.

Chapter 3
Quantitative analysis of bacterial resistance elements in the bovine fecal metagenome: chlortetracycline use rapidly expands cephalosporin resistance elements in a randomized controlled trial

3.1. Abstract

The objective of this study was to determine the effects of two treatment regimens on the quantities of ceftiofur and tetracycline resistance genes in feedlot cattle. The two regimens consisted of ceftiofur crystalline-free acid administration (CCFA) at either one or all the steers within a pen, and subsequent feeding/not feeding of therapeutic doses of chlortetracycline. In a 26 day randomized controlled field trial, 176 steers were allocated to 16 pens of 11 steers each. The two treatment strategies were randomly assigned to the pens in a two-way full factorial manner resulting in four treatment groups. Quantitative real-time PCR was used to quantify $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), *tet*(B), and 16S rRNA gene copies per gram of feces from fecal community DNA. Pens in which all the steers received the CCFA treatment showed an increase in ceftiofur resistance in terms of $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$ $\log_{10}$ gene copies per gram wet feces; this was in contrast to the pens where CCFA was administered to one steer among 11 pen mates. There was a significant decrease in both tetracycline resistance genes in those pens in which all animals received CCFA treatment. Thereafter, chlortetracycline administration led to rapid expansion of both ceftiofur ($bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$) and tetracycline [*tet*(A) and *tet*(B)] $\log_{10}$ gene copies per gram of feces. Chlortetracycline administration did not lead to a hypothesized decrease in ceftiofur resistance determinants in feedlot cattle; in fact, our data suggest that

chlortetracycline use is contraindicated when attempting to avoid expansion of resistance to the critically important 3rd generation cephalosporins.

3.2. Introduction

Cephalosporins are used in both animals and humans to treat a wide variety of bacterial diseases. Cephalosporins sold or distributed in the United States for use in food-producing animals for the year 2011 amounted to 26,611kgs [23]. Ceftiofur, a third generation cephalosporin, is used in beef cattle to treat bovine respiratory disease and acute bovine interdigital necrobacillosis [121]. Ceftiofur is classified as a critically important antibiotic by the World Health Organization (WHO) [5]. It belongs to the same general class of antibiotics as ceftriaxone, which has been highly valued in human medicine for treating serious and life threatening infections. Ceftriaxone is one of the few drugs accepted for use in children for treating invasive salmonellosis [122-123]. Emergence and spread of *Salmonella* resistant to 3rd generation cephalosporins among humans have been linked by some authors to ceftiofur use in veterinary practice [76, 106, 124-126]. Data from the 2010 U.S. National Antibiotic Resistance Monitoring System (NARMS) indicate that 2.8% of the non-Typhi *Salmonella* isolates arising from human clinical cases were resistant to both ceftiofur and ceftriaxone. At the same time, 21.5% of the non-Typhi *Salmonella* isolates, obtained from food-producing animals at slaughter, were resistant to both ceftiofur and ceftiaxone. This is a significant increase in comparison to the 14.5% of the non-Typhi *Salmonella* animal isolates that were resistant in 2009. The prevalence of ceftriaxone resistance among human isolates has been observed to be relatively stable since 2004; however, in 2010 ceftriaxone resistance among non-Typhi *Salmonella* cattle isolates (22%) was observed to be at its highest since NARMS testing began in 1997 [107]. In response to the observed increases in cephalosporin resistance among food animal non-Typhi *Salmonella*

isolates and to preserve antibiotic efficacy in both veterinary and human medicine, in April 2012 the U.S. Food and Drug Administration released an order restricting certain extra-label uses of cephalosporin drugs in food-producing animals [127].

Restricting or banning the use of some antimicrobial classes in animal agriculture may result in unintended consequences, such as an increase in therapeutic use of antimicrobials that may be critical for human medicine [20, 128]. There is a need to design and test intervention strategies to control ceftiofur resistance at the farm level in order to protect both animal and human health. Studies in human medicine have demonstrated a protective effect of fluoroquinolone use as an alternative intervention against the emergence of cephalosporin resistance [129-130]. Our previous work suggested that chlortetracycline in feed significantly decreased the prevalence of ceftiofur resistant *E. coli* [11]. The outcome measure in that study was strictly phenotypic utilizing a culture-based approaches; that is, the proportion of bacteria resistant to a panel of antimicrobials (NARMS CMV1AGNF Gram negative panel, Trek Diagnostics, Cleveland, OH). Further, the total bacterial load was not quantified so the effect of chlortetracycline on the background population could not be determined. This current study aims to evaluate the effects of chlortetracycline on ceftiofur and tetracycline resistance gene copies and also the 16S rRNA gene copies using quantitative RT-PCR assay, in the fecal metagenome. Relying strictly on phenotypic, or even genotypic, data from cultured isolates restricts the focus to a very small subset of the gut microbiome. Rarely are resistance elements restricted to one genus (e.g., *Escherichia* or *Salmonella*) or even family (e.g., Enterobacteriaceae). If results from our previous work [11] prove to be robust across the entire metagenome, chlortetracycline might be expected to minimize the proliferation and accumulation of ceftiofur resistant bacteria and their resistance elements in production-animal agriculture settings. Overall, in the present study,

samples were evaluated both at phenotypic [131] and genotypic levels; the latter involving both analysis of fecal bacterial isolates [131-133] as well as the broader metagenome. This chapter focuses on the results obtained from the analysis of resistance genes present in the metagenome. This quantitative study further explores the differential selection and co-selection of the resistance genes based in fecal metagenome, upon antimicrobial treatments. Several studies including this study on *E. coli* isolates demonstrated the phenomenon of differential selection of resistance genes based on chlortetracycline exposure [118, 131-132]. Other studies have likewise reported associations among various tetracycline and ceftiofur resistance genes [42-43, 64, 134].

Although not universally accepted, many scientists believe that resistance genes are associated with a fitness cost to the host, at least initially after the introduction of the drug in the market for clinical practice [9-10, 135-136]. The animals within a pen are suggested to exchange bacterial strains/resistant determinants (horizontal transmission) among each other [8]. The un-treated animals may become a source of antimicrobial susceptible bacterial strains to the treated animal harboring resistant bacteria/determinants in the pen. It has been proposed that readily available susceptible bacteria may help to promote the rapid re-colonization of the animal gut, helping to outcompete the resistant bacteria [137].

The primary objective of this study was to investigate the effects of two treatment strategies, i.e., 1) the ratio of housing of CCFA-treated with untreated animals on the levels of ceftiofur and tetracycline resistance genes copies in the fecal metagenome and, 2) the feeding of therapeutic doses of chlortetracycline following treatment with ceftiofur crystalline-free acid (CCFA). Quantitative real time PCR was performed on fecal community DNA to determine the changes in microbial populations as measured by variations in target gene copies [$bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A),

tet(B), and 16S rRNA genes] under the influence of various treatments. The study results are essentially quantitative; that is, the results are expressed in terms of both absolute resistance genes copies or standardized to the 16S rRNA gene copies representing the total bacterial population. Our primary hypothesis was that both strategies would help to decrease the levels of ceftiofur resistance elements in the fecal metagenome.

3.3. Materials and Methods

Study design

Bovine fecal samples were obtained from feeder cattle in a 26-day field trial. The randomized controlled longitudinal study was conducted on 176 steers housed in two replicates of 88 steers each. For each replicate, steers were assigned to 8 pens of 11 steers such that the average pen weights were similar. A total of four treatment groups resulted from the random allocation of two intervention strategies in a complete two-way full factorial manner. The two intervention strategies were: 1) differential pen-level CCFA treatment ratios (Mix) and, 2) subsequent pen-level chlortetracycline treatment (CTC). Pen-level CCFA treatment ratios (Mix) referred to whether long acting CCFA (Excede®, Zoetis Animal Health, NJ, U.S.A.) was administered to either one or else all 11animals within pens and pen-level CTC treatment referred to whether all cattle in a pen received chlortetracycline treatment top-dressed in feed subsequent to the CCFA treatment, respectively. The study day (Day) was considered as the third factor in the statistical models. This accounted for the effect of day of trial on the quantities of resistance genes detected in feces from the steers.

A single dose of CCFA treatment (Excede®, Zoetis Animal Health, NJ, U.S.A.) was injected subcutaneously in all steers in 8 of the 16 pens at a dose rate of 6.6 mg/kg, on Day 0. Subsequently at the base of the ear, 4 of these 8 pens received chlortetracycline treatment at the

labeled dose rate of 22mg/kg body weight (Aureomycin®, chlortracycline complex equivalent to 220.5g of chlortetracycline/kg, Alpharma, Bridgewater, NJ). Chlortetracycline was administered during three separate 5-day periods (with a one-day break in between). It was top-dressed after the morning feed was delivered, according to the label directions starting at Day 4. In the remaining 8 pens, one steer among 11 pen-mates within the pen received CCFA treatment. Subsequent chlortetracycline treatment among these 8 pens was likewise given to all animals in just 4 of the pens (Table 3-1). All allocations of animals to pens, pens to treatments, and treatments to animals within pen (where indicated; differentially CCFA treated pens) were *via* randomization protocols.

Sample collection and qPCR methods

Fecal grab samples were collected per rectum every other day from Day 0 to Day 26, after restraining the animals in a squeeze chute. A new obstetric sleeve was used to collect fecal sample from each animal. All samples were transported on ice to the laboratory on the day of sample collection. A total of 2,424 samples were collected. Five milliliter vials were filled to the 4 ml level with fresh feces. These fecal samples were stored at -70°C for further genotypic analysis.

Community DNA Extraction

Total community DNA was extracted from 200mg feces *via* the QIAamp DNA Stool Mini Kit ™ (Qiagen, Valencia, CA) according to manufacturer's instructions. The extraction was performed in the QIAcube robot ™ (Qiagen, Valencia, CA). The quality and concentration of DNA samples were determined *via* NanoDrop® ND-1000 UV-Vis Spectrophotometer

(NanoDrop Technologies, Wilmington, DE). The DNA concentration in ng/µl was recorded. These DNA samples were used for further genotypic quantification.

Genotypic quantification of target genes in community DNA

Gene copies/g wet feces of *bla*$_{CMY-2}$, *bla*$_{CTX-M}$, *tet*(A), *tet*(B), and 16S rRNA genes were determined in fecal community DNA using quantitative real time PCR (qPCR). All 4 resistance gene copies were standardized to the 16S rRNA gene, used as the reference gene in this study. The PCR plates were set up using an automated robot (QIAgility^{TM}, Qiagen, Valencia, CA). The fecal community DNA was used directly as a template in qPCR reactions for quantification of the genes using a Stratagene Mx3005P^{TM} real-time qPCR system (Agilent Technologies, La Jolla, CA). The real time qPCR reactions for *bla*$_{CMY-2}$ and 16S rRNA genes were adapted from a technique previously described [106]; qPCR reactions for *tet*(A), and *tet*(B) genes were adapted from [138]; and qPCR reactions for *bla*$_{CTX-M}$ were adapted from [133]. All 2,424 samples were quantified for *bla*$_{CMY-2}$ and 16S rRNA genes per gram; however, only samples from Days 0, 4, 12, and 26 were chosen for *tet*(A), *tet*(B), and *bla*$_{CTX-M}$ gene quantification. All community DNA qRT-PCR assays were run in triplicate.

Primers

Nested PCR was used to quantify two of the target genes. Broad range primer sets (585F and 1038R) and (1056F and 1456R) for *bla*$_{CMY-2}$ and 16S rRNA genes, respectively, were used to obtain the PCR products. These PCR products were used further as template for the standard curves in the nested runs. The second primer sets (675F and 738R) for *bla*$_{CMY-2}$ and (P201 and P1370) for 16S rRNA genes, respectively, were used in the nested amplification to determine the target gene copies in the unknown samples. A single pair of primers specific for each of *tet*(A),

tet(B), and *bla*$_{CTX-M}$ genes were used for both PCR template generation as well as the quantification of the unknown samples. Primers set details are listed in Table 3-2.

Controls

Negative control: Two negative controls were run with each PCR plate. One was the no-template control and the other had water as a template.

Positive Control: *E. coli* strain (M1) was used as the positive control for the *bla*$_{CMY-2}$ and 16S rRNA gene. It was obtained from the University of Illinois, Chicago [139]. XL1-Blue *E. coli* strain (Agilent Technologies, Santa Clara, CA) and *E. coli* ATCC 47042 were used as the positive controls for *tet*(A) and *tet*(B) genes, respectively. The *bla*$_{CTX-M}$ positive control was an *E. coli* isolate internal control strain obtained from this study, which was positive on PCR with the specific primers. The PCR product from this strain was sequenced for confirmation of the *bla*$_{CTX-M-32}$ gene and has been published elsewhere [133]. The positive control strains were plated on blood agar (Thermo Fisher Scientific, Lenexa, KS) and were incubated at 37°C for 18 hours. DNA isolation was carried out by suspending a colony in 500µl of nuclease free water (Qiagen, Valencia, CA) and then by heating the suspension at 95°C for 10 minutes and storing at -20°C. One positive and two negative controls were run with each plate. These controls were also used for the generation of template for the standard curve in the 1st amplification reaction.

Template for the standard curve generation- 1st amplification reaction

Real time qPCR reactions were performed with broad range primers for 2 genes (*bla*$_{CMY-2}$ and 16S rRNA) in a total volume of 22µl. Each reaction contained 12.5µl of 2×Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA), 4.75µl of nuclease

free water (Qiagen, Valencia, CA), 1µl of each primer, 0.75µl of reference dye, and 2µl of the DNA extracted from M1 strain (positive control). For the *bla*$_{CMY-2}$ gene, the thermal profile for amplification was 95°C for 10 minutes, 35 cycles of 95°C for 10seconds, 55°C for 45 seconds, and 84°C for 20 seconds. Similarly, for the 16S rRNA gene, the thermal profile for amplification was 95°C for 10 minutes, 35 cycles of 95°C for 30seconds, 60°C for 1minute, and 72°C for 30seconds. Both *tet*(A) and *tet*(B) gene quantification reactions were run in a total volume of 20µl. Each reaction contained 10µl of 2×Brilliant II SYBR® Green QPCR Master Mix, 6.25µl of nuclease free water, 0.5µl of each primer, 0.75µl of reference dye, and 2µl of the positive control DNA [XL1-Blue *E. coli* strain-*tet*(A) and *E. coli* ATCC 47042-*tet*(B)]. The thermal profile for amplification for both *tet* genes was 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 28 seconds. The *bla*$_{CTX-M}$ gene quantification reactions were run in a total volume of 25µl. Each reaction contained 12.5µl of 2×Brilliant SYBR® Green II QPCR Master Mix, 9.5µl of nuclease free water, 0.625µl of each primer, 0.75µl of reference dye, and 1µl of the positive control DNA. The thermal profile of these reactions was 94°C for 5 minutes, 40 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute. Thermal profiles for all genes targets ended with one last single cycle to obtain a dissociation curve, which consisted of 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds. All real time PCR reactions were carried in the Mx3005P™ (Agilent Technologies, Santa Clara, CA).

The PCR products were purified using a DNA purification Kit (Promega Corporation, Madison, WI). This purified DNA was used as the template for generating standard curves, after determining its concentration *via* NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at wavelengths of 260 and 280nm. The quantities of target gene

copies µl-1 for standard curve generation were determined under consideration of the molecular weight of the amplicons, weight of one mole of base pairs, and concentration of PCR product. Standard curves used the 10 fold serial dilution of the 2µl purified PCR products (with known number of gene copies) to 18µl of nuclease-free water to a minimum concentration of 10^0 for *bla*$_{\text{CMY-2,}}$ both tetracycline genes, and *bla*$_{\text{CTX-M}}$ and a minimum concentration of 10^3 for 16S rRNA gene. The lowest five concentrations were used to build each standard curve. The standard curve was run in triplicate for every plate.

Quantification of target genes in unknown samples-2nd amplification reaction

Narrow range primers for *bla*$_{\text{CMY-2}}$ and 16S rRNA genes and the same set of 1st amplification reaction primers for the *tet*(A), *tet*(B), and *bla*$_{\text{CTX-M}}$ resistance genes were used to quantify the gene copies in the unknown samples along with the standard curve. All reactions, including standards, were run in triplicate in a total volume of 22µl for *bla*$_{\text{CMY-2}}$ and 16S rRNA genes, 20µl for *tet* genes, and 25 µl for the *bla*$_{\text{CTX-M}}$ gene. The reaction mixture composition was same as that of the 1st amplification reaction for all respective genes. The thermal profile for amplification in the Stratagene Mx3005P^{TM} system (Agilent Technologies, Santa Clara, CA) for the *bla*$_{\text{CMY-2}}$ gene was 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 52°C for 30 seconds, and 79°C for 17 seconds. Likewise, the thermal profile for the16S rRNA gene was 94°C for 10 minutes, 40 cycles of 94°C for 2 minutes, 60°C for 1 minute, and 72°C for 17 seconds. Thermal profiles for the *tet*(A) and *tet*(B) and *bla*$_{\text{CTX-M}}$ resistance genes were the same as for the 1st amplification reaction. Dissociation curves were run with every plate to examine the specificity of the amplifications. Sequencing of PCR products was carried out on an ongoing basis for a random subset of reaction runs to further confirm the specificity of the products.

Comparisons against published online Basic Local Alignment Search Tool database at the National Center for Biotechnology were conducted.

Statistical Analysis:

The gene copy number estimates were derived from either 1or 2 µl of the sample community DNA depending on the gene being quantified. The gene quantities per microliter were back calculated to gene copies per gram wet feces. The factor determined for back calculation to gene copies per gram of wet feces was 7,000 or 3,500 depending on whether 1 or 2 µl community DNA template was used in final assay. Community DNA was extracted from 200mg feces (one gram fecal sample would be expected to have 5 times greater gene quantities). The DNA extraction process had a total calculated DNA loss by a factor of 7. The final DNA suspension obtained was 200 µl. Only 1-2µl DNA was used in the final assay depending on the gene quantified; therefore, the final volume used was expected to have 200 (or 100) times less DNA as compared to the 200 µl final volume. The final factor was the product of all the factors (5, 7, 100 (or 200)) constituting the total loss of DNA during the process. Non-standardized $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), and *tet*(B) gene copies per gram wet feces and the quantities obtained by standardizing them in a ratio with the 16S rRNA gene were used for the outcomes. All standardized and non-standardized quantities were logarithmically transformed to log base 10 for use as a dependent variable in both imputation and linear mixed models.

A missing value was recorded in instances where qPCR amplification was below the quantification limit of the assay or else was below the threshold limit determined by the assay. These observations were considered missing. These observations were assumed to have values more than zero but below the limit of quantification of the assay utilized. Estimates of missing

values were imputed using multiple imputation procedures in STATA® SE Release 12.1 (STATA Corp., College Station, TX). This is a simulation-based method to handle the missing values. Several plausible imputed datasets are created in this procedure and results from each are combined by taking the average over the distribution of these missing data. This permits accounting for the uncertainty linked with the missing data [140]. These imputed datasets for missing values were sampled from a predictive distribution which was based on the observed data. Therefore, this procedure is based on a Bayesian approach [140]. A multiple regression method was utilized for the imputation process. Importantly, multiple imputation procedures utilize the known covariates that are believed to be associated with the missing values [141]. Our imputation model was based on the unique animal id (repeats within animal), sampling day, and the number of observations missing among the triplicate for each sample (i.e., 1, 2 or all 3 triplicates were missing). Twenty imputations were performed to reduce the sampling variability from the imputation process [142]. The descriptive statistics of several imputed datasets (out of 20 datasets) were compared to the non-imputed dataset and also results of the main linear regression models before and after imputation were compared to rule out any bias that might introduced during the imputation procedure.

To determine the effect of treatment on gene quantities, a multi-level mixed linear model was utilized on the datasets following imputation. The three explanatory variables tested in the model were pen-level CCFA treatment (ratio at which CCFA was administered within a pen), pen-level CTC administration, and the sampling days (period). The relationships among the outcome and the explanatory variables were assessed using the mixed linear regression model with three-way full factorial design of the three explanatory variables (fixed effects) in STATA® SE Release 12.1 (STATA Corp., College Station, TX). The models accounted for pen level

dependencies as random effects. However, due to convergence issues, the animal level dependencies could not be accounted for within the linear regression model.

3.4. Results

The *bla*$_{\text{CMY-2}}$ and 16S rRNA gene copies per gram of wet feces were determined from community DNA extracted from all 2,424 fecal samples obtained from the trial. There were 176 steers studied in this trial for 26 days. Fecal samples were collected every other day. Forty samples from 7 animals could not be collected as the animal either became sick or died during the trial. Further, *bla*$_{\text{CTX-M}}$, *tet*(A), and *tet*(B) genes copies per gram of wet feces were determined from only 694 fecal samples obtained from Days 0, 4, 12, and 26 during the trial. These 4 days represent pre-treatment, effect of differential CCFA administration, effect of chlortetracycline administration, and the post-treatment washout phases, respectively. All community DNA qPCR assays were run in triplicate.

The multiple imputation technique was efficient in imputing reasonable values to the missing observations in the dataset. This technique avoids a left-truncated distribution that is commonly observed since the assay cannot provide gene quantities values below the limit of quantification (LOQ); the LOQ of the assay being the lowest value observed among DNA samples as shown in Figures 3.1 and 3.2. In our study, depending on the gene, there were as few as zero and as many as 435 missing observations. The *bla*$_{\text{CMY-2}}$ and 16S rRNA gene quantity datasets derived from all 2,424 community DNA samples (7,272 total observations) had 179 and 0 missing observations, respectively. The data obtained from a subset of community DNA samples from the selected four days had 44, 435, 2, and 431 missing observations for *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M}}$, *tet*(A), *tet*(B) genes, respectively, from a the total of 2,084 observations. In Figure 3.1

(i, ii), the histograms demonstrate the $\log_{10}$ *bla*$_{\text{CTX-M}}$ gene quantity distribution from raw and imputed data, graphed by the number of observations missing (n=0, 1, 2, or 3) among the triplicates of one community DNA extract (Figure 3.1 i, ii; sub graphs: A, B, C, and D). Out of the 20 imputations performed, the histograms shown here are from the results of the single imputation to demonstrate the distribution of the gene copies before and after imputation procedure. Sub graphs A, B, C, and D show the distributions of the observations that had no missing triplicates, one missing triplicate, two missing triplicates and all three missing triplicate, respectively. The peak at -1.15 $\log_{10}$ gene copies in the non-imputed histograms represents the observations that were missing in each category. The limit of quantification for the *bla*$_{\text{CTX-M}}$ gene real time PCR quantification assay was 1.82 $\log_{10}$ gene copies per gram. The distribution is therefore left truncated at this quantification limit (Figure 3.1 i; subgraphs A, B, and C). Figure 3.1i; subgraph D shows the distribution of observations that had three missing triplicates. It can be seen that the imputation process distributed these observations in a way that yielded many more observations below the 1.82 $\log_{10}$ gene copies quantification limit as compared to the single or double missing triplicates. Similarly, raw and imputed gene distributions for the *tet*(B) gene are shown in Figure 3.2 i, ii. The limit of quantification for the *tet*(B) gene was -0.56 $\log_{10}$ gene copies. Overall, the distribution of missing observations was shifted much more to the left (i.e., fewer gene copies) since the number of missing observations among triplicates increased.

The marginal predicted means of *bla*$_{\text{CMY-2}}$ $\log_{10}$ gene copy numbers per gram wet feces alone, and standardized to 16S rRNA gene copy numbers, stratified by the four treatment groups are shown in Figure 3.3A and B. The baseline, Day 0, predicted mean $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram wet feces (± SE) for the four treatment groups were 4.29±0.12, 4.52±0.12, 4.55±0.12, and 4.70±0.12. The two treatment groups to which CCFA was administered to all steers within a

pen on Day 0 showed an increase on Day 4 in the $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram as compared to the other two groups as shown in Figure 3.3 (sub graphs A and B). Chlortetracycline treatment starting after Day 4 led to further expansion of $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram, both in the CCFA treated and non-treated group, as seen on Day 12.

Figure 3.3C and Figure 3.4A and B shows the16S rRNA gene copies alone. The baseline, Day 0, predicted mean $\log_{10}$ 16S rRNA gene copies per gram ± SE for the four treatment groups were 10.06±0.05, 10.13±0.05, 10.10±0.05, and 10.01±0.05. CCFA administration at different proportions did not lead to any significant changes in the total 16S rRNA gene copies per gram feces. CTC seemed to increase the total 16S rRNA gene copies per gram feces. The effect was more pronounced in the pens in which all steers had prior CCFA treatment. The two way interactions of CTC and 6 sampling days (10 to 20) in the mixed linear model were highly significant ($p<0.05$).

Effects of treatments on the absolute and standardized ceftiofur resistance (*bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M}}$) gene copy numbers per gram wet feces for Days 0, 4, 12, and 26 are represented in Figure 3.4A and B, respectively. Day 4 illustrates the effect only of CCFA on the ceftiofur resistance gene copies. Fecal samples collected from animals in pens in which CCFA was administered to all the animals within the pen (Figure 3.4A and B, Day 4, depicted by dark navy and maroon lines) had a greater number of ceftiofur resistance determinants, *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M}}$ gene copies per gram, as compared to the other two treatment groups where CCFA was administered to one steer among 11 pen-mates. In the latter pens ceftiofur resistance seemed to decrease mildly at the pen level on Day 4. Day 12 in Figure 3.4A and B illustrate the effect of chlortetracycline on the absolute and standardized ceftiofur resistance gene copies, respectively. Chlortetracycline treatment was found to increase both *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M}}$ gene copies per

gram as compared to the pens which did not receive chlortetracycline treatment (Figure 3.4A and B, Day12, depicted by dark navy and green lines). The chlortetracycline effect was consistent across pens receiving CCFA at different proportions, suggesting that priming of animals/pens with CCFA was not required for chlortetracycline to have an effect.

Effect of treatments on absolute and standardized tetracycline resistance [*tet*(A) and *tet*(B)] gene copy numbers per gram wet feces for the four days tested are represented in Figure 3.5A and B, respectively. CCFA significantly decreased both *tet*(A) and *tet*(B) genes copies per gram feces (Figure 3.5A and B, Day 4, depicted by dark navy and maroon lines) at the pen level as compared to the pens in which CCFA was administered to one steers among 11 pen-mates. As expected, there was also a marked increase in each of the tetracycline resistance *tet*(A) and *tet*(B) gene copies per gram feces following chlortetracycline treatment (Figure 3.5A and B, Day12, depicted by dark navy and green lines).

3.5. Discussion

The hypothesis for this study was based on previous work that suggested that sparing chlortetracycline lowered the proportion of ceftiofur resistant *E. coli* isolates found in bovine feces [11]. In that study, chlortetracycline appeared to differentially favor *E. coli* population that were singly, doubly or triply resistant to tetracycline versus those in which ceftiofur and tetracycline resistance were both present and always with at least 6 other resistance types [11]. The results of that study suggested the intriguing possibility for using chlortetracycline as an intervention to control ceftiofur, if only in the short term.

In the present study, samples were evaluated by both culture-based [131-133] as well as metagenome-based assays. This chapter focuses on the results obtained from the analysis of

resistance genes copies present in the entire fecal metagenome. Also, the 16S rRNA gene copies were quantified to take into account the effect of treatment strategies on the total bacterial population.

In the present study, chlortetracycline did not reduce ceftiofur resistance in the fecal metagenome; in fact, gene copy numbers of both *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M}}$ genes significantly increased following chlortetracycline treatment. There are aspects of this study design that could have caused this discrepancy with the results of Platt *et al*.(2008) [11]. In the present study, either one or all animals within each of 8/16 pens received ceftiofur treatment prior to chlortetracycline treatment. Initial ceftiofur exposure to animals might have changed the microbial gut population at the pen level enough to have modified the effect of CTC [132]. Importantly, any chlortetracycline effect that would have been expected, unlike the previous study, would have been observed on a microbial population at disequilibrium.

The *bla*$_{\text{CMY-2}}$ gene has previously been quantified in two longitudinal trials; one in beef cattle [106] and one in dairy cattle [143], to evaluate the effect of ceftiofur treatment. The former trial was an experimental study in which three different dose regimens of ceftiofur were compared. The results of that study suggested that repeated regimens (three doses of 6.6mg/kg versus a single dose of 4.4 mg/kg) favored greater expansion of *bla*$_{\text{CMY-2}}$ gene; however, there also was a concurrent reduction in the bacterial population among the total microbial fecal flora. Therefore, in these two regimens, higher group means of standardized $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram were observed while lower group-means of non-standardized $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram were observed following ceftiofur treatment. The third regimen was more similar to the one used in the current study. It consisted of a single dose of 6.6mg/kg. Alali *et al.* (2009) showed [106] that the ceftiofur treatment significantly increased both the standardized as well as

non-standardized $\log_{10}$ *bla*$_{\text{CMY-2}}$ gene copies per gram feces; in fact, this was similar to what was observed in our study. The quantity of *bla*$_{\text{CMY-2}}$ gene in our study also was comparable to earlier feedlot cattle study [106]; however, the *bla*$_{\text{CMY-2}}$ gene quantities were lower for the dairy cattle study reported by Boyer *et al.*(2012) [143]. Those authors attributed this difference to the possibility that feedlot cattle likely harbor higher *bla*$_{\text{CMY-2}}$ gene copies as compared to the dairy cattle because of the variation in management and environment. All three studies have recorded a temporary increase in *bla*$_{\text{CMY-2}}$ gene copies immediately following ceftiofur treatment. We did not observe any significant effects on the 16S rRNA gene copies upon ceftiofur treatment. In the earlier feedlot cattle study [106], the treatment group that received a similar ceftiofur dose to this study had overall higher predicted mean $\log_{10}$16S rRNA gene copies per gram when compared to the control group. Results from both studies indicate that there was a selective advantage favoring expansion of ceftiofur resistance elements (and likely bacteria harboring same) as opposed to a significant reduction of overall bacterial community following ceftiofur treatment. The dairy cattle study [143] did not report quantification of a bacterial reference gene.

The *bla*$_{\text{CTX-M}}$ gene was detected in *E. coli* [132-133] as reported earlier, and was also quantified from community DNA samples derived from feces in this study. For both, the targeted ceftiofur resistance genes, *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M}}$ gene copies per gram feces significantly increased in treatment groups where all animals were administered CCFA as compared to the pens where only one animal received CCFA treatment. The short-term selection of *bla*$_{\text{CMY-2}}$ gene copies following CCFA administration has been documented as mentioned before. However, this is the first study to quantify *bla*$_{\text{CTX-M}}$ gene in feedlot cattle in the United States. The quantities of tetracycline genes *tet*(A) and *tet*(B) significantly decreased in treatment pens where all animals were administered CCFA as compared to the pens where only one

animals received CCFA treatment. Several phenotypic studies on *E. coli* in the past have recorded that all ceftiofur resistant isolates found in those study were co-resistant to tetracycline [11, 116, 124]. Therefore, the decrease in copies per gram wet feces of both of the tetracycline gene types from community DNA was not expected; however, it is readily explainable. First, *E. coli* constitutes a small fraction of the entire microbial community. That is, the microbial community constitutes of both culturable and non-culturable organisms that may or may not carry ceftiofur and tetracycline determinants together on one plasmid. Secondly, even though those *E. coli* that remain following ceftiofur treatment are resistant to both ceftiofur and tetracycline, those bacteria that are resistant only to tetracycline do not survive, or else are suppressed. The complexity of the co-selection or co-resistance mechanisms has been discussed by Doyle *et al.* (2013) [39]. Microbial population may evolve in a different ways under specific selection pressure in different niches depending on the complexity of the niches and the selection pressure, particularly if the starting bacterial population is not exactly the same. Competitive bacterial interactions plays a critical role in niches like host gut harboring large numbers of varied microbes [39]. The $bla_{\text{CTX-M}}$ gene found among 29 out of these 88 study animals was reported to be harbored on IncN plasmid, among the *E. coli* isolates. This plasmid was shown to be self-transmissible without conferring any additional resistance to the recipient *E. coli* [133]. This indicated the presence of *E. coli* isolates that harbored plasmids which possessed resistance determinants towards ceftiofur alone.

There were distinct differences observed between the *E. coli* (culture-based assay) results [131-132] and the gene quantification results obtained in this study. The qPCR results from the community DNA indicated an increase in both $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$ gene copies per gram wet feces following chlortetracycline treatment. This runs counter to the results obtained from *E.*

coli isolates where *bla*$_{\text{CMY-2}}$ gene was differentially favored over the *bla*$_{\text{CTX-M}}$ gene [132]. Similarly, *tet*(A) and *tet*(B) gene copies per gram wet feces significantly decreased in contrast to an increase in proportion of *E. coli* isolates harboring *tet*(A) and *tet*(B), following ceftiofur treatment [131]. These results clearly indicate the bias that can be introduced if conclusions regarding microbial populations are based solely on particular culturable organisms. There is a huge unculturable microbial population that might well be expected to behave differently under similar selective pressures. Further, it has been indicated before that the bacterial cells may also lose the entire plasmid or parts of the plasmid carrying resistance determinants, or the resistance genes may not be expressed under culturing stress [106]. Therefore, results from specific culturable organisms may incorrectly estimate the prevalence of target resistance gene among the gut flora. The results from metagenome-based assays also have limitations; there may be multiple and varying amount of target gene copies per bacterial cell (relative abundance) among different bacterial species [106, 144]. The metagenome-based assay gives information about the overall target gene load in community DNA. The information about variation of specific bacterial species carrying target genes is not provided from this assay. Most antimicrobial resistance monitoring and surveillance systems across the world today test isolates and/or samples for antimicrobial resistance using culture-based methods. Extrapolating the results obtained from specific culturable organisms (e.g. *E. coli, Salmonella*, or *Campylobacter*) to the entire microbial community may not be valid. This can heavily influence the policies formulated by the regulatory bodies to control antimicrobial resistance in humans and animals. Therefore, a metagenome-based component should be considered as part of a more holistic monitoring system to identify when contradictory or paradoxical results are seen in order to avoid such bias.

A multiple imputation procedure was used to deal with missing data in this study. Multiple imputation has been suggested to produce less-biased estimates and more accurate standard errors as compared to other approaches like single imputation, mean imputation, and complete case analysis [145]. However in a recent study, it was proposed that multiple imputation techniques did not improve a linear mixed model when missing data represented up to 40% of the sample set [146]. However, these authors acknowledged that multiple imputation procedure may be useful if such models included relevant predictor variables, which were not included in their linear mixed models. We based our imputation model on unique animal identifier, sampling day, and the number of observations missing among the triplicate of each sample (i.e., 1, 2, or all 3 triplicates were missing). The number of missing observations among the triplicates was considered as an important predictor similar to the previous study to impute missing quantitative real-time PCR data [143]. The histograms resulting from the imputed datasets, demonstrating $\log_{10}$ distributions of gene copies per wet gram feces, and based on the number of missing observations per triplicate, reveal that the distribution of missing observations shifts towards the left (including additional imputed observations at lower gene copies) as the number of missing values among triplicate increases. For those observations where all three data points among the triplicates were missing were more likely to be assigned a lower value and also a value below the assay limit of quantification when contrasted to observations with either double or single missing values among the triplicates. It was found to be a useful tool to handle missing data in order to obtain valid inference from parametric statistical models similar to the ones used in this study, whose assumptions depend on the integrity of underlying distributions. It is an effective procedure to analyze qRT-PCR data, which has moderate levels of missing values.

Overall, our study results indicate that chlortetracycline should not be used to control ceftiofur resistance. Chlortetracycline treatment led to significant expansion of both $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$ genes copies per gram wet feces in cattle pen previously exposed to ceftiofur in varying ratios of treatment. Pen-level ceftiofur resistance elements were present in lower numbers in pens where one CCFA treated animal was co-housed with other non-treated pen-mates. Further studies with mixing at different treatment ratios should be conducted in order to establish the threshold of the co-mingling effect on levels of antimicrobial resistance. In addition, there were distinct differences observed between culture-based results reported elsewhere [131-132] and the metagenome-based results obtained herein; this warrants further investigation as current worldwide antimicrobial resistance monitoring and surveillance systems tend to be based solely on culture-based approaches. These may provide a biased picture of the changes occurring in the microbiome, depending largely on the organisms being observed. The incorporation of gene quantification and other metagenome-based approaches in such surveillance systems may dramatically increase a more holistic understanding in this sphere of antimicrobial resistance.

176 steers; 16 pens; 4 treatment groups			
Group 1	**Group 2**	**Group 3**	**Group 4**
4 pens, 11 steers each - All 11 steers receive CCFA; 6.6 mg/kg once Day 0 - CTC 22mg/kg; 3 treatment regimens- 5 day each	4 pens, 11 steers each -All 11 steers receive CCFA; 6.6 mg/kg once Day 0 -No CTC Treatment	4 pens, 11 steers each -One among 11 steers receive CCFA; 6.6 mg/kg once Day 0 -CTC 22mg/kg; 3 treatment regimens- 5 day each	4 pens, 11 steers each -One among 11 steers receive CCFA; 6.6 mg/kg once Day 0 -No CTC treatment

Table 3-1 Schematic representation of the study design.
The two interventions were assigned in a two-way full factorial manner. Number of pens assigned to each treatment and number of animals within each of the pens are shown above.

Gene name	Primer	Primer Sequence	Expected Product Size (bp)	GenBank Accession no.*
bla$_{CMY-2}$	585F	5'- CAG ACG CGT CCT GCA ACC ATT AAA -3'	454 [a,1]	AB212086
	1038R	5'- TAC GTA GCT GCC AAA TCC ACC AGT -3'		
	675F	5'- AGG GAA GCC CGT ACA CGT T -3'	64 [a,2]	
	738R	5'- GCT GGA TTT CAC GCC ATA GG -3'		
16S rRNA	1056F	5' - AAT GTT GGG TTA AGT CCC GCA ACG - 3'	400 [a,1]	EU014689
	1456R	5' - ATG ATC ACA AAG TGG TAA GCG CCC - 3'		
	P201	5'- GAG GAA GGI GIG GAI GAC GT - 3'	216 [b,2]	
	P1370	5' - AGI CCC GIG AAC GTA TTC AC - 3'		
tet(A)	*tet*(A)(F)	5' -GCTACATCCTGCTTGCCTTC- 3'	210 [c]	X61367
	tet(A)(R)	5' -CATAGATCGCCGTGAAGAGG- 3'		
tet(B)	*tet*(B)(F)	5' -TTGGTTAGGGGCAAGTTTTG- 3'	659 [c]	J01830
	tet(B)(R)	5' -GTAATGGGCCAATAACACCG- 3'		
bla$_{CTX-M}$	*CTX-M*(F)	5' -ATGTGCAGYACCAGTAA- 3'	536[d]	AY143430_CTX-M-24
	CTX-M(R)	5' -CCGCTGCCGGTYTTATC- 3'		

Table 3-2 Primers used for PCR reactions

[a] Primer set used are from Alali *et al.* [106]

[b] Primer set used are from Steinman *et al.* [147]

[c] Primer set used are from Ng *et al.* [148]

[d] Primer set used are from Cottell *et al.* [133]

[1] Broad range primers to generate template for the standard curve

[2] Narrow range primers for sample quantification

* Sequence used for primer design

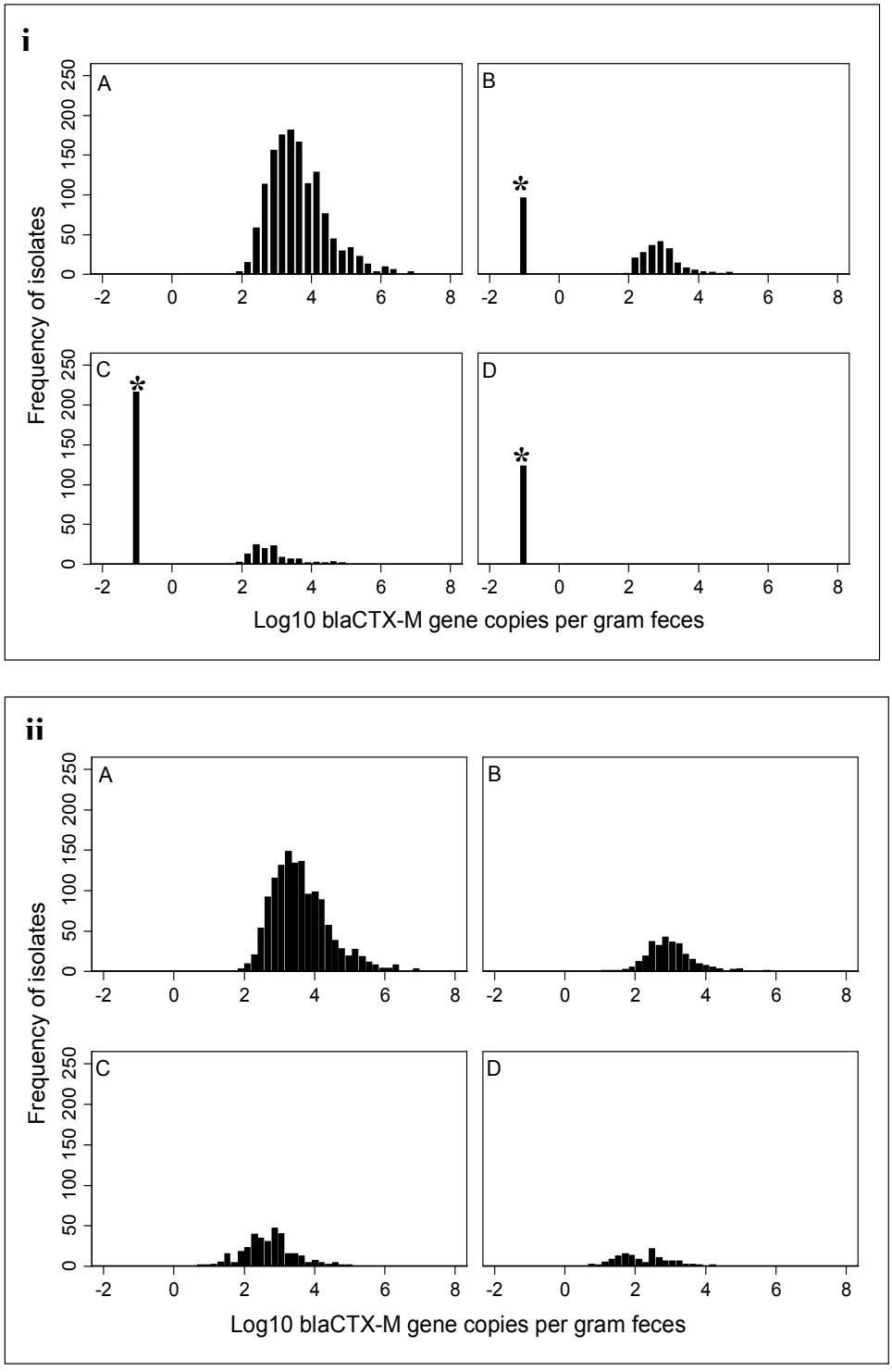


Figure 3.1 Histograms illustrating (i) overall raw distribution of $\log_{10}$ *bla*$_{\text{CTX-M}}$ gene copies per gram feces and, (ii) distribution of $\log_{10}$ *bla*$_{\text{CTX-M}}$ gene copies per gram feces with a single imputation, subgraphed by the number of missing observations (A=0, B=1, C=2, D=3) among the PCR triplicate assays. Note: * represents missing observations.

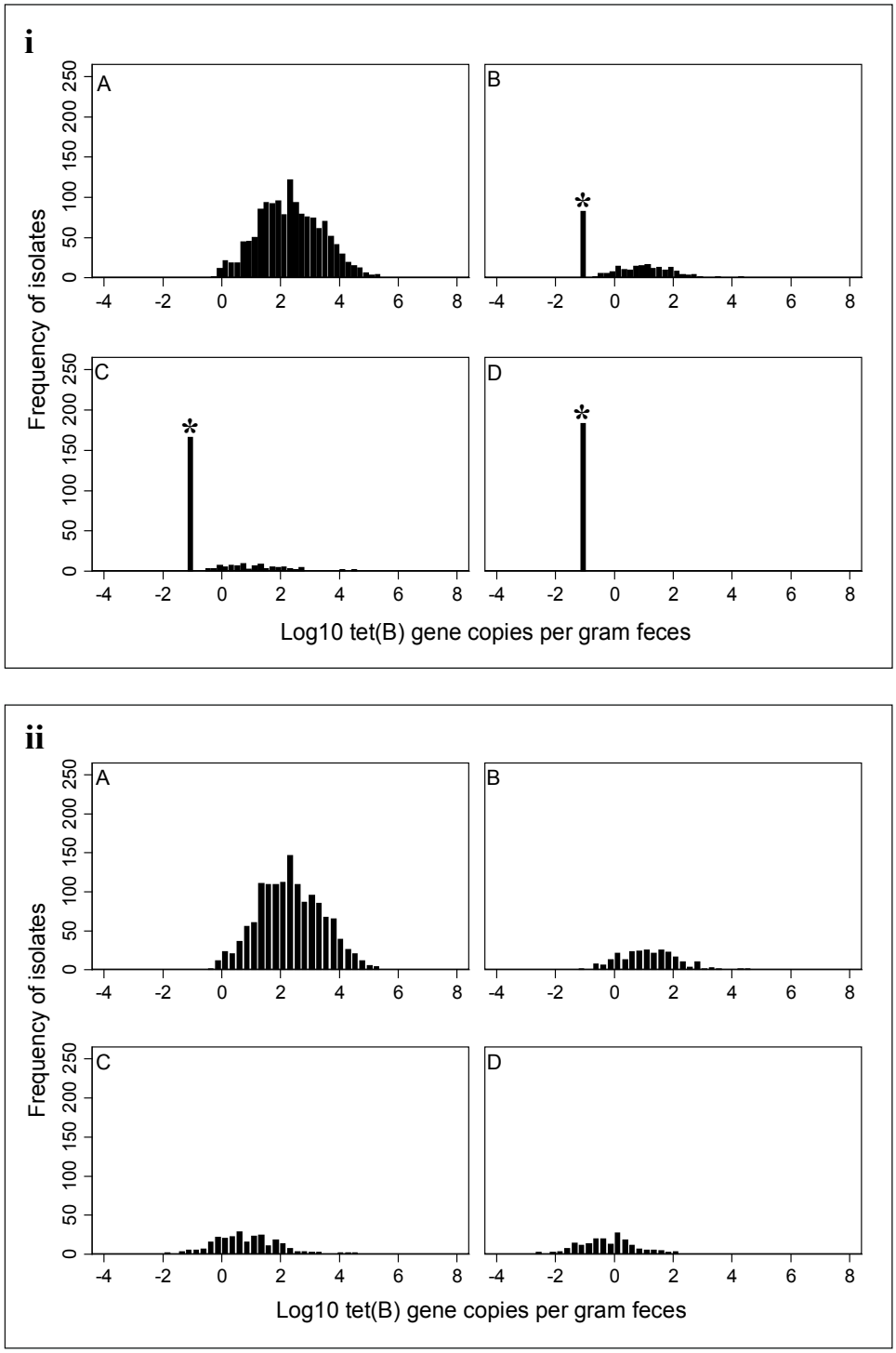
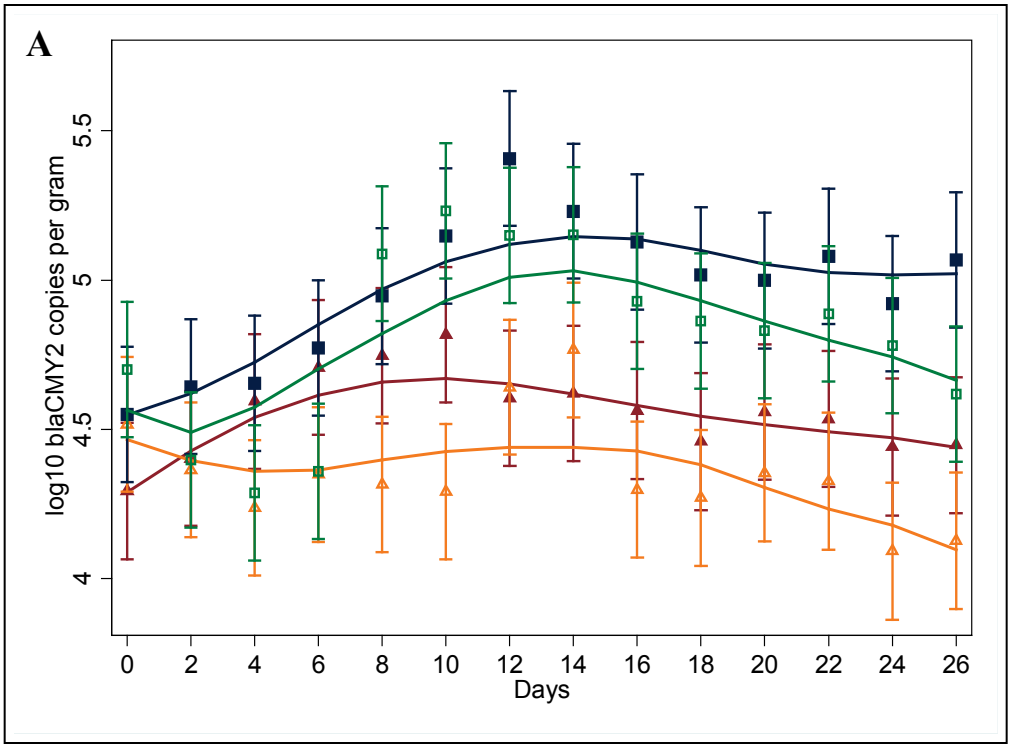
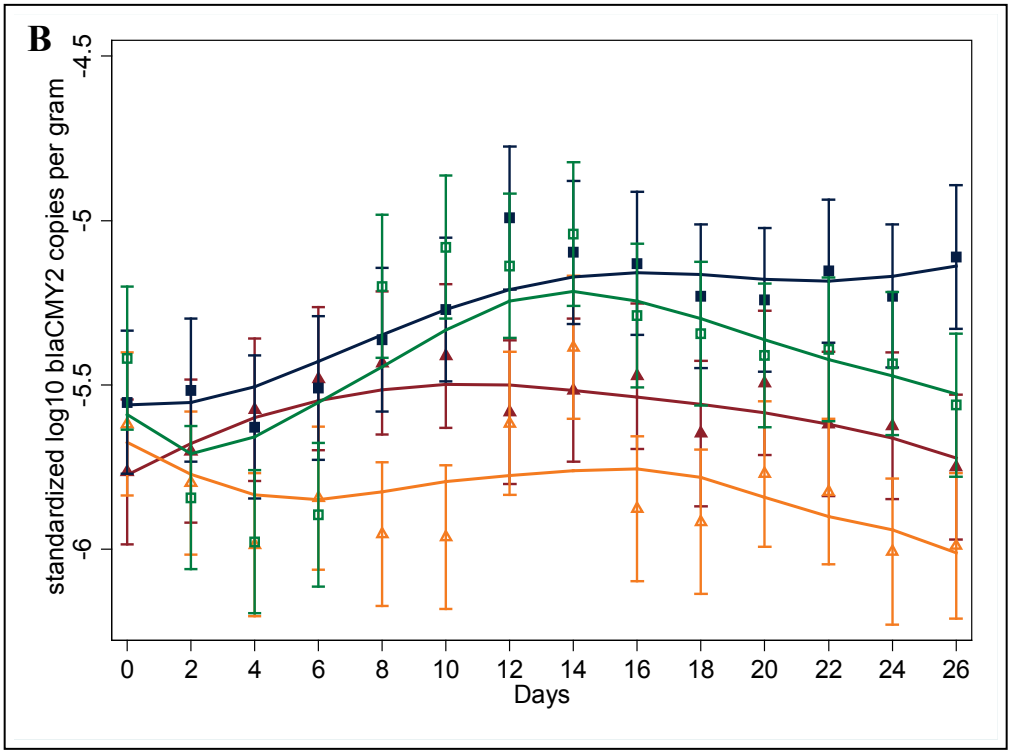


Figure 3.2 Histograms illustrating: (i) overall raw distribution of $\log_{10}$ *tet*(B) gene copies per gram feces and, (ii) distribution of $\log_{10}$ *tet*(B) gene copies per gram feces with a single imputation, subgraphed by the number of missing observations (A=0, B=1, C=2, D=3) among the PCR triplicate assays. Note: * represents missing observations.


A
log10 blaCMY2 copies per gram
4
4.5
5
5.5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Days


B
standardized log10 blaCMY2 copies per gram
-6
-5.5
-5
-4.5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Days

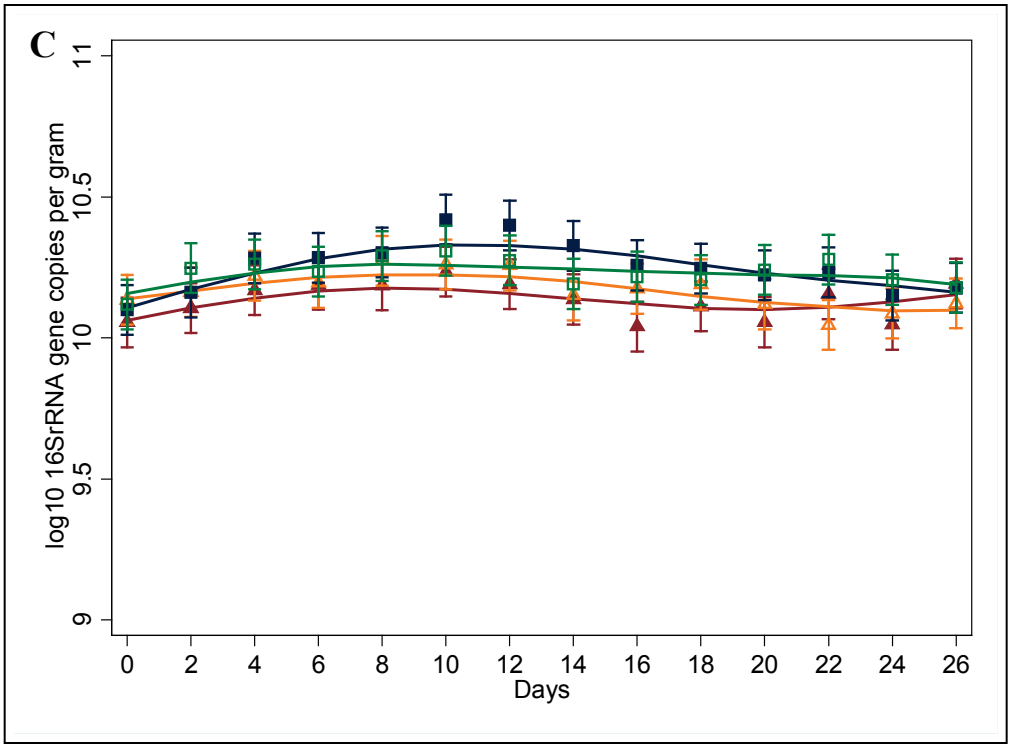


Figure 3.3 Line graphs illustrating (A) non-standardized $\log_{10}$ *bla*$_{\text{CMY-2}}$ (B) standardized (as ratio to $\log_{10}$ 16S rRNA) $\log_{10}$ *bla*$_{\text{CMY-2}}$, and (C) $\log_{10}$ 16S rRNA gene copies per gram wet feces, by treatment groups and over days.
Dark navy line (with solid square) is for the pens in which CCFA was administered to all the steers within pens followed by CTC administration at the pen level; green (with hollow square) is for the pens in which CCFA was administered to a single steer within the pens followed by CTC administration at the pen level; maroon line (with solid triangle) represents pens in which CCFA was administered to all steers within pens without subsequent CTC administration at the pen level; and orange lines (with hollow triangle) represents pens in which CCFA was administered to a single steer within the pens without subsequent CTC administration at the pen level.

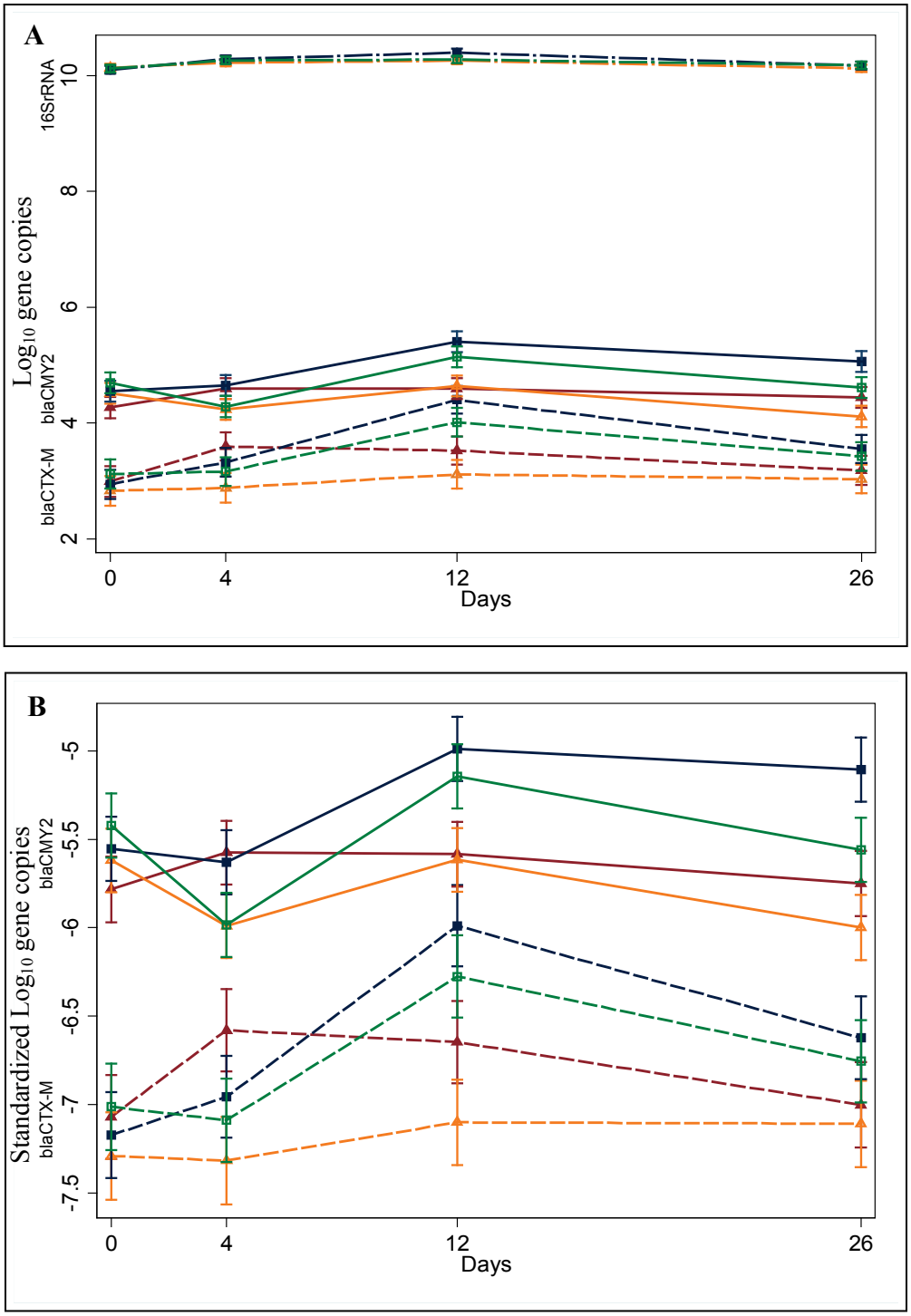


Figure 3.4 Line graphs illustrating (A) non-standardized $\log_{10}$ *bla*$_{\text{CTX-M}}$ (dashed lines), non-standardized $\log_{10}$ *bla*$_{\text{CMY-2}}$(solid lines), and $\log_{10}$ 16S rRNA (dashed and dotted lines) gene copies per gram wet feces (B) standardized (as ratio to $\log_{10}$ 16S rRNA) $\log_{10}$ *bla*$_{\text{CTX-M}}$ (dashed lines) and $\log_{10}$ *bla*$_{\text{CMY-2}}$ (solid lines) gene copies per gram wet feces, by treatment groups and over days. See Figure 3.3 for treatment groups key.

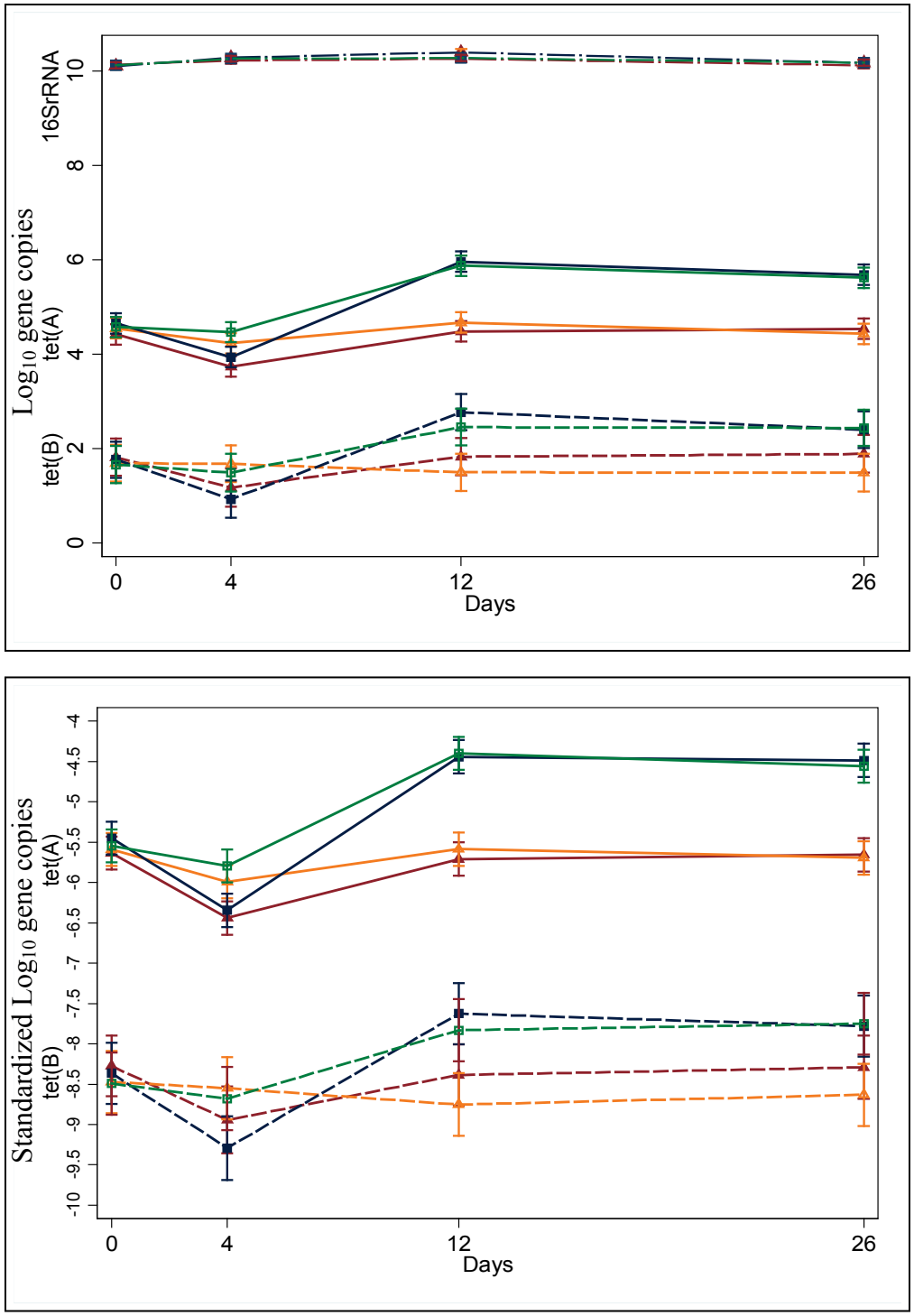


Figure 3.5 Line graphs illustrating (A) non-standardized $\log_{10}$ *tet*(B) (dashed lines), non-standardized $\log_{10}$ *tet*(A) (solid lines), and $\log_{10}$ 16S rRNA (dashed and dotted lines) gene copies per gram wet feces (B) standardized (as ratio to $\log_{10}$ 16S rRNA) $\log_{10}$ *tet*(B) (dashed lines) and $\log_{10}$ *tet*(A) (solid lines) gene copies per gram wet feces, by treatment groups and over days. See Figure 3.3 for treatment groups key.

Chapter 4

Effects of ceftiofur and chlortetracycline on antimicrobial susceptibility and on *tet*(A), *tet*(B), and bla_{CMY-2} genes among *E. coli* isolated from cattle feces

4.1. Abstract

A randomized controlled field trial was conducted to evaluate the effects of two treatment strategies on ceftiofur and tetracycline resistance in feedlot cattle. The strategies consisted of ceftiofur crystalline-free acid (CCFA) administered either to one or else to all of the steers within a pen; followed by feeding, or else not feeding, a therapeutic dose of chlortetracycline (CTC). Eighty-eight steers were randomly allocated to 8 pens of 11 steers each. Both treatment regimens were randomly assigned to the pens in a two-way full factorial design. Non-type-specific (NTS) *E. coli* (n=1,050) were isolated from fecal samples gathered on Days 0, 4, 12, and 26. Antimicrobial susceptibility profiles were determined using a microbroth dilution technique. PCR was used to detect *tet*(A), *tet*(B), and bla_{CMY-2} genes within each isolate. Chlortetracycline administration greatly exacerbated the already increased levels of both phenotypic and genotypic ceftiofur resistance favored by prior CCFA treatment ($P<0.05$). The 4 treatment regimens also influenced the phenotypic multidrug resistance count of NTS *E. coli* populations. Chlortetracycline treatment by itself was associated with an increased probability of selecting isolates that harbored *tet*(B) versus *tet*(A) ($P<0.05$); meanwhile, there was an inverse association between finding *tet*(A) versus *tet*(B) genes for any given regimen ($P<0.05$). The presence of a *tet*(A) gene was associated with an isolate exhibiting reduced phenotypic susceptibility to a higher median number of antimicrobials (n=289, median=6; 95% CI= 4-8) as compared to the *tet*(B) gene (n=208, median=3; 95% CI= 3-4). Results indicate that chlortetracycline can

exacerbate ceftiofur resistance following CCFA therapy and therefore should be avoided, especially when considering their use in sequence. Further studies are required to establish the animal-level effects of co-housing antimicrobial-treated and non-treated animals together.

4.2. Introduction

Antimicrobial resistance is of global public health concern as it can exert enormous clinical and financial burdens on health care systems worldwide [149-150]. Antimicrobials are widely used in animal agriculture as therapeutic, prevention, control, and growth promotion agents [151]. Though not without controversy, several reports have indicated that antimicrobial use in food animals has been associated with subsequent development of resistance to antimicrobials in bacterial pathogens from humans [74-76]. Various intervention strategies have been proposed or else established by regulatory organizations around the world in an attempt to address this problem. Such strategies include: 1) banning of antibiotics as agricultural growth promoters [152-153], 2) removing certain antibiotic classes from the market and, 3) recommending that some classes of antibiotics never be approved for food animal use [154]. While an outright ban or removal of antimicrobials might eventually result in reduced resistance, such actions may also impede the veterinarian's or producer's ability to prevent, control, and treat diseases; paradoxically, this could actually increase public health risk [155-156]. There is an urgent need to better understand those factors that contribute to the dissemination, propagation, and persistence of antimicrobial resistance determinants among both commensal and pathogenic enteric bacteria and to design treatment strategies at the animal, pen, and farm levels to control and mitigate this global problem [157].

Ceftiofur, a 3rd generation cephalosporin, belongs to the same general class of antibiotics as ceftriaxone and is classified as a critically important antibiotic by the World Health Organization [5]. Ceftriaxone is highly valued in human medicine, especially for treating invasive salmonellosis in children [122-123]. Resistance to ceftiofur is regarded as problematic since shared resistance determinants may confer resistance to ceftriaxone. This paper describes a study designed to evaluate the effects of two different treatment strategies on phenotypic and genotypic ceftiofur resistance among non-type-specific *Escherichia coli* isolates in feedlot cattle. The first treatment strategy was to evaluate the differential effect of whole-pen versus individual-animal level ceftiofur treatment (ceftiofur crystalline-free acid: CCFA, a long acting ceftiofur formulation). The whole-pen treatments with CCFA were meant to mimic a 'metaphylaxis' or 'control' label use such as for a bovine respiratory disease (BRD) outbreak in feeder cattle. On the other hand, single individual-animal treatment in a pen of otherwise untreated and healthy cattle were meant to mimic the sporadic treatment of BRD cases. Among pens in which only a single animal received the CCFA therapy, the remaining animals were expected to serve as a ready source of more susceptible enteric bacteria, useful for repopulating the gut flora of treated cattle. Though far from unanimous agreement, many scientists accept that antibiotic-resistant bacteria carry resistance genes at a relative fitness cost [10, 136]. Readily available susceptible bacteria – bacteria devoid of the resistance gene – may help to promote rapid re-colonization of the host gut (treated animal) by outcompeting resistant bacteria that tend to dominate post-treatment periods [137]. This study exploited these principles to determine if re-colonization was affected by higher levels of exposure to susceptible bacteria, or even to those bacteria resistant to other antimicrobials.

The second treatment strategy was to feed chlortetracycline (CTC) at therapeutic doses following CCFA treatment. Our previous work had demonstrated that chlortetracycline resulted in a temporary decrease in the prevalence of ceftiofur resistant *E. coli*, especially while it was being administered in the feed [11]. Chlortetracycline treatment in the study by Platt *et al.* (2008) appeared to differentially favor *E. coli* isolates that were singly resistant to tetracycline versus those that exhibited both ceftiofur and tetracycline resistance. Those earlier results suggested that chlortetracycline might help to minimize the proliferation and accumulation of ceftiofur resistant bacteria in animal agriculture settings. Our second treatment strategy was designed based on those results. We hypothesized that chlortetracycline would expedite the return to baseline levels of ceftiofur resistance among non-type-specific NTS *E. coli*, whether following metaphylaxis or individual therapy indications. Our focus was on pen-level interpretations: both for treatments and for outcomes. This is consistent with aiming to reduce the overall carriage of resistant bacteria in truckloads of cattle shipped to slaughter.

The effects of these 2 treatment strategies (i.e., differential CCFA treatment and subsequent chlortetracycline administration) were determined by examining the susceptibility profiles and the differential selection and co-selection of ceftiofur and tetracycline resistance genes among NTS *E. coli* isolates from cattle feces. Further, associations of the resistance genes among themselves and with the various phenotypic multidrug resistant (MDR) counts obtained were evaluated. The phenotypic MDR counts in this study are defined as the number of antimicrobials (present on a single 15-drug panel) towards which an isolate exhibits phenotypic resistance. These observations were explored further in this study to better explain the phenomena observed in the current as well as in the earlier trial by Platt *et al.* [11]. This aided in

understanding the factors behind the development and accumulation of multidrug resistance determinants.

4.3. Methods

Study Design A 26-day randomized controlled trial was conducted at a research feedlot located at West Texas A&M University in Canyon, TX. All procedures used were reviewed and approved by the Amarillo-Area Cooperative Research, Education, and Extension Triangle Animal Care and Use Committee (Protocol No. 2008-07), and by the Clinical Research Review Committee at Texas A&M University (CRRC # 09-35).

Eighty-eight steers were allocated to 8 pens of 11 steers each, such that the average pen steer weights were similar. The 2 pen-level treatment strategies were randomly assigned to these 8 pens in a complete two-way full factorial design resulting in four different treatment groups. Factor 1 was whether all 11 animals in a pen versus one out of 11 animals were treated with CCFA. Factor 2 was a follow-up chlortetracycline regimen; referring to cattle in pens receiving chlortetracycline in feed following the CCFA regimens. A third factor (not randomized) in the statistical models was the effect of day of study (period) on the level of antimicrobial resistance measured in each of the phenotypic and genotypic endpoints. The drugs, dosages, routes of administration, and treatment regimens are presented in Table 4-1.

All steers in 4 out of 8 pens were given CCFA treatment (Excede®, Pharmacia & Upjohn Co, Division of Pfizer Inc, New York, NY) as a single dose regimen of 6.6 mg/kg administered subcutaneously at the base of the ear) on Day 0. This is a recommended labeled dose and route of administration for treatment of bovine respiratory disease (BRD) and bovine foot rot and for the control of BRD. Two of these pens each received 3 separate 5-day regimens (with a one-day

break in between) of 22mg/kg chlortetracycline (Aureomycin®, chlortracycline complex equivalent to 220.5 g of chlortetracycline/ kg, Alpharma, Bridgewater, NJ) *via* topdressing after the morning feed was delivered, according to the label directions and starting at Day 4. A therapeutic labeled dose of chlortetracycline, recommended for the control and treatment of bacterial conditions like pneumonia caused by *Pasteurella multocida*, was used. The three consecutive 5-day treatment regimens were administered in a similar manner to our previous study [11] to better aid in inter-study comparisons. In the remaining 4 pens, CCFA was administered only to 1 out of 11 steers within the pen. In just 2 of these pens, chlortetracycline was likewise given to all animals on the same schedule and dosing regimen as described above (Figure 4.1).

Sample collection

At 06:00 every other day, steers were restrained in a squeeze chute. Fecal grab samples of approximately 50 g were collected per rectum with a new obstetric sleeve glove and placed in individual plastic cups. All samples were transported on ice to the laboratory on the day of sample collection. Fecal samples were mixed with glycerol at a 1:1 ratio; 4 ml of the mixture was added to 5 ml cryo-vials and stored at -70°C for further bacterial culture and isolation as well as phenotypic and genotypic analysis of same. Two fecal samples were not collected due to the death of a single steer late in the study period.

***Isolation of non-type-specific* E. coli**

A microbiological culture-based method was used for NTS *E. coli* isolation from the glycerol-preserved frozen samples. The procedures for the NTS *E. coli* isolation and antimicrobial susceptibility testing were adapted from previous work [11]. Briefly, 200

milligrams of fecal sample was mixed with 1.8 milliliter of buffered peptone water and the suspension was streaked onto MacConkey agar (BD Difco™, Sparks, MD) for NTS *E. coli* isolation. Plates were incubated at 37° for 18-24 hours. Further, 3 separate and distinct colonies (slightly convex, magenta-colored colonies surrounded by a dark pink area) were streaked on 3 separate MacConkey plates and were incubated for 18-24 hours. This step was added to the protocol in order to assure we obtained pure cultures of NTS *E. coli*.

We performed a quality control experiment to test if this single extra passage would lead to the loss of plasmid/resistant determinants and thereby cause significant differences in the susceptibility results. The head-to-head experiment was conducted on 33 *E. coli* isolates derived from Day 4 fecal samples. These samples were obtained from 3 pens in which all animals received CCFA treatment. The results revealed an extremely high level of agreement (median κ =0.93) between the susceptibility results obtained from either a single, or double passage for all 15 antimicrobials. The paired t-test, comparing the MIC values and testing resistant proportions between the two groups, also revealed no significant differences between the results obtained from the two passage approaches ($p>0.05$). Therefore, we concluded that an extra passage did not lead to significant differences in antimicrobial susceptibility results, but aided in assuring we had pure cultures to perform further phenotypic and genotypic analyses. An indole spot test was performed on each isolate. While not definitive for *E. coli*, when combined with the prior probabilities that arise from the selective medium and the morphological selection (including lactose fermentation) the post-test probabilities are well in excess of 99% [116], Previous work [116] has suggested that there is little advantage of biochemical confirmation of NTS *E. coli* isolates over simple morphological selection of a typical colony obtained from MacConkey agar; in that study, biochemical assays confirmed 99.9% of the typical colonies on MacConkey agar to

be *E. coli*. A single colony from each of the 3 MacConkey plates was streaked to 3 separate Tryptic soy agar plates (BD Difco™, Sparks, MD) and was incubated for 18-24 hours. The NTS *E. coli* isolates from the TSA plates were further used for the antibiotic susceptibility testing.

DNA from NTS *E. coli* isolates was extracted for genotypic analysis by suspending a colony in 500µl of nuclease free water (Qiagen, Valencia, CA) and then heating the suspension at 95°C for 10 minutes. The NTS *E. coli* DNA samples were stored at -20°C for further qualitative detection of *tet*(A), *tet*(B), and $bla_{\text{CMY-2}}$ genes. The plasmids encoding ($bla_{\text{CMY-2}}$) gene is predominantly associated with ceftiofur resistance from both humans and animals isolates in the United States [31-32, 158]. Therefore, the $bla_{\text{CMY-2}}$ gene was chosen to predict the genotypic ceftiofur resistance among the isolates. The two tetracycline genes [*tet*(A) and *tet*(B)] were chosen as they have been reported to be the most abundant tetracycline resistance genes detected among *E. coli* in cattle in the United States [61].

Antimicrobial susceptibility testing of non-type-specific* E. coli *isolates

Fecal samples from all animals in all 8 pens representing all 4 treatment combinations on Days 0, 4, 12, and 26 were analyzed. Three NTS *E. coli* isolates from a total number of 350 fecal samples each (1,050 NTS *E. coli* isolates) were tested for their antimicrobial susceptibility profile and gene presence.

Two to three distinct NTS *E. coli* colonies were picked from the TSA plates, mixed and then suspended into 4 ml of sterile deionized water to adjust to a 0.5 McFarland standard. Ten microliters of the suspension was mixed with Mueller-Hinton broth and 50µl of the suspension was inoculated to each well of a Sensititre plate using the Sensititre™ automated inoculator (Trek Diagnostic Systems, Cleveland, OH). The plates were incubated at 37°C for 18hours.

Minimum inhibitory concentrations (MIC) were determined for 15 different antibiotics *via* the broth micro-dilution method using the gram negative NARMS panel CMV1AGNF (Trek Diagnostic Systems, Cleveland, OH) [159]. The plates were read by the Sensititre ARIS® automated system (Trek Diagnostic Systems, Cleveland, OH). The Sensititre ARIS® automated system interprets isolates as susceptible, intermediate, or resistant according to Clinical and Laboratory Standards Institute (CLSI) guidelines [160]. For our purposes, those isolates demonstrating intermediate susceptibility towards antimicrobials were re-classified as susceptible so as to utilize a binary classification in the data analysis. For those antibiotics without breakpoints established by the CLSI guidelines, we instead used 'consensus' breakpoints established by the National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria [161] (see Table 4-3). *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection, Manassas, VA) were used as quality control strains for susceptibility testing. The MIC results obtained from these quality control strains were compared to the quality control ranges recommended by the Clinical and Laboratory Standards Institute [162]. The quality control was performed for every new batch of Mueller-Hinton broth used and also for every new batch of microbroth dilution susceptibility plates.

Genotypic qualitative detection of resistance genes

A duplex PCR assay to detect both *tet*(A) and *tet*(B) was performed as previously described [163] using DNA extracted from the same 1,050 NTS *E. coli* isolates for which antibiotic susceptibility test was conducted. The $bla_{\text{CMY-2}}$ PCR was performed as previously described [106]. The primers used for all PCR reactions are listed in Table 4-2. Promega® PCR

mastermix (Promega Corp., Madison, WI) was used for both assays. All reactions were carried out in Eppendorf Mastercycler® gradient thermal cyclers (USA Scientific, Inc., Ocala, FL). Automated capillary electrophoresis analysis of the PCR product for all 3 resistance genes was performed *via* the QIAxcel System (QIAgen, Valencia, CA).

Controls: The positive control used for the duplex reaction was a 1:1 mixture of the DNA obtained from *E. coli* ATCC 47042 and the XL1-Blue *E. coli* strain. *E. coli* ATCC 47042 is known to harbor the *tet*(B) gene and XL1-Blue *E. coli* strain harbors the *tet*(A) gene. *E. coli* strain (M1) was used as the positive control for the $bla_{\text{CMY-2}}$ gene. It was previously obtained from the University of Illinois, Chicago [139]. This strain is known to harbor the $bla_{\text{CMY-2}}$ gene. The negative control consisted of the master mix alone.

Statistical Methods:

Descriptive Statistics: The outcome measures (and data types) were: 1) proportion of resistant (versus susceptible) NTS *E. coli* isolates for each of 15 antimicrobials (binary), 2) $\log_2$ MIC for each of 15 antimicrobials (truncated integer), and 3) presence (or absence) of 3 different resistance genes (binary). Basic descriptive statistics were computed by cross-tabulating each of these outcomes across 4 sampling days for each treatment group. The phenotypic MDR count of an isolate was determined by establishing the total number of antimicrobials to which an isolate was phenotypically resistant, out of the 15 antimicrobials tested on the NARMS panel. The overall frequency distribution of the phenotypic MDR counts among isolates in all 4 groups was examined. These distributions were compared to evaluate the treatment effect. Similarly, the three resistance genes' presence (or, absence) was cross-tabulated by treatment and day, as well as with the phenotypic MDR counts obtained from the NARMS panel. Significance of

associations was determined by likelihood ratio chi-square test. Distributions of MIC for each of the 15 antimicrobials, cross-tabulated by treatment and day, were also examined.

Multivariable analysis to evaluate the treatment effects on phenotypic and genotypic resistance among non-type-specific* E. coli*:

Generalized estimating equations (GEE) with binomial error distribution and logit link functions were used to analyze the data (STATA® SE Release 12.1; STATA Corp., College Station, TX). This approach was used to simultaneously evaluate the risk factors for isolates exhibiting phenotypic expression of ceftiofur resistance and also for isolates harboring $bla_{\text{CMY-2}}$, *tet*(A), and *tet*(B) genes separately. All GEE models were adjusted for the pen-level dependencies assuming exchangeable correlation structures at the pen level. There also were dependencies expected among the three isolates derived from a single fecal sample on each day; however, pen- and animal-level dependencies both could not simultaneously be accounted for due to convergence problems in the multi-level mixed logistic model.

The factors that resulted in isolates with higher phenotypic MDR counts (number of antimicrobials towards which an isolate exhibited resistance) were analyzed using ordinal logistic models. There were a maximum of 12 out of 15 antimicrobials against which resistances were exhibited by isolates in this study. Therefore, there were 13 different categories (e.g. pan-susceptible, single-, double-, penta-, deca-, dodeca-resistant) depending on the number of antimicrobials to which an isolate exhibited resistance.

Logistic regression models for discrete-time survival analysis were used to model treatment factor effects on the ability of NTS *E. coli* isolates to grow/survive over each of the increasing ceftiofur concentrations, as tested on the NARMS panel [164]. This approach

allowed for right-censoring of the MIC data at the highest recorded concentration present on the panel.

4.4. Results

Descriptive statistics

The distributions of minimum inhibitory concentrations for all 15 antibiotics among the 1,050 NTS *E. coli* isolates tested are shown in Table 4-3. Out of 1,050 NTS *E. coli* isolates, resistance to at least 1 antimicrobial was detected in 710 (67.62%) isolates; 340 (32.38%) isolates were found to be susceptible to all 15 antimicrobials tested. The most common resistance was detected against tetracycline (642 [61.14%] isolates), ampicillin (391 [37.24%] isolates), sulfisoxazole (329 [31.33%] isolates), streptomycin (308 [29.33%] isolates), ceftriaxone (267 [25.43%] isolates), chloramphenicol (246 [23.43%] isolates), and ceftiofur (241 [22.95%] isolates) as shown in Table 4-3.

The frequency distribution of the *E. coli* isolates by the phenotypic MDR count for all 4 treatment groups is shown in Figure 4.2. The pens in which CCFA was administered to 1 out of 11 steers within the pens and chlortetracycline treatment was not administered experienced the least antimicrobial selection pressure (Figure 4.2B). Within this treatment group, 55.1% of the isolates were pan-susceptible to the panel of 15 antimicrobials; in addition, the distribution was highly right-skewed indicating decreasing numbers of greater phenotypic MDR counts in this group (Figure 4.2B). A higher prevalence of greater phenotypic MDR counts was identified in isolates from animals within pens where CCFA was administered to all the animals within the pens, but likewise were not subsequently fed chlortetracycline in feed (Figure 4.2A and B). The frequency distribution of phenotypic MDR counts in this treatment group (illustrating CCFA

treatment alone) was more uniformly distributed over the full range of multidrug resistant numbers (0-15); in this case, 41% of the isolates were found to be pan-susceptible to all 15 antimicrobials (Figure 4.2A). The effect of chlortetracycline alone was best illustrated by the contrasts among the pens in which only 1 among 11 animals had prior CCFA exposure (Figure 4.2B and D). Chlortetracycline exposure increased the prevalence of higher phenotypic MDR counts (Figure 4.2D); however, chlortetracycline tended to select for lower MDR categories (isolates resistant to one or two antimicrobials) rather than categories with ≥ 5 antimicrobials. The chlortetracycline effect was much more profound when it followed CCFA treatment applied to all animals in a pen (Figure 4.2C). These pens illustrated the maximum CCFA and chlortetracycline treatment effects (Figure 4.2C) and isolate profiles illustrated a highly uniform distribution with a quite remarkable 3.03% of NTS *E. coli* isolates resistant to 12 antimicrobials.

Overall, *tet*(A), *tet*(B), and $bla_{\text{CMY-2}}$ genes were harbored by 289, 208, and 139 isolates, respectively, among the 1,050 NTS *E. coli* isolates (Figure 4.3); 564 isolates were negative for all 3 resistance genes. There were only 3 isolates that harbored $bla_{\text{CMY-2}}$ alone; that is, without *tet*(A) or *tet*(B). The majority of $bla_{\text{CMY-2}}$ positive isolates also harbored the *tet*(A) gene (n=120), while the $bla_{\text{CMY-2}}$ gene was much less prevalent among the isolates also harboring the *tet*(B) gene (n=23). There were only 14 isolates that harbored *tet*(A)and *tet*(B) genes together; further, there were 7 isolates that were positive for all 3 resistance genes (Figure 4.3).

The association of a particular gene, or gene combination, with the phenotypic MDR count was evaluated and illustrated by way of the Box-plot (Figure 4.4). Overall, the presence of the *tet*(A) gene (n=289) was associated with isolate phenotypes exhibiting reduced susceptibility to a higher median number of antimicrobials (median=6, 95% CI= 4-8) as compared to the *tet*(B) gene (n=208; median=3, 95% CI= 3-4). Similarly, $bla_{\text{CMY-2}}$ gene-positive isolates were

associated with very high phenotypic MDR count (n=139; median= 9). The 564 isolates that were found to be negative for all three resistance genes were generally pan-susceptible (median number of antimicrobials = 0) (Figure 4.4). The 162 isolates harboring only *tet*(A), in the absence of *tet*(B) and $bla_{\text{CMY-2}}$, exhibited phenotypic resistance towards a median number of 2 antimicrobials. Isolates, when positive to both *tet*(A) and $bla_{\text{CMY-2}}$ and in the absence of *tet*(B) (n=113), exhibited a higher phenotypic MDR count (median = 9 antimicrobials); on the other hand, isolates positive for only *tet*(B), but in the absence of *tet*(A) and $bla_{\text{CMY-2}}$ (n=178), were resistant to a median of 3 antimicrobials. In Figure 4.4, the Box-plot graphic clearly demonstrates that the presence of $bla_{\text{CMY-2}}$ gene was associated with higher phenotypic MDR counts.

Multivariable analysis to evaluate the treatment effects on phenotypic and genotypic resistance among non-type-specific E. coli:

Generalized estimating equation (GEE) with three-way full factorial designs were used to evaluate the effects of the treatment strategies on resistance, measured both at genotypic and phenotypic levels. The three factors corresponded to: CCFA administered to either one or all the animals within pens (Mix; binary variable), chlortetracycline administration to all animals within a pen (CTC; binary variable), and study period (Day 0, 4, 12, or 26; categories coded as an indicator variable with 0 as referent) respectively. Importantly, results are interpreted at the pen-level, not at the individual-animal level.

Figure 4.5 illustrates the prevalence of NTS *E. coli* isolates exhibiting both phenotypic resistance to ceftiofur (shown by the solid line) and the presence of $bla_{\text{CMY-2}}$ (shown by the dashed line). The likelihood of recovering ceftiofur-resistant NTS *E. coli* or $bla_{\text{CMY-2}}$ positive

isolates tended to increase following CCFA administration (Figure 4.5 A, C; Day 4). Chlortetracycline treatment delayed the return of ceftiofur resistance to the baseline (Figure 4.5 C, D; Day12). In fact, chlortetracycline appeared to have favored the expansion of the ceftiofur resistant population, fully independent of the prior CCFA regimen. Importantly, phenotypic ceftiofur resistance was not completely associated with the presence of $bla_{\text{CMY-2}}$ gene at all time points, nor in all 4 treatment groups. There were multiple isolates (n=102) that exhibited phenotypic ceftiofur resistance but did not harbor the $bla_{\text{CMY-2}}$ gene. This can best be appreciated by the gap between the line graphs illustrating phenotypic versus genotypic ($bla_{\text{CMY-2}}$) ceftiofur resistance in Figure 4.5. Further characterization of the non-$bla_{\text{CMY-2}}$ isolates has been carried out to explain this difference and results were reported elsewhere [133].

The likelihood of recovering isolates expressing tetracycline resistance, as well as isolates harboring tetracycline resistance genes, increased with CCFA administration (Figure 4.6 A, C; Day 4). As was expected, chlortetracycline treatment further increased tetracycline resistance, both at phenotypic and genotypic levels (Figure 4.6 C, D; Day 12). Importantly, there was a differential selection favoring isolates harboring *tet*(A) over *tet*(B) following CCFA treatment administered to all steers within a pen (Figure 4.6C; Day 4). However, when only 1 steer in a pen of 11 animals received prior CCFA treatment, there was a clear preferential selection favoring isolates harboring *tet*(B) gene over *tet*(A) once chlortetracycline was administered in the feed (Figure 4.6 D; Day 12).

The association between treatment strategies and the phenotypic MDR count was assessed using an ordinal logistic model with a three-way full factorial design (Mix, CTC, and Day) as outlined above. Phenotypic MDR count was defined in this study as the number of

antimicrobials towards which an isolate exhibited (binary) resistance, out of the panel of 15 antimicrobials tested on the NARMS panel. The treatment groups in which all steers received CCFA were observed to have a significant decrease in the proportion of isolates that were pan-susceptible (Figure 4.7 A, Day 4). There also was a significant effect of CCFA treatment on increasing the likelihood of recovering penta- or deca-resistant isolates (Figure 4.7 B & C, Day 4). This was in marked contrast to the groups in which only 1 animal among 11 in a pen received CCFA treatment. Less markedly, though also significant, feeding of chlortetracycline lowered the probability of isolates being pan-susceptible when compared to pens of cattle that did not receive chlortetracycline (Figure 4.7 A, Day 12). Chlortetracycline administration was also associated with increased odds for recovering both penta- and deca-resistant isolates, especially notable when prior CCFA treatment was administered to only one among 11 steers within the pen (Figure 4.7 B & C, Day 12). When CCFA was administered to all steers, subsequent chlortetracycline administration appeared to sustain the penta-resistant proportion while further increasing the deca-resistant NTS *E. coli* proportion of isolates. Overall, CCFA appeared to have the more dramatic effect on selection of higher phenotypic MDR counts (penta- or deca- resistant) than chlortetracycline alone; however, chlortetracycline greatly exacerbated the prior effects of CCFA on phenotypic MDR counts.

A discrete-time logistic model was used to compare among the 4 treatment groups the proportion of NTS *E. coli* isolates that were able to grow (i.e., were not inhibited) over each of the increasing ceftiofur concentrations on the NARMS panel. The fixed effects for the model consisted of Mix, CTC, and 'survival time' (survival time here was an analog for each increasing concentration of ceftiofur tested on NARMS panel). The two-way interactions with 'time' were

statistically insignificant in the model and therefore were excluded from the final model. Survival curves represent the predicted probabilities of NTS *E. coli* isolates not being inhibited at each increasing concentration of ceftiofur; ceftiofur concentration is graphed as zero-adjusted and ordered $\log_2$ transformed (MIC) values for all 4 groups (Figure 4.8). The two reference lines in Figure 4.8 indicate that the two treatment groups in which all steers received CCFA treatment had 26% and 17% of isolates that were not inhibited even at the highest concentrations of the ceftiofur tested on the NARMS plate ($\log_2$ (8 μg/ml) + 4 = 7). These proportions were considered right-censored and were significantly higher than in the groups in which only one animal among 11 in a pen that received ceftiofur treatment. Similarly, the steers in chlortetracycline-administered pens had a higher proportion of isolates that were not inhibited at higher concentrations of ceftiofur when compared to the isolates derived from pens in which chlortetracycline was not administered.

4.5. Discussion

This study was designed to evaluate the effects of co-housing ceftiofur treated and non-treated animals together, and also the effect of chlortetracycline treatment following ceftiofur treatment. The effects were measured in terms of the phenotypic susceptibility profiles of NTS *E. coli* isolates and by evaluating the genotypic prevalence of specific resistance genes among these same NTS *E. coli* isolates.

CCFA treatment on Day 0 selected for isolates with reduced susceptibility towards ceftiofur, both at genotypic and phenotypic levels. There have been four other major published studies that have evaluated the effect of ceftiofur on cephalosporin resistance among enteric bacteria in cattle [115-117, 120]. Our results were quite similar to two of these earlier studies

[115-116]. The results from these two earlier studies indicated that CCFA was associated with a significant decrease in the total *E. coli* $\log_{10}$ CFU/g of feces in cattle. This combined with a concurrent, though transient, expansion of the ceftiofur-resistant *E. coli* fraction following CCFA administration, resulted in the observed increase in prevalence [115-116]. In our study, the total *E. coli* CFU/g of feces also dropped by approximately 2 logs following CCFA treatment (data not shown) and remained lower than baseline for approximately 8-12 days [165]. A third study [120] also reported a similar transient increase in the animal-level prevalence of ceftiofur resistant *E. coli* immediately following ceftiofur treatment. However, they did not observe a herd-level association between levels of ceftiofur use and increased shedding of extended spectrum cephalosporin resistant *E. coli* isolates. These authors did not report the variations in the total *E. coli* load among samples collected during the study period; thus, their results could have been biased by ignoring the decrease in the background susceptible bacterial population. The fourth study [117] did not observe the animal-level associations between the ceftiofur use and prevalence of *E. coli* isolates with reduced susceptibility to ceftriaxone; however, while a significant herd-level association was associated with use versus non-use, no dose-response was detected among herds that used ceftiofur. This was a cross-sectional prevalence study conducted in only 18 dairy herds. Herds reporting ceftiofur use had a significantly higher prevalence of ceftriaxone resistance isolates among cattle fecal samples as compared to the herds that did not use ceftiofur. The individual ceftiofur-treated cattle themselves did not exhibit an increased risk of shedding of *E. coli* isolates that had reduced susceptibility to ceftriaxone. This may easily be attributed to the study design; the time duration between ceftiofur treatment and fecal sample collection was not controlled. The increase in shedding of resistant isolates is typically found to be transitory and resistance levels usually return to baseline levels soon after selection pressures

are removed [115-116]. Therefore, the probability of finding a significant relationship between ceftiofur treatment and resistant bacteria post antimicrobial washout phase is low within a pen or herd. On the other hand, herd-level associations suggest that a significant treatment- and time-dependent shift in ceftriaxone resistance baseline levels could occur once a product is first introduced into a herd [116].

In contrast to our hypothesis, subsequent chlortetracycline treatment on Day 4 was not associated with a decrease in ceftiofur resistance as was seen in a previous study [11]. Instead, chlortetracycline greatly delayed the return of ceftiofur resistance to baseline levels following CCFA treatment. Steers from both of the two studies were housed in the same research feedlot at West Texas A&M University. The bacterial load was not quantified in the previous study so it was not possible to determine if chlortetracycline administration was associated with increasing the ceftiofur-resistant bacterial counts, or if there was merely a decrease in the susceptible bacterial population numbers. In the present study, the total NTS *E. coli* load was quantified by measuring CFU counts (data not shown). It was determined that, overall, chlortetracycline treatment slightly increased the NTS *E. coli* population. Therefore, chlortetracycline treatment might also have led to an expansion of the ceftiofur resistant population instead. This was in direct contrast to the hypothesis of the present study.

Though the results were unexpected, they can best be explained based on simple mechanistic considerations of co-selection. The genotypic analysis of the isolates obtained from this study revealed certain associations: chlortetracycline treatment in the absence of prior CCFA treatment significantly increased selection of the *tet*(B) gene over the *tet*(A) gene; the 2 *tet* genes were found to be negatively associated with each other; and *tet*(A) gene was found to be strongly associated with $bla_{\text{CMY-2}}$ gene. The chlortetracycline treatment in the previous study [11] may

have differentially selected for isolates with *tet*(B) genes over *tet*(A) gene. The *tet*(B) and bla_{CMY-2} genes, not being significantly associated with each other, may have led to the finding of the previous study suggesting that chlortetracycline treatment resulted in a preferential selection of tetracycline resistant isolates over isolates co-resistant to both tetracycline and ceftiofur [11]. However, in the present study, pens (or animals) receiving CCFA treatment selected for bla_{CMY-2} positive isolates. This expanded population likely also favored the *tet*(A) over *tet*(B) gene. Subsequent chlortetracycline treatment in the present trial may have further expanded this population (isolates harboring *tet*(A) along with bla_{CMY-2} genes); therefore, the reduction in ceftiofur resistance was not seen in these pens. A major difference between the present study and that of Platt *et al.* (2008) was that the latter did not include prior 'priming' of the bacterial population with CCFA, either at the pen-level or of single individuals within those pens. Further, the 'baseline' prevalence of resistance to ceftiofur in the experimental feedlot has steadily increased over the years from 2003 through the present as traced from Lowrance *et al.* (2007) [116] through Platt *et al.* (2008) [11]. While one of our treatment groups very closely mimicked the previous study [11]; that is, where CCFA was administered to one among the 11 steers within pens and then later on Day 4 all animals were exposed to chlortetracycline treatment, chlortetracycline in those pens too was not observed to reduce ceftiofur resistance. While the present study was designed to evaluate the effect of chlortetracycline as an intervention strategy to control ceftiofur resistance, the difference in study design when compared to Platt *et al.* may have caused some of the disparity. However, it is extremely unlikely that it caused all of the disparity. The *E. coli* isolates from both studies are being further characterized to better explain the study discrepancies, especially as relates to the earlier assumptions about the expected associations among *tet*(A), *tet*(B), and bla_{CMY-2} genes.

Over the entire study period, the frequency of phenotypically ceftiofur-resistant NTS *E. coli* isolates was always higher than the prevalence of isolates harboring $bla_{\text{CMY-2}}$. All earlier work in North America suggested that in feeder cattle populations the $bla_{\text{CMY-2}}$ gene dominated and explained almost 100% of the resistance to ceftiofur [32, 158]. Obviously, as time moves forward there can always be other genetic determinants such as ESBL genes that will contribute to explain the disparity between phenotypic expression and genotype. One previous study [64] indicated that the phenotypic and genotypic disparity could be attributed to other resistance determinants that were not tested for, or else could be due to the inability of the resistance genes to be turned on, in order to express phenotypic resistance. The isolates in the present trial were further characterized to explain the observed discrepancy between phenotypic expression and genotype. Twenty nine out of 88 steers tested positive for the $bla_{\text{CTX-M-32}}$ gene over the study period [133]. The presence of the $bla_{\text{CTX-M-32}}$ gene (28.8%) and *ampC* promoter mutation (0.5%) among NTS *E. coli* isolates almost completely explains the higher frequency of phenotypic ceftiofur resistance observed among isolates than was predicted by $bla_{\text{CMY-2}}$ alone.

CCFA treatment on Day 0 also selected for isolates with reduced susceptibility towards tetracycline. A similar effect has been observed elsewhere [116, 166]. Also at the genotypic level, the prevalence of isolates harboring tetracycline resistance genes increased with CCFA treatment. Chlortetracycline treatment further increased the frequency of tetracycline resistance. In this study, in the situation where the vast majority of steers in a pen were not primed with CCFA before chlortetracycline treatment, there was a differential selection of isolates harboring the *tet*(B) gene over *tet*(A) gene (Figure 4.6 D). Chlortetracycline treatment effects on tetracycline resistance prevalence have been previously investigated [11, 167]. One of those studies [167] detected no significant increase in tetracycline resistance following

chlortetracycline treatment. The authors contributed this to the gap in sampling time and a low initial prevalence of tetracycline resistance. The other study [11] observed a significant increase in the likelihood of recovering tetracycline resistant isolates during and immediately following chlortetracycline administration. The effect was transitory and the prevalence of the isolates with reduced susceptibility returned to baseline levels by 17 days post treatment.

Pens in which all animals received CCFA treatment had significantly higher pen-level ceftiofur resistance as compared to pens in which only 1 among 11 animals were CCFA-treated. A previous study [8] in which ceftiofur treated and non-treated dairy cattle were co-housed together, reported a small increase in the $bla_{\text{CMY-2}}$ gene copy numbers in fecal community DNA of the non-treated animals. The authors attributed this effect to co-mingling of treated and non-treated animals together. Those results suggested horizontal transmission of bacterial strains/resistant determinants among the cattle that were housed together. In our study, the non-treated animals were expected to supply susceptible enteric bacteria into the environment within pens when treated and non-treated animals were housed together. The treated animals also were in constant exposure to these susceptible bacteria. These bacteria were expected to improve the microbial ecology of the treated animals by more rapidly returning the gut flora to baseline or equilibrium levels of resistance. However, this present study was not designed to evaluate the animal-level effects of co-housing of treated and non-treated animals together; rather, the effects were assessed solely at the pen-level with sufficient statistical power to meet our stated objectives. Further studies are required to establish any individual animal-level effects since insufficient statistical power is present in this study design to evaluate 'mixing' effects on the individual steers (n=4 total) receiving CCFA among 11 in a pen.

Antimicrobial pressure exerted both by CCFA and chlortetracycline selected for isolates with higher phenotypic MDR counts. The effect of CCFA on co-selecting for other phenotypic antimicrobial resistances besides cephalosporins was more profound than was seen with chlortetracycline treatment. At genotypic level, the presence of *bla*$_{\text{CMY-2}}$ gene seemed to have a similar co-selection effect. Isolates harboring the *bla*$_{\text{CMY-2}}$ gene showed phenotypic resistance to a higher median number of antimicrobials when directly compared to isolates that did not harbor this gene. Studies in the United States on *Salmonella* isolates derived from bovine, porcine, and human origin have indicated that the *bla*$_{\text{CMY-2}}$ gene is usually located on a large IncA/C plasmid which harbors several other resistance genes [39-41]. Overall across all treatment groups and days, isolates harboring the *tet*(A) gene also showed phenotypic resistance against a higher median number of antimicrobials than those isolates carrying the *tet*(B) gene. One previous study indicated instead an association of *tet*(B) genes with carriage more multiple resistance when compared to isolates harboring the *tet*(A) gene [63]. However, that was a cross-sectional prevalence study conducted at the farm-level. In this randomized controlled trial, pretreatment Day 0 results suggested no significant difference between these 2 *tet* genes with respect to the carriage of multiple phenotypic antimicrobial resistance. CCFA administered on Day 0 selected for isolates harboring *bla*$_{\text{CMY-2}}$ and this gene was associated with resistance against many other antimicrobials. Importantly, the *bla*$_{\text{CMY-2}}$ gene exhibited a positive association with the *tet*(A) gene. There was likely to have been co-selection of isolates harboring *bla*$_{\text{CMY-2}}$ and *tet*(A) genes because of the initial CCFA treatment. It is also possible that isolates harboring the *tet*(A) gene demonstrated a higher multidrug phenotypic resistance count due to prior CCFA exposure and selection. Therefore, in our study *tet*(A) positive isolates exhibited an overall higher multi-drug resistance profile when compared to *tet*(B) positive isolates.

A negative association was found between *tet*(A) and *tet*(B) genes among NTS *E. coli* isolates. There were only 0.01 % (14 isolates) that harbored both the *tet*(A) and *tet*(B) gene. Previous studies have also indicated a negative association between these 2 *tet* genes [62-64]. The negative association between the tetracycline resistance determinants has been suggested by some to be due to the incompatibility of the plasmids that carry these genes [168]. This has not been explored here, but work is underway in another laboratory that specializes in plasmid-typing to examine this possibility using these 1,050 isolates. There were only 3 isolates that harbored only the $bla_{\text{CMY-2}}$ gene without the presence of either the *tet*(A) or *tet*(B) gene. Earlier studies in North American cattle have reported that the $bla_{\text{CMY-2}}$ gene is usually present with at least 1 other resistance gene. However, we must acknowledge the high probability that there were other unexamined resistance factors present with the gene (beyond those few we examined); in fact, the phenotypic resistance profile suggests this was likely to have been the case, though not always to have been associated with the usual IncA/C plasmid [133, 169].

The present system of classifying antimicrobial susceptibility data into sensitive (susceptible) or resistant has been critiqued before [167, 170]. Such a system does not easily allow for analyzing the trends of changes in MIC values statistically. The changes in the MIC values, both above and below an internationally accepted cut point (e.g. CLSI in North America; EUCAST in Europe), cannot readily be evaluated by the binary coding system. In addition, such cut points are subject to change, especially when not based on epidemiological breakpoints. Survival analyses using non-parametric assumptions such as the Kaplan-Meier method [171-172] or else using Cox proportional hazards model [170] have been proposed as alternative approaches for analyzing MIC data. These methods use the entire dilution range of antimicrobial being tested and also deal effectively with the large number of right-censored observations (i.e.,

those isolates that grow beyond the upper limit of antibiotic concentration included on commercially available plates). The resulting survival curves illustrate and compare the proportion of bacteria that are uninhibited at each specific concentration of antimicrobial used, given that these bacteria have survived up to that concentration. In addition, statistics such as the MIC_{50} (median MIC) and MIC_{90} (90^{th} percentile MIC) are readily visible. The drawback of a traditional survival approach is that the proportional hazard and the continuous time data assumptions are typically not met. We instead analyzed our MIC data using a logistic model adapted for discrete-time survival data [164, 173-175]. Discrete time in our analysis was analogous to the specific concentrations (dilutions) of the antimicrobials on a $\log_2$ transformed scale (plus 4 to avoid negative values). The recorded event was the inhibition of the bacterial growth at an observed minimum concentration.

Fitted survival curves from the discrete-time regression model indicated that administration of both chlortetracycline and CCFA selected for higher proportions of isolates that could grow (i.e., were not inhibited) at higher *in vitro* ceftiofur concentrations. The CCFA treatment effect on MIC distributions was much more profound than the chlortetracycline treatment effect. Pens in which all animals received both CCFA and chlortetracycline treatments had 26% of the isolates that were still able to grow at the highest ceftiofur concentration on the NARMS panel (right censored on survival curve, MIC above the highest concentration used (8 μg/ml)). Meanwhile, isolates obtained from pens in which only 1 animal received CCFA treatment, and without subsequent chlortetracycline treatment, had almost all of their isolates inhibited by the highest ceftiofur concentration. Differences also emerged among the treatment groups at much lower ceftiofur concentrations (see Figure 4.8: 2 and 3 on the X-axis, corresponding to concentrations of 0.25 and 0.5 μg/ml, respectively). These curves provide

useful information and permit direct comparison of overall trends of the MIC distribution over the entire antimicrobial dilution range for all 4 groups, rather than simply comparing the proportion resistant/susceptible among 4 the treatment groups.

In conclusion, chlortetracycline treatment resulted in an increased probability of recovering ceftiofur resistant isolates both at phenotypic and genotypic levels. Chlortetracycline appears to greatly exacerbate ceftiofur resistance levels following CCFA therapy and therefore should be avoided, especially when used in sequence. Unsurprisingly, pen-level ceftiofur resistance was lower in the groups with individual CCFA-treated and other non-treated animals co-housed together. Further studies are required to establish the effect on the levels of antimicrobial resistance in individuals animals of co-housing antimicrobial-treated and non-treated animals together at these and other varying ratios. Such information will assist in determining some of the risks/benefits of individual- versus mass-therapy in production agriculture settings.

Drug Name	Brand Name	Dose (by BW)	Route of administration	Treatment regimen(s)	Days in regimen
Ceftiofur crystalline-free acid (CCFA)	Excede®	6.6 mg/kg	Subcutaneous, base of ear	1 (Day 0)	1
Chlortetracycline (CTC)	Aureomycin®	22 mg/kg	Top-dressed on feed	3 (Days 4-8,10-14,16-20)	5

Table 4-1 Drugs, dosages, routes of administration, and treatment regimens.
BW=body weight
Excede®, (Zoetis Animal Health, NJ, U.S.A)
Aureomycin®, chlortetracycline complex equivalent to 220.5 g of chlortetracycline/kg of premix, Alpharma™, Bridgewater, NJ

Gene name	Primer	Primer Sequence	Expected Product Size (bp)	GenBank Accession no. *
bla$_{CMY-2}$	585F	5'- CAG ACG CGT CCT GCA ACC ATT AAA -3'	454 [a]	AB212086
	1038R	5'- TAC GTA GCT GCC AAA TCC ACC AGT -3'		
tet(A)	*tet*(A)(F)	5' -GCTACATCCTGCTTGCCTTC- 3'	210 [b]	X61367
	tet(A)(R)	5' -CATAGATCGCCGTGAAGAGG- 3'		
tet(B)	*tet*(B) (F)	5' -TTGGTTAGGGGCAAGTTTTG- 3'	659 [b]	J01830
	tet(B) (R)	5' -GTAATGGGCCAATAACACCG- 3'		

Table 4-2 PCR primers used for PCR reactions.
[a] Primer set used is from [106]
[b] Primer set used is from [148]
*Sequence used for primer design.

NARMS Code	Antimicrobial	% Resistant[1]	95% CI[2]	Distribution of MICs in ug/ml (%)																
				<0.015	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
AMI	Amikacin	0.00	0-0.004[Δ]							0.10	5.43	80.29	14.00	0.19	0.00	0.00	0.00					
AMP	Ampicillin	37.24	34.31-40.24								8.57	27.33	22.10	2.57	2.19	3.24	34.00					
AUG	Amoxicillin /Clavulanic Acid*	18.67	16.35-21.16								5.81	15.05	43.43	13.52	3.52	15.52	3.14					
AXO	Ceftriaxone	25.43	22.82-28.18						70.38	2.67	0.86	0.67	4.48	9.05	3.24	1.81	4.95	1.90				
CHL	Chloramphenicol	23.43	20.90-26.11									5.05	47.33	20.57	3.62	2.67	20.76					
CIP	Ciprofloxacin	3.14	2.17-4.39		87.62	1.90	0.38	1.05	5.33	0.48	0.10	0.00	0.10	3.05								
SXT	Trimethoprim /Sulfamethoxazole**	4.00	2.90-5.37					54.00	37.81	4.00	0.00	0.19	0.29	3.71								
FOX	Cefoxitin	19.05	16.71-21.56							0.00	3.33	11.14	43.52	19.71	3.24	10.86	8.19					
GEN	Gentamicin	0.95	0.46-1.74						1.24	67.33	22.10	0.95	3.52	3.90	0.29	0.67						
KAN	Kanamycin	4.57	3.39-6.02											86.95	3.05	5.43	2.10	2.48				
NAL	Nalidixic Acid	9.62	7.90-11.57							0.19	7.90	75.71	4.86	0.86	0.86	0.95	8.67					
FIS	Sulfisoxazole	31.33	28.54-34.24												61.52	4.57	0.76	0.86	0.95	31.33		
STR	Streptomycin	29.33	26.59-32.19													70.67	9.24	20.10				
TET	Tetracycline	61.14	58.12-64.10										30.67	8.19	5.62	12.19	43.33					
TIO	Ceftiofur	22.95	20.44-25.62					4.86	18.38	47.43	2.10	1.14	3.14	9.43	13.52							

Table 4-3 Distributions of minimum inhibitory concentrations of 1,050 non-type-specific *E. coli* isolates against 15 antibiotics.

* Amoxicillin shown, clavulanic acid at 1/2X concentration that of amoxicillin.

** Trimethoprim shown, sulfamethoxazole at 19X concentration that of trimethoprim.

Unshaded areas indicate the dilution range of the Sensititre® plate used to test isolates.

Vertical bars indicate the CLSI resistance breakpoint when available or else NARMS consensus breakpoint.

Sum of numbers beyond vertical bar represents the percent of isolates which grew beyond the CLSI breakpoint (or, NARMS consensus breakpoint). These were considered as resistant in this study.

Numbers in the shaded area indicate the percent of isolates that had an MIC greater than the highest concentration tested.

[1] Percent of the isolates that were resistant out of the total 1,050 non-type-specific *E. coli* isolates tested.

[2] 95% Confidence interval was calculated using exact binomial method.

[Δ] One-sided 97.5% confidence interval; used only when estimate was zero.

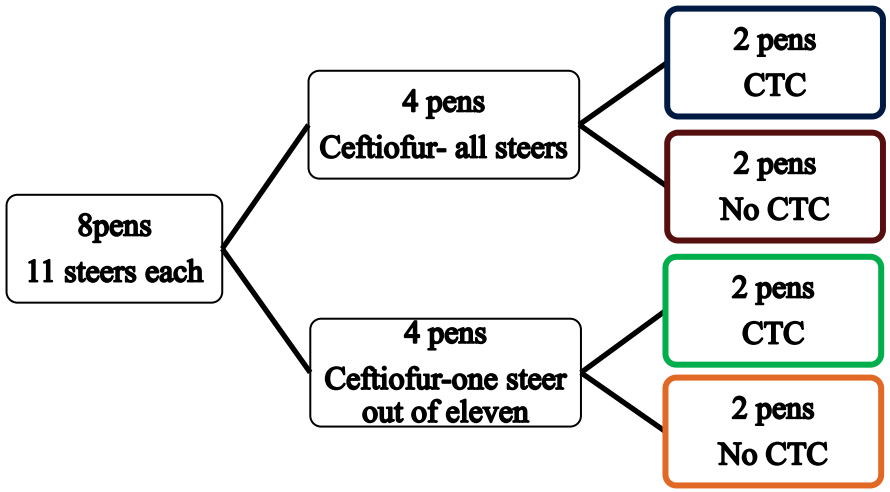


Figure 4.1 Schematic diagram of the study design.
The two interventions were assigned in a two-way full factorial manner. Number of pens to each treatment and number of animals within each pen are shown above.

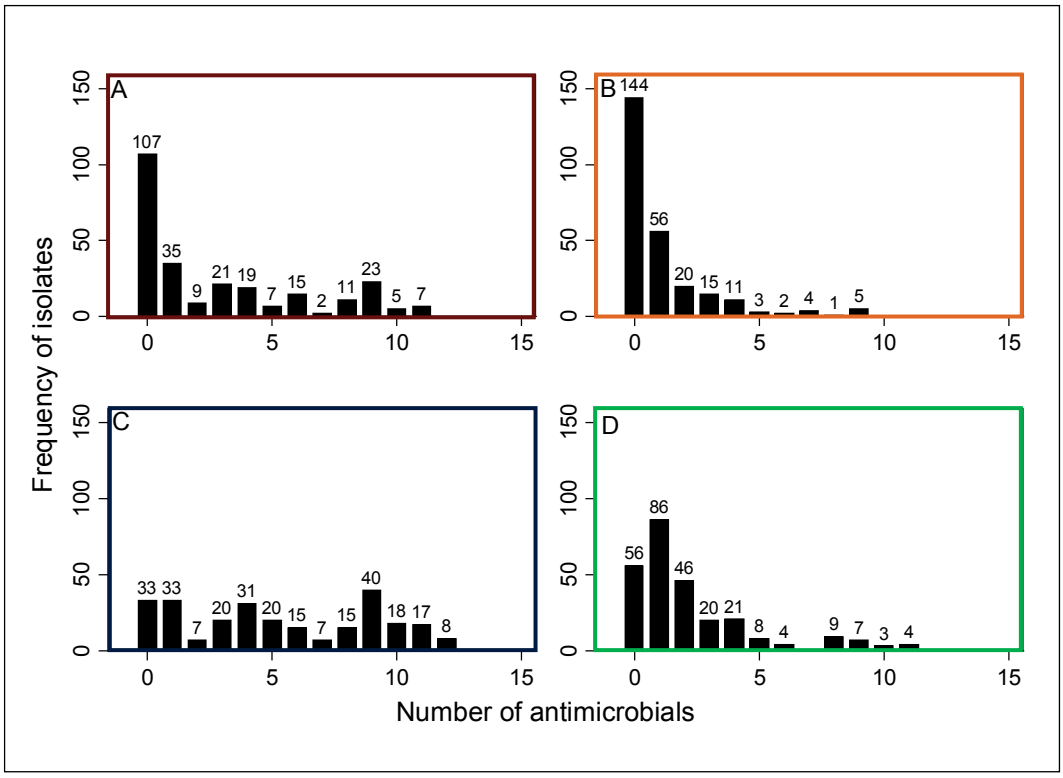


Figure 4.2 Frequency distribution of NTS *E. coli* by phenotypic multidrug resistance counts for the four treatment groups.
(A) CCFA administered to all steers within pens without subsequent CTC administration at the pen level; (B) CCFA administered to one out of 11 steers within pens without subsequent CTC administration at the pen level; (C) CCFA administered to all steers within pens followed by CTC administered at the pen level; (D) CCFA administered to one out of 11 steers within pens followed by CTC administered at the pen level.

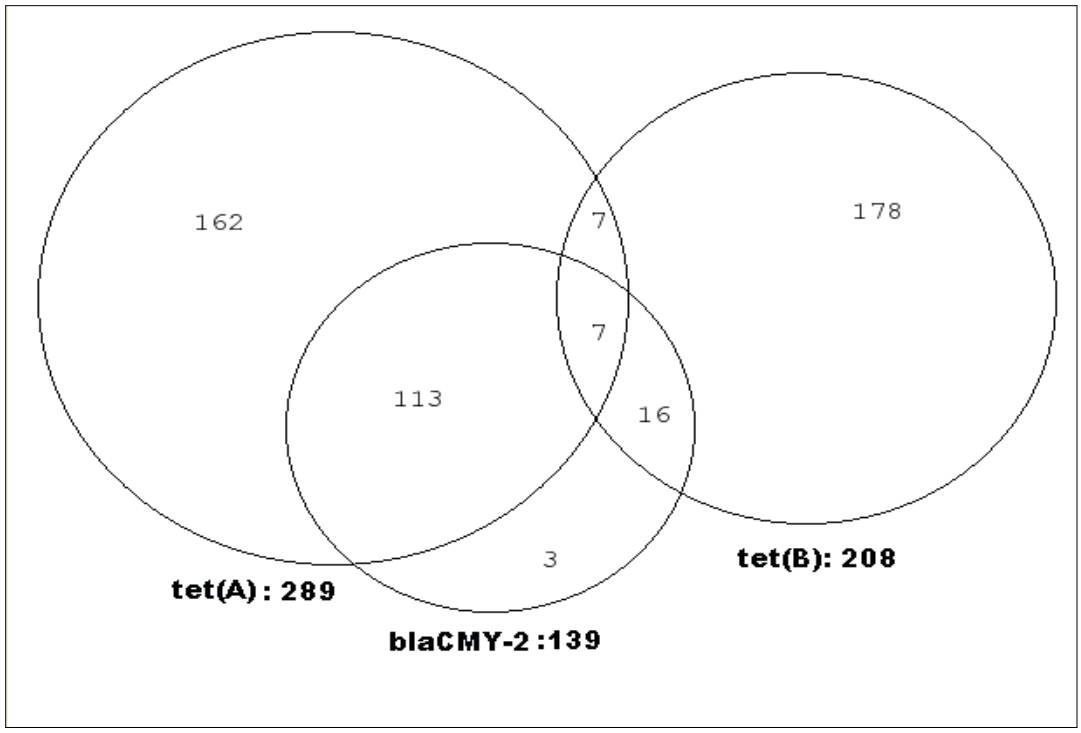


Figure 4.3 Proportional Venn diagram illustrating the joint frequencies of three resistance genes among NTS *E. coli* isolates.
Bolded numbers represent the marginal totals for each of the *tet*(A), *tet*(B), and bla_{CMY-2} genes among 1,050 NTS *E. coli* isolates. There was a total of 564 isolates that did not harbor any of the three genes.

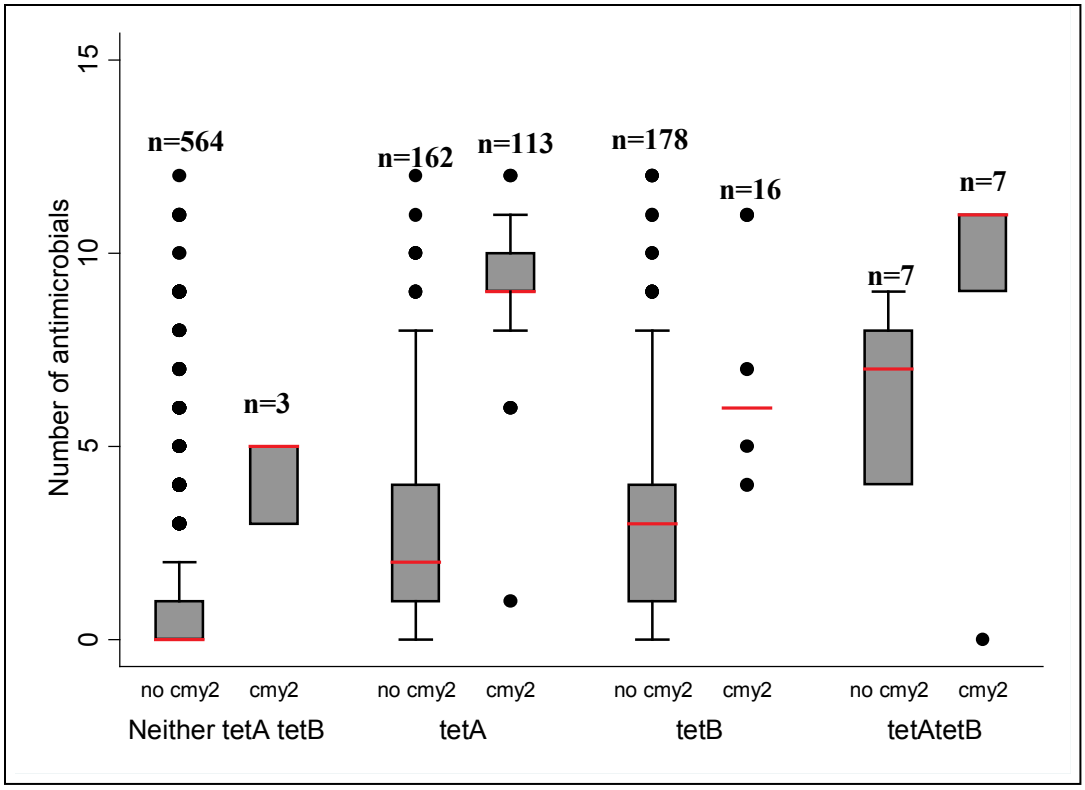


Figure 4.4 Boxplot of the phenotypic multidrug resistance count among NTS *E. coli* cross-tabulated by different resistance gene combinations.
Legend terms- cmy2, no cmy2, tetA, tetB, tetAtetB, neither tetA tetB in the figure are used for isolates that were found to be positive for *bla*$_{CMY-2}$, negative for *bla*$_{CMY-2}$, positive for *tet*(A), positive for *tet(*B), positive for both *tet*(A) and *tet*(B), and negative for *tet*(A) and *tet*(B) genes, respectively.
Horizontal red bars indicate the median number of antimicrobials among each of gene combinations

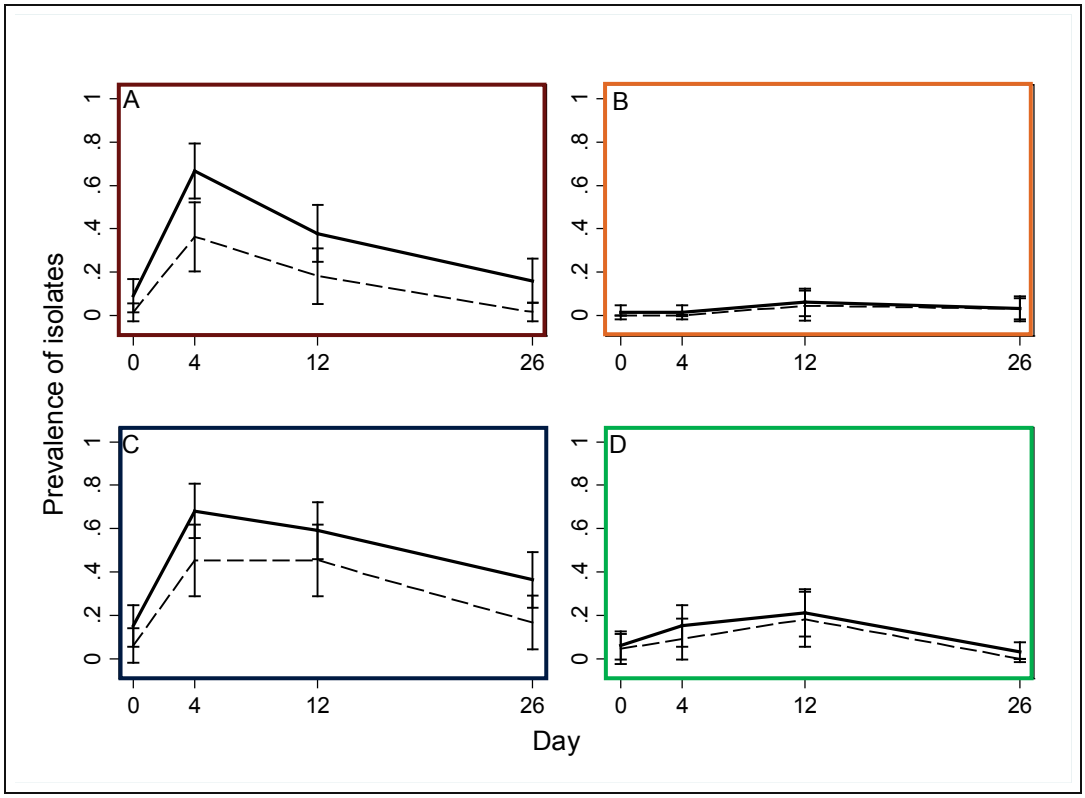


Figure 4.5 Prevalence of ceftiofur-resistant NTS *E. coli* isolates, modeled as marginal predicted probabilities, over days.
Solid line represents the proportion of NTS *E. coli* isolates phenotypically resistant to ceftiofur at ≥ 8 µg/ml. Dashed line represents the proportion of isolates harboring the bla_{CMY-2} gene. The four treatment groups are (A) CCFA administered to all steers within pens without subsequent CTC administration at pen level; (B) CCFA administered to one out of 11 steers within pens without subsequent CTC administration at pen level; (C) CCFA administered to all steers within pens followed by CTC administered at pen level; (D) CCFA administered to one out of 11 steers within pens followed by CTC administered at pen level.

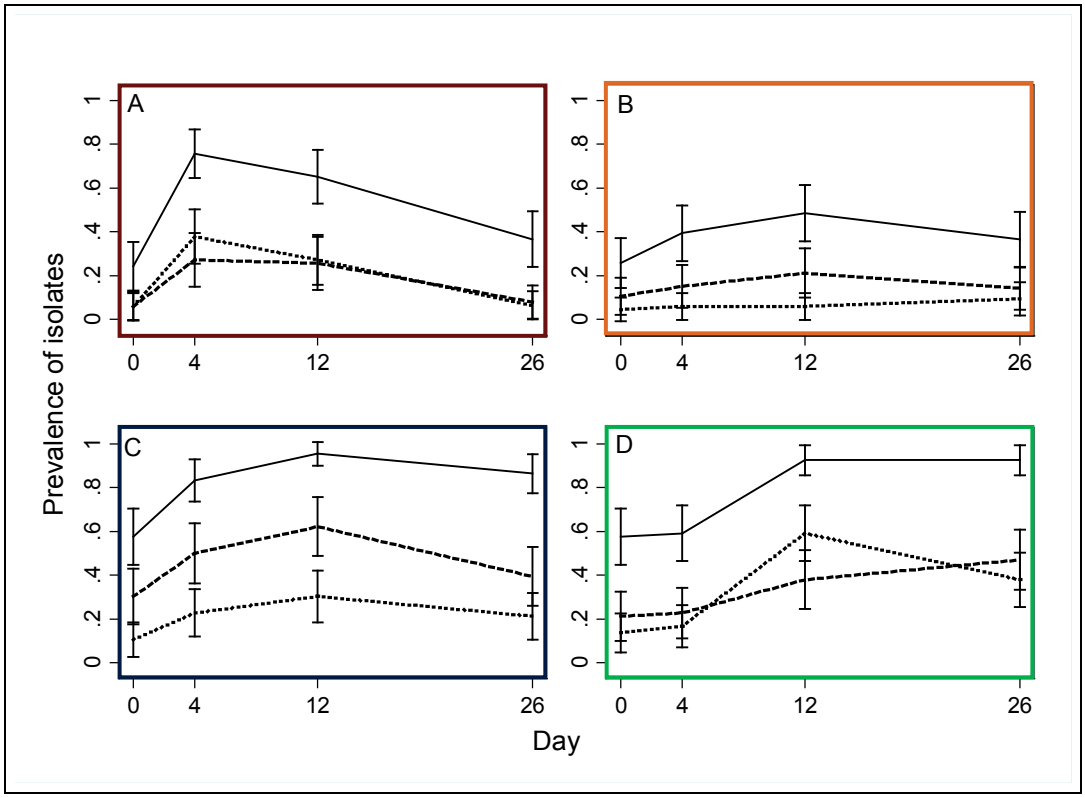


Figure 4.6 Prevalence of tetracycline-resistant NTS *E. coli* isolates, modeled as marginal predicted probabilities, over days.
Solid line represents the proportion of NTS *E. coli* isolates expressing phenotypic tetracycline resistance; dashed line represents the proportion of NTS *E. coli* isolates harboring the *tet*(A) gene; dotted line represents the proportion of NTS *E. coli* isolates harboring the *tet*(B) gene. The four treatment groups are: (A) CCFA administered to all steers within pens without subsequent CTC administration at pen level; (B) CCFA administered to one out of 11 steers within pens without subsequent CTC administration at pen level; (C) CCFA administered to all steers within pens followed by CTC administered at pen level; (D) CCFA administered to one out of 11 steers within pens followed by CTC administered at pen level.

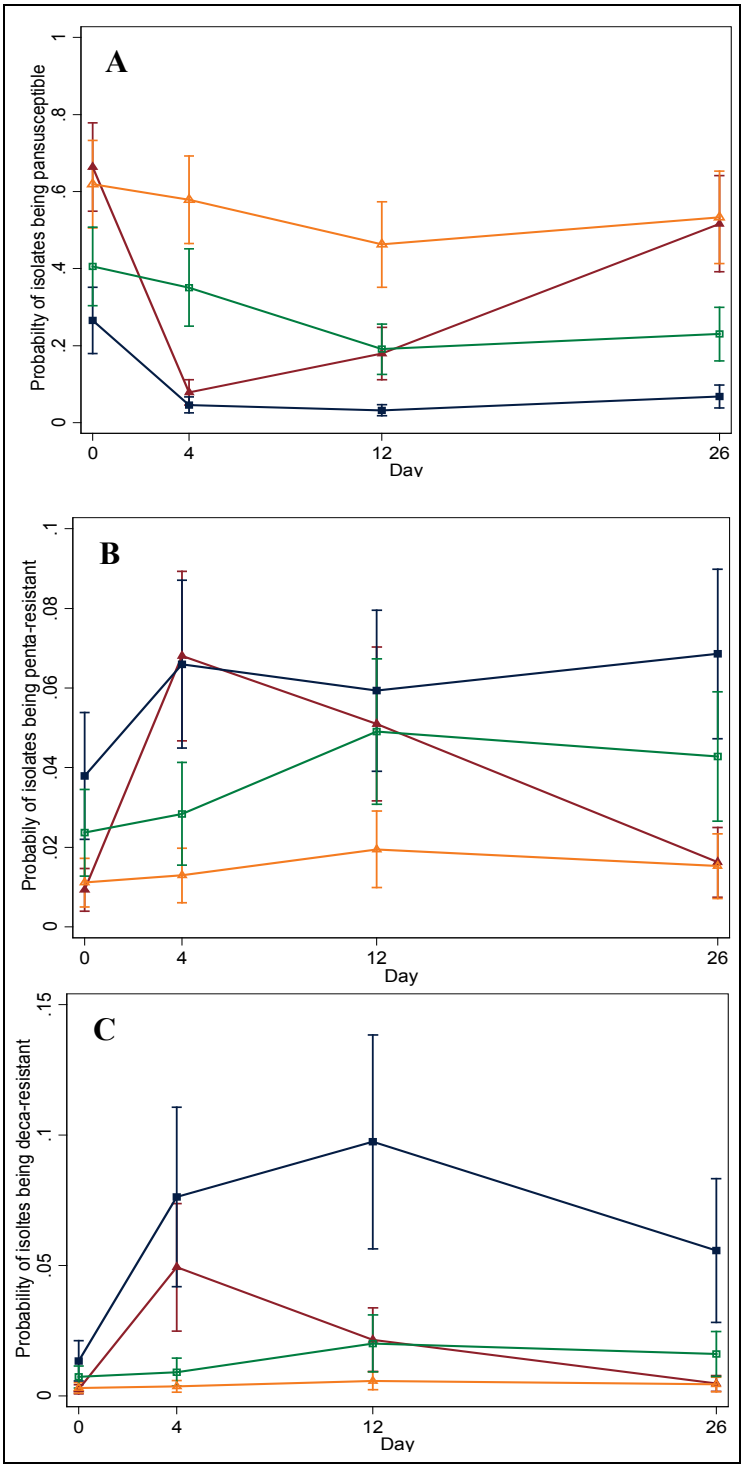


Figure 4.7 Marginal predicted probabilities of NTS *E. coli* isolates to be (A) pan-susceptible, (B) penta-resistant, and (C) resistant to 10 antimicrobials among the four treatment groups. The four treatment groups are represented by four lines in all three sub graphs. See Figure 3.3 for treatment groups key.

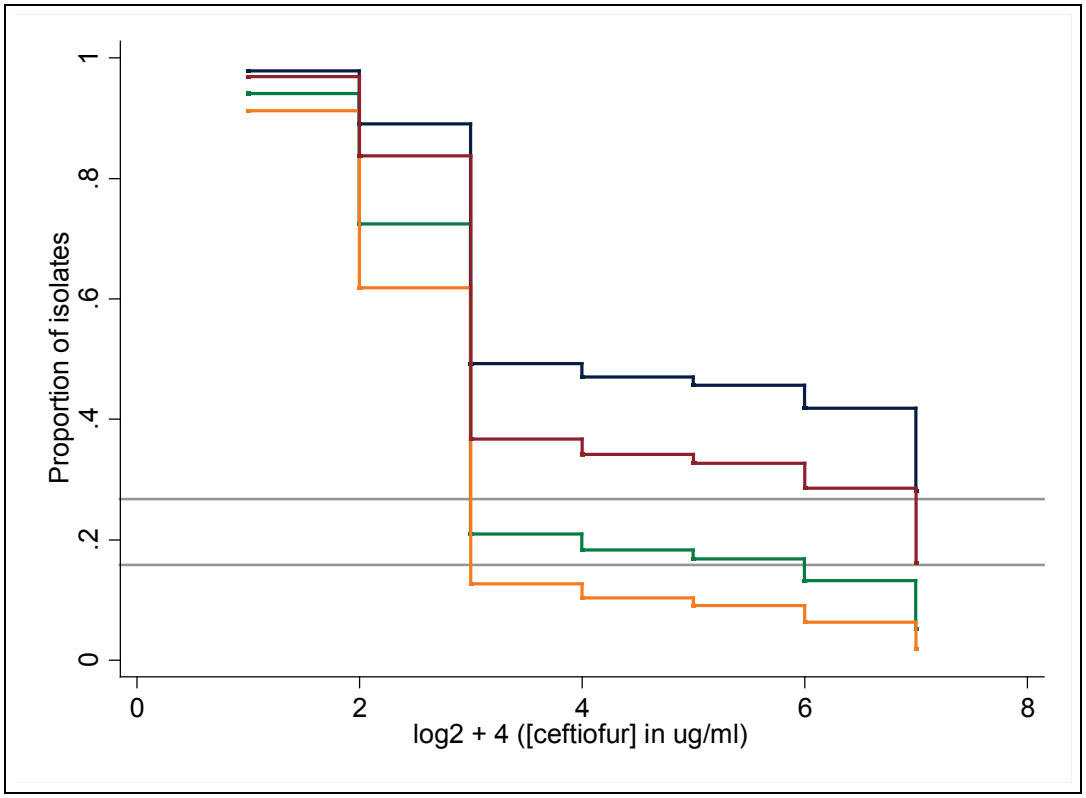


Figure 4.8 Survival curve (predicted probabilities) of NTS *E. coli* isolates over the entire range of the ceftiofur concentration tested on a log scale. See Figure 3.3 for treatment groups key.

Chapter 5
Summary and Conclusions

The research described in this dissertation focused on evaluating the effect of two treatment strategies on ceftiofur and tetracycline resistance among *E. coli* in feedlot cattle. Ceftiofur has been categorized as a critically important antimicrobial by the WHO because resistance to ceftiofur typically imparts ceftriaxone resistance among bacteria in humans; this is, due to shared resistance determinants. In order to safeguard human health, the FDA in April 2012 released an order of prohibition restricting certain extra-label uses of ceftiofur in animal agriculture. In general, such prohibition orders may reduce the ability of the veterinarian or producer to prevent, control, and treat diseases among animals and potentially decrease the safety of food. As a result, such actions may inadvertently affect both animal and human health.

There is an urgent need to better understand those resistance dynamics which contribute to the emergence, dissemination, propagation, and persistence of resistance determinants among bovine gut microflora. This understanding is essential for designing intervention strategies at animal, pen, and farm levels to best control or mitigate resistance against antimicrobials, especially those that are critically important for use in human medicine.

This study was one effort towards evaluating the impact of two treatment strategies to better understand the ceftiofur resistance dynamics at both phenotypic and genotypic levels *via* culture-based and metagenome-based assays. The first treatment strategy was evaluating pen versus individual-level CCFA treatments. The CCFA treatment regimens employed mimicked either metaphylatic (pen-level) or therapeutic (individual animal) use in feedlot cattle to control or treat bovine respiratory disease, respectively. Previous studies have reported a transient increase in the quantities of the ceftiofur resistance determinant $bla_{\text{CMY-2}}$ gene among untreated

control cattle during the ceftiofur treatment phase; that is, when control and treated cattle were allowed to comingle at a 1:1 ratio. This suggested that co-housed cattle can readily exchange bacteria or resistant determinants among themselves. The non-treated animals in the pens in which individual animal received CCFA treatment were expected to serve as a ready source of susceptible bacterial strains and thus help to promote the re-colonization of treated animal with the 'baseline' microflora. The second treatment strategy was based on either feeding or not feeding therapeutic doses of chlortetracycline, subsequent to the differential CCFA treatment. Chlortetracycline in our previous study [11] was found to significantly decrease the prevalence of ceftiofur resistant *E. coli* among the fecal bacteria populations. Chlortetracycline treatment was expected to expedite the return of ceftiofur resistance to baseline levels among the enteric bacterial population, following differential CCFA administration. The two strategies (co-housing CCFA-treated with non-treated animals and chlortetracycline treatment) each were hypothesized to reduce both phenotypic and genotypic ceftiofur resistance among gut microflora in cattle.

As would be expected, ceftiofur resistance was indeed lower in the pens where only one animal was treated among the 11 pen-mates. However, further studies are required to establish the animal-level effect of co-housing antimicrobial treated and non-treated animals together at varying ratios on the levels of antimicrobial resistance. Pens in which all the animals received CCFA treatment demonstrated a significant increase in ceftiofur resistance, both in terms of ceftiofur resistance ($bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$) gene copies as well as the proportion of *E. coli* isolates that exhibited phenotypic ceftiofur resistance and also proportion of *E. coli* isolates that harbored $bla_{\text{CMY-2}}$ gene. CCFA treatment led to a significant decrease in the tetracycline resistance [*tet*(A) and *tet*(B)] gene copy numbers; however, there was a significant increase in

the proportion of *E. coli* isolates that exhibited phenotypic tetracycline resistance as well as the isolates that harbored the *tet*(A) and *tet*(B) genes.

Pens receiving tetracycline treatment demonstrated a significant increase in the ceftiofur (bla_{CMY-2} and bla_{CTX-M}) and tetracycline resistance [*tet*(A) and *tet*(B)] gene copies, irrespective of the prior differential CCFA treatment. There was a significant increase in the proportion of *E. coli* isolates that exhibited phenotypic resistant to ceftiofur and tetracycline and also isolates that harbored bla_{CMY-2}, *tet*(A), and *tet*(B) genes. These study results demonstrate that chlortetracycline use did not lead to the hypothesized decrease in ceftiofur resistance in feedlot cattle. Therefore, chlortetracycline is clearly contraindicated as an intervention when attempting to control resistance to critically important 3rd generation cephalosporins in feedlot cattle.

Phenotypic and genotypic discrepancies in terms of ceftiofur resistance were observed among the *E. coli* isolates. The proportion of *E. coli* isolates exhibiting phenotypic ceftiofur resistance was to found higher than the proportion of isolates that harbored the expected ceftiofur resistance determinant – the bla_{CMY-2} gene. This bla_{CMY-2} gene had been previously been predominantly associated with, and was found to explain, almost 100% of the ceftiofur resistance in cattle in the United States. These phenotypic and genotypic discrepancies were almost completely explained by the presence of bla_{CTX-M} gene and ampC promoter mutations among the remaining phenotypically resistant *E. coli* isolates [133]. Both bla_{CMY-2} and bla_{CTX-M} genes were quantified from fecal community DNA. The bla_{CTX-M} gene belongs to the family of genes encoding for extended-spectrum β-lactamases (ESBL). This gene is widespread in Europe and the rest of the world, but reports of this gene in food-producing animals in the United States were rare and required enhanced broth enrichment techniques until now [52-53].

Distinct differences were also observed between results obtained from culture-based and metagenome-based assays. Among fecal community DNA, CCFA treatment significantly decreased tetracycline resistance gene copies [*tet*(A) and *tet*(B)] whereas chlortetracycline treatment led to an increase in both of the ceftiofur resistance gene copies ($bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M}}$). Among *E. coli* isolates, contrary to community DNA results, an increase in proportion of isolates harboring both tetracycline resistance genes (A and B) was observed following CCFA treatment and there was preferential selection of isolates harboring $bla_{\text{CMY-2}}$ gene over $bla_{\text{CTX-M}}$ gene while receiving chlortetracycline treatment [132].

These differences indicate the biases that could be introduced if conclusions pertaining to entire microbial populations are based solely on certain sentinel and culturable organisms. Presently, most antimicrobial resistance monitoring and surveillance systems across the world today test isolates and samples for antimicrobial resistance using culture-based methods. Extrapolating the results obtained from specific culturable organisms to the entire microbial community is unlikely to be valid as these results could incorrectly estimate the true target resistance load in the fecal microbiome. The results from metagenome-based assays also have limitations; there may be multiple and varying amounts of target gene copies per bacterial cell (relative abundance) among different bacterial species. The metagenome-based assay gives information about the overall target gene load in total community DNA. The information about variation of specific bacterial species carrying target genes is not provided from this assay. The results from either of the two approaches alone may not cover all aspects of antimicrobial resistance development, propagation and dissemination process. Therefore, solely relying on either one of the approaches for making policies to control antimicrobial resistance in humans and animals by the regulatory bodies may not be appropriate. A metagenome-based component

should be considered as part of a more holistic monitoring system in order to identify when contradictory or paradoxical results might be seen and in order to avoid bias. Further, detection-based methods (such as with broth enrichment or quanititative metagenome-based assays) should complement the prevalence-based assays to identify new and emerging threats.

A quantitative real-time PCR performed on the community DNA enabled us to determine the effect of treatments on the resistance gene quantities in the entire microbial gene pool, and over the entire study period. One of the challenges of this metagenome-based approach arises when the target gene quantities are below the quantification limit of the quantitative real-time PCR assay. There are several ways proposed to deal with these missing data, such as single imputation, mean imputation, and complete case analysis. A multiple imputation approach was found to be a useful tool to handle missing data in order to obtain valid inference from parametric statistical models whose assumptions depended on the integrity of underlying distributions. We based our imputation model on unique animal identifier, sampling day, and the number of observations missing among the triplicate of each sample (i.e., 1, 2, or all 3 triplicates were missing).

In addition to the associations among the antimicrobial treatment, resistance genes, and phenotypic resistance expression, the associations among resistance genes themselves also played an important role in understanding the emergence, persistence, and propagation of unrelated resistance determinants. The complex resistance dynamics makes it necessary to deeply understand the system in order to design and implement effective intervention strategies to control antimicrobial resistance. Overall, results from this study indicate that chlortetracycline use following CCFA treatment can readily exacerbate ceftiofur resistance and therefore should be avoided when used in sequence.

Bibliography

1. **U. S. Food and Drug Administration**. 2013. Fighting the impact of antibiotic-resistant bacteria. http://www.fda.gov/downloads/ForConsumers/ConsumerUpdates/UCM350090.pdf.
2. **Cookson C**. June 2013. G8 ministers pledge to act on bacterial antibiotic resistance: Finanacial Times. London.
3. **Centers for Disease Control and Prevention.** . April 2011. Antimicrobial resistance posing growing health threat- CDC and partners celebrate World Health Day 2011 to draw attention to the issue. http://www.cdc.gov/media/releases/2011/p0407_antimicrobialresistance.html.
4. **Spellberg B**. 2012. New antibiotic development: barriers and opportunities in 2012. Alliance for the prudent use of antibiotics. Newsletter Vol. 30 No. 1.
5. **World Health Organization (WHO)**. 2012. Critically important antimicrobials for human medicine (3rd revision). http://www.who.int/foodborne_disease/resistance/cia/en/.
6. **Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, Bourgault AM, Cole L, Daignault D, Desruisseau A, Demczuk W, Hoang L, Horsman GB, Ismail J, Jamieson F, Maki A, Pacagnella A, and Pillai DR**. 2010. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. Emerging Infectious Diseases. **16**(1): p. 48-54.
7. **U. S. Food and Drug Administration**. 2012. New animal drugs; cephalosporin drugs; extralabel animal drug use; order of prohibition. Final rule. Fed Reg 77(4): 735-45. http://www.gpo.gov/fdsys/pkg/FR-2012-01-06/pdf/2012-35.pdf.

8. **Boyer TC and Singer RS**. 2012. Quantitative measurement of *bla*$_{(CMY-2)}$ in a longitudinal observational study of dairy cattle treated with ceftiofur. Foodborne Pathogens and Disease. **9**(11): p. 1022-1027.

9. **Subbiah M, Top EM, Shah DH, and Call DR**. 2011. Selection pressure required for long-term persistence of *bla*$_{CMY-2}$-positive IncA/C plasmids. Applied and Environmental Microbiology. **77**(13): p. 4486-93.

10. **Sun Z, Jiao X, Peng Q, Jiang F, Huang Y, Zhang J, and Yao F**. 2013. Antibiotic resistance in *Pseudomonas Aeruginosa* is associated with decreased fitness. Cellular Physiology and Biochemistry. **31**(2-3): p. 347-354.

11. **Platt T, Loneragan G, Scott HM, Norby B, Thomson D, Brown M, Brown M, Ives S, and Brashears M**. 2008. Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed. American Journal of Veterinary Research. **69**(8): p. 988-996.

12. **National Animal Health Monitoring System, USDA**. Overview of U.S. livestock, poultry, and aquaculture production in 2011 and statistics on major commodities. http://www.aphis.usda.gov/animal_health/nahms/downloads/Demographics2011.pdf.

13. **McEwen SA and Fedorka-Cray PJ**. 2002. Antimicrobial use and resistance in animals. Clinical Infectious Diseases. **34**(Supplement 3): p. S93-S106.

14. **United States Department of Agriculture**. 2013. Feedlot 2011 Part I: Management practices on U.S. feedlots with a capacity of 1,000 or more head. http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_dr_PartI.pdf.

15. **United States Department of Agriculture**. 2000. Health management and biosecurity in U.S. feedlots, 1999. Part 3. http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot99/Feedlot99_dr_PartIII.pdf.

16. **U. S. Food and Drug Administration**. January-February 2006, The centennial edition. Animal health and consumer protection. FDA consumer magazine. http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/AnimalHealthandConsumerProtection/.

17. **U. S. Food and Drug Administration**. 2011. Summary report on antimicrobials sold or distributed for use in food-producing animals. http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf.

18. **Mathew AG, Cissell R, and Liamthong S**. 2007. Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. Foodborne Pathogens and Disease. **4**(2): p. 115-33.

19. **Regassa TH, Koelsch RK, Wortmann CS, Randle RF, and Abunyewa AA**. Use of antibiotics in animal production: environmental concerns. Universityof Nebraska-Lincoln Extension RP196. (http://ianrpubs.unl.edu/epublic/live/rp196/build/rp196.pdf).

20. **Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, and Waddell J**. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy. **53**(1): p. 28-52.

21. **National Research Council IoM**, 1999.The use of drugs in food animals: Benefits and risks. Washington, D.C.

22. **United States Department of Agriculture**. 2002. National agricultural statistics database -1992 to 2001-U.S. totals. http://www.nass.usda.gov/research/.

23. **U. S. Food and Drug Administration**. 2011. Summary report on antimicrobials sold or distributed for use in food-producing animals. http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf.

24. **U. S. Food and Drug Administration**. 2009. Summary report on antimicrobials sold or distributed for use in food-producing animals. http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM231851.pdf.

25. **U. S. Food and Drug Administration**. 2010. Summary report on antimicrobials sold or distributed for use in food-producing animals. http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM277657.pdf.

26. **Department of Health and Human Services. U. S. Food and Drug Administration**. 2012. Estimates of antibacterial drug sales for use in humans. http://www.fda.gov/downloads/Drugs/DrugSafety/InformationbyDrugClass/UCM319435.pdf.

27. **Department of Health and Human Services. U. S. Food and Drug Administration**. 2010. Sales of antibacterial drugs in kilograms.

http://www.fda.gov/downloads/Drugs/DrugSafety/InformationbyDrugClass/UCM261174.pdf.

28. **U. S. Food and Drug Administration**. 2012. Estimates of Antibacterial Drug Sales in Human Medicine. http://www.fda.gov/Drugs/DrugSafety/InformationbyDrugClass/ucm261160.htm.

29. **Hicks LA, Taylor TH, and Hunkler RJ**. 2013. U.S. outpatient antibiotic prescribing, 2010. New England Journal of Medicine. **368**(15): p. 1461-1462.

30. **Parry CM and Threlfall EJ**. 2008. Antimicrobial resistance in typhoidal and nontyphoidal *Salmonellae*. Current Opinion in Infectious Diseases. **21**(5): p. 531-8.

31. **Frye JG and Fedorka-Cray PJ**. 2007. Prevalence, distribution and characterisation of ceftiofur resistance in *Salmonella enterica* isolated from animals in the USA from 1999 to 2003. International Journal of Antimicrobial Agents. **30**(2): p. 134-142.

32. **Donaldson SC, Straley BA, Hegde NV, Sawant AA, DebRoy C, and Jayarao BM**. 2006. Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. Applied and Environmental Microbiology. **72**(6): p. 3940-3948.

33. **Frye JG and Jackson CR**. 2013. Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica*, *Escherichia coli*, and *Enteroccocus* spp. isolated from U.S. food animals. Frontiers in Microbiology. **4**(135): p. 135.

34. **Li XZ, Mehrotra M, Ghimire S, and Adewoye L**. 2007. Beta-Lactam resistance and beta-lactamases in bacteria of animal origin. Veterinary Microbiology. **121**(3-4): p. 197-214.

35. **Livermore DM**. 1995. Beta-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews. **8**(4): p. 557-84.

36. **Bradford PA**. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Reviews. **14**(4): p. 933-51, table of contents.

37. **Center for Disease Control and Prevention**. 2003. National Antimicrobial Resistance Monitoring System-enteric bacteria (NARMS): 2003 executive summary.

38. **Glenn LM, Lindsey RL, Frank JF, Meinersmann RJ, Englen MD, Fedorka-Cray PJ, and Frye JG**. 2011. Analysis of antimicrobial resistance genes detected in multidrug-resistant *Salmonella enterica* serovar Typhimurium isolated from food animals. Microbial Drug Resistance. **17**(3): p. 407-418.

39. **Doyle M, Loneragan GH, Scott HM and Singer RS**. 2013. Antimicrobial Resistance: Challenges and Perspectives. Comprehensive Reviews in Food Science and Food Safety. **12**(2): p. 234-248.

40. **Winokur PL, Brueggemann A, DeSalvo DL, Hoffmann L, Apley MD, Uhlenhopp EK, Pfaller MA, and Doern GV**. 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrobial Agents and Chemotherapy. **44**(10): p. 2777-83.

41. **Rankin SC, Aceto H, Cassidy J, Holt J, Young S, Love B, Tewari D, Munro DS, and Benson CE**. 2002. Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvania. Journal of Clinical Microbiology. **40**(12): p. 4679-84.

42. **Winokur PL, Canton R, Casellas J-M, and Legakis N**. 2001. Variations in the prevalence of strains expressing an extended-spectrum β-Lactamase phenotype and

characterization of isolates from Europe, the Americas, and the Western Pacific Region. Clinical Infectious Diseases. **32**(Supplement 2): p. S94-S103.

43. **Poppe C, Martin L, Muckle A, Archambault M, McEwen S, and Weir E**. 2006. Characterization of antimicrobial resistance of *Salmonella* Newport isolated from animals, the environment, and animal food products in Canada. Canadian Journal of Veterinary Research. **70**(2): p. 105-14.

44. **Daniels JB, Call DR, and Besser TE**. 2007. Molecular epidemiology of $bla_{\text{CMY-2}}$ plasmids carried by *Salmonella enterica* and *Escherichia coli* isolates from cattle in the pacific northwest. Applied and Environmental Microbiology. **73**(24): p. 8005-8011.

45. **Fernández-Alarcón C, Singer RS, and Johnson TJ**. 2011. Comparative genomics of multidrug resistance-encoding IncA/C plasmids from commensal and pathogenic *Escherichia coli* from multiple animal sources. PLoS ONE. **6**(8): p. e23415.

46. **Burman LG, Park JT, Lindstrom EB, and Boman HG**. 1973. Resistance of *Escherichia coli* to penicillins: identification of the structural gene for the chromosomal penicillinase. Journal of Bacteriology. **116**(1): p. 123-30.

47. **Jacoby GA**. 2009. AmpC beta-lactamases. Clinical Microbiology Reviews. **22**(1): p. 161-82.

48. **Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz I, Luzzaro F, Poirel L, and Woodford N**. 2007. CTX-M: changing the face of ESBLs in Europe. The Journal of Antimicrobial Chemotherapy. **59**(2): p. 165-74.

49. **Canton R, Gonzalez-Alba JM, and Galan JC**. 2012. CTX-M enzymes: origin and diffusion. Frontiers in Microbiology. **3**(110): p. 110.

50. **Bush K**. 2008. Extended-spectrum beta-lactamases in North America, 1987-2006. Clinical Microbiology and Infection. **14 Suppl 1**: p. 134-43.

51. **Moland ES, Black JA, Hossain A, Hanson ND, Thomson KS, and Pottumarthy S**. 2003. Discovery of CTX-M-like extended-spectrum beta-lactamases in *Escherichia coli* isolates from five US States. Antimicrobial Agents and Chemotherapy. **47**(7): p. 2382-3.

52. **Mollenkopf DF, Weeman MF, Daniels JB, Abley MJ, Mathews JL, Gebreyes WA, and Wittum TE**. 2012. Variable within- and between-herd diversity of CTX-M cephalosporinase-bearing *Escherichia coli* isolates from dairy cattle. Applied and Environmental Microbiology. **78**(13): p. 4552-4560.

53. **Wittum TE, Mollenkopf DF, Daniels JB, Parkinson AE, Mathews JL, Fry PR, Abley MJ, and Gebreyes WA**. 2010. CTX-M-type extended-spectrum beta-lactamases present in *Escherichia coli* from the feces of cattle in Ohio, United States. Foodborne Pathogens and Disease. **7**(12): p. 1575-9.

54. **Boyd DA, Tyler S, Christianson S, McGeer A, Muller MP, Willey BM, Bryce E, Gardam M, Nordmann P, and Mulvey MR**. 2004. Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrobial Agents and Chemotherapy. **48**(10): p. 3758-64.

55. **Bonnet R**. 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrobial Agents and Chemotherapy. **48**(1): p. 1-14.

56. **Costa D, Poeta P, Brinas L, Saenz Y, Rodrigues J, and Torres C**. 2004. Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. The Journal of Antimicrobial Chemotherapy. **54**(5): p. 960-1.

57. **Nelson ML and Levy SB**. 2011. The history of the tetracyclines. Annals of the New York Academy of Sciences. **1241**: p. 17-32.

58. **Chopra I and Roberts M**. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews. **65**(2): p. 232-60

59. **Walsh C**. 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature. **406**(6797).

60. **Mathers JJ, Flick SC, and Cox LA, Jr.** 2011. Longer-duration uses of tetracyclines and penicillins in U.S. food-producing animals: Indications and microbiologic effects. Environment International. **37**(5): p. 991-1004.

61. **Bryan A, Shapir N, and Sadowsky MJ**. 2004. Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. Applied and Environmental Microbiology. **70**(4): p. 2503-2507.

62. **Sengeløv G, Halling-Sørensen B, and Aarestrup FM**. 2003. Susceptibility of *Escherichia coli* and *Enterococcus faecium* isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in *E. coli* from food animals. Veterinary Microbiology. **95**(1–2): p. 91-101.

63. **Blake DP, Humphry RW, Scott KP, Hillman K, Fenlon DR, and Low JC**. 2003. Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal *Escherichia coli* populations. Journal of Applied Microbiology. **94**(6): p. 1087-1097.

64. **Gow SP, Waldner CL, Harel J, and Boerlin P**. 2008. Associations between antimicrobial resistance genes in fecal generic *Escherichia coli* isolates from cow-calf herds in western Canada. Applied and Environmental Microbiology. **74**(12): p. 3658-66.

65. **Watanabe T**. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriological reviews. **27**: p. 87-115.

66. **Ochiai K, Yamanaka T, Kimura K, and O. S**. 1959. Studies on inheritance of drug resistance between *Shigella* strains and *Escherichia coli* strains. Nippon Iji Shimpo. **Vol. 1861**: p. 34-46.

67. **Harada K, Kameda M, Suzuki M, Hashimoto H, and Mitsuhashi S**. 1961. [On the drug resistance of enteric bacteria. 6. Transmissibility of multiple drug resistance among *Citrobacter*, *Shigella* and *E. coli*]. Nihon Saikingaku Zasshi. **16**: p. 137-41.

68. **Guest GB**. 1976. Status of FDA's program on the use of antibiotics in animal feeds. Journal of Animal Science. **42**(4): p. 1052-7.

69. **Swann M. M**. 1969. Report of joint committee on the use of antibiotics in animal husbandry and veterinary medicine. Cmnd. 4190. London. Her Majesty's Stationery Office London.

70. **Landers T, Cohen B, Wittum T, and Larson E**. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public health reports. **127**(1): p. 4-22.

71. **Haapapuro ER, Barnard ND, and Simon M**. 1997. Review--animal waste used as livestock feed: dangers to human health. Preventive Medicine. **26**(5 Pt 1): p. 599-602.

72. **Levy SB**, 1992.The antibiotic paradox. : New York: Plenum Press. Pp. 137–156.

73. **Witte W and Klare I**. 1995. Glycopeptide-resistant *Enterococcus faecium* outside hospitals: a commentary. Microbial Drug Resistance. **1**(3): p. 259-63.

74. **Lyons RW, Samples CL, DeSilva HN, Ross KA, Julian EM, and Checko PJ**. 1980. An epidemic of resistant *Salmonella* in a nursery. Animal-to-human spread. Journal of the American Medical Association. **243**(6): p. 546-7.

75. **Angulo FJ, Nargund VN, and Chiller TC**. 2004. Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance. Journal of Veterinary Medicine. Series B. **51**(8-9): p. 374-379.

76. **Fey PD, Safranek TJ, Rupp ME, Dunne EF, Ribot E, Iwen PC, Bradford PA, Angulo FJ, and Hinrichs SH**. 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. New England Journal of Medicine. **342**(17): p. 1242-1249.

77. **Ferber D**. 2000. Superbugs on the Hoof? Science. **288**(5467): p. 792-794.

78. **Salyers A and Shoemaker NB**. 2006. Reservoirs of antibiotic resistance genes. Animal Biotechnology. **17**(2): p. 137-46.

79. **Aarestrup FM, Collignon P, and Wegener HC**. 2008. Resistance in bacteria of the food chain: epidemiology and control strategies. Expert Review of Anti-infective Therapy. **6**(5): p. 733.

80. **Linton AH**. 1977. Animal to man transmission of Enterobacteriaceae. Royal Society of Health journal. **97**(3): p. 115-118.

81. **Corpet DE**. 1988. Antibiotic resistance from food. New England Journal of Medicine. **318**(18): p. 1206-7.

82. **Linton AH, Howe K, Bennett PM, Richmond MH, and Whiteside EJ**. 1977. The colonization of the human gut by antibiotic resistant *Escherichia coli* from chickens. Journal of Applied Bacteriology. **43**(3): p. 465-9.

83. **Winokur PL, Vonstein DL, Hoffman LJ, Uhlenhopp EK, and Doern GV**. 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrobial Agents and Chemotherapy. **45**(10): p. 2716-22.

84. **Salyers AA, Gupta A, and Wang Y**. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology. **12**(9): p. 412-416.

85. **Lester CH, Frimodt-Moller N, Sorensen TL, Monnet DL, and Hammerum AM**. 2006. In vivo transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrobial Agents and Chemotherapy. **50**(2): p. 596-9.

86. **Shoemaker NB, Vlamakis H, Hayes K, and Salyers AA**. 2001. Evidence for Extensive Resistance Gene Transfer amongBacteroides spp. and among Bacteroides and Other Genera in the Human Colon. Applied and Environmental Microbiology. **67**(2): p. 561-568.

87. **Service FaI**. **1994**. Nationwide beef biological baseline data collection program: steers and heifers, USDA SaT, Microbiology Division, Editor.

88. **Bryan FL and Doyle MP**. 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. Journal of Food Protection. **58**(3): p. 326-344.

89. **Scott HM, Campbell LD, Harvey RB, Bischoff KM, Alali WQ, Barling KS, and Anderson RC**. 2005. Patterns of antimicrobial resistance among commensal *Escherichia coli* isolated from integrated multi-site housing and worker cohorts of humans and swine. Foodborne Pathogens and Disease. **2**(1): p. 24-37.

90. **National Research Council, Institute of Medicine**. 1999. The use of drugs in food animals: Benefits and risks. Washington, D.C.

91. **Singer R, Finch R, Wegener H, Bywater R, Walters J, and Lipsitch M**. 2003. Antibiotic resistance--the interplay between antibiotic use in animals and human beings. The Lancet Infectious Diseases. **3**(1): p. 47-51.

92. **Vieira AR, Collignon P, Aarestrup FM, McEwen SA, Hendriksen RS, Hald T, and Wegener HC**. 2011. Association between antimicrobial resistance in *Escherichia coli* isolates from food animals and blood stream isolates from humans in Europe: an ecological study. Foodborne Pathogens and Disease. **8**(12): p. 1295-301.

93. **Cox L and Singer R**. 2012. Confusion over antibiotic resistance: ecological correlation is not evidence of causation. Foodborne Pathogens and Disease. **9**(8): p. 776-776.

94. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, and Griffin PM**. 2011. Foodborne illness acquired in the United States--major pathogens. Emerging Infectious Diseases. **17**(1): p. 7-15.

95. **U. S. Food and Drug Administration**. Judicious use of antimicrobials for beef cattle veterinarians. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/JudiciousUseofAntimicrobials/ucm2007130.htm.

96. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, and Griffin PM**. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerging Infectious Diseases. **19**(3): p. 407-15.

97. **Scallan E, Griffin PM, Angulo FJ, Tauxe RV, and Hoekstra RM**. 2011. Foodborne illness acquired in the United States--unspecified agents. Emerging Infectious Diseases. **17**(1): p. 16-22.

98. **Scharff RL**. 2012. Economic burden from health losses due to foodborne illness in the United States. Journal of Food Protection. **75**(1): p. 123-131.

99. **Capita R and Alonso Calleja C**. 2013. Antibiotic-resistant bacteria: a challenge for the food industry. Critical reviews in food science and nutrition. **53**(1): p. 11-48.

100. **Institute of Medicine**. 1998. Antimicrobial drug resistance: issues and options. Workshop report. Washington: National Academy Press.

101. **Elbasha EH**. 2003. Deadweight loss of bacterial resistance due to overtreatment. Health economics. **12**(2): p. 125-38.

102. **Roberts RR, Hota B, Ahmad I, Scott RD, Foster SD, Abbasi F, Schabowski S, Kampe LM, Ciavarella GG, Supino M, Naples J, Cordell R, Levy SB, and Weinstein RA**. 2009. Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: Implications for antibiotic stewardship. Clinical Infectious Diseases. **49**(8): p. 1175-1184.

103. **Tollefson L**. 1996. FDA reveals plans for antimicrobial susceptibility monitoring. Journal of the American Veterinary Medical Association. **208**(4): p. 459-60.

104. **U. S. Food and Drug Administration**. (FDA/SAB). 2007. National Antimicrobial Resistance Monitoring System (NARMS) program review. http://www.fda.gov/ohrms/dockets/ac/07/briefing/2007-4329b_02_06_NARMS%20Review%20Update.pdf.

105. **Patterson SK and Singer RS**. 2006. Development of a polymerase chain reaction assay for the detection of antibiotic resistance genes in community DNA. Journal of Veterinary Diagnostic Investigation. **18**(2): p. 172-181.

106. **Alali WQ, Scott HM, Norby B, Gebreyes W, and Loneragan GH**. 2009. Quantification of the *bla*$_{(CMY-2)}$ in feces from beef feedlot cattle administered three different doses of ceftiofur in a longitudinal controlled field trial. Foodborne Pathogens and Disease. **6**(8): p. 917-924.

107. **United States Department of Agriculture**. 2011. National Antimicrobial Resistance Monitoring System (NARMS) 2010 Executive report, D.C. W, Editor: Washington D.C.

108. **U. S. Food and Drug Administration**. 2012. Guidance for industry (GFI#209). The judicious use of medically important antimicrobial drugs in food-producing animals. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM216936.pdf.

109. **Cattlemen's Beef Board and National Cattlemen's Beef Association**. 2009. Antibiotic use in cattle production: Factsheet. http://www.explorebeef.org/CMDocs/ExploreBeef/FactSheet_AntibioticUseInCattleProduction.pdf.

110. **U. S. Food and Drug Administration**. 1997. Extralabel animal drug use; fluoroquinolones and glycopeptides; order of prohibition. Fed Reg 62(99):27944-7. http://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm127904.htm.

111. **U. S. Food and drug Administration**. 2003. Guidance for industry (GFI#152). Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern.

http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052519.pdf.

112. **U. S. Food and Drug Administration**. 2012. Guidance for industry (GFI #159). Studies to evaluate the safety of residues in veterinary drugs in human food: General approach to establish a microbiological ADI, VICH GL36(R). http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM124674.pdf.

113. **U. S. Food and Drug Administration**. 2008. New animal drugs; cephalosporin drugs; extralabel animal drug use; order of prohibition. Fed Reg 73(129): 38110-13. https://www.avma.org/Advocacy/National/Federal/Pages/Extralabel-Use-of-Cephalosporins-in-Food-Producing-Animals.aspx.

114. **Alliance for the Prudent Use of Antibiotics**. 2012. Preservation of Antibiotics for Medical Treatment Act (PAMTA) reintroduced to US Congress. http://www.tufts.edu/med/apua/news/news-newsletter-vol-29-no--1-11.shtml.

115. **Singer RS, Patterson SK, and Wallace RL**. 2008. Effects of therapeutic ceftiofur administration to dairy cattle on *Escherichia coli* dynamics in the intestinal tract. Applied and Environmental Microbiology. **74**(22): p. 6956-6962.

116. **Lowrance TC, Loneragan GH, Kunze DJ, Platt TM, Ives SE, Scott HM, Norby B, Echeverry A, and Brashears MM**. 2007. Changes in antimicrobial susceptibility in a population of *Escherichia coli* isolated from feedlot cattle administered ceftiofur crystalline-free acid. American Journal of Veterinary Research. **68**(5): p. 501-507.

117. **Tragesser LA, Wittum TE, Funk JA, Winokur PL, and Rajala-Schultz PJ**. 2006. Association between ceftiofur use and isolation of *Escherichia coli* with reduced

susceptibility to ceftriaxone from fecal samples of dairy cows. American Journal of Veterinary Research. **67**(10): p. 1696-700.

118. **Alexander TW, Reuter T, Sharma R, Yanke LJ, Topp E, and McAllister TA**. 2009. Longitudinal characterization of resistant *Escherichia coli* in fecal deposits from cattle fed subtherapeutic levels of antimicrobials. Applied and Environmental Microbiology. **75**(22): p. 7125-7134.

119. **Daniels JB, Call DR, Hancock D, Sischo WM, Baker K, and Besser TE**. 2009. Role of ceftiofur in selection and dissemination of $bla_{\text{CMY-2}}$-mediated cephalosporin resistance in *Salmonella enterica* and commensal *Escherichia coli* isolates from cattle. Applied and Environmental Microbiology. **75**(11): p. 3648-55.

120. **Schmidt JW, Griffin D, Kuehn LA, and Brichta-Harhay DM**. 2013. Influence of therapeutic ceftiofur treatments of feedlot cattle on fecal and hide prevalences of commensal *Escherichia coli* resistant to expanded-spectrum cephalosporins, and molecular characterization of resistant isolates. Applied and Environmental Microbiology. **79**(7): p. 2273-2283.

121. **Pfizer**. Excenel® RTU. Pfizer animal health. Available at: https://animalhealth.pfizer.com/sites/pahweb/US/EN/Products/Pages/Excenel.aspx, accessed October 23, 2012.

122. **Mann S, Siler J, Jordan D, and Warnick L**. 2011. Antimicrobial susceptibility of fecal *Escherichia coli* isolates in dairy cows following systemic treatment with ceftiofur or penicillin. Foodborne Pathogens and Disease. **8**(8): p. 861-867.

123. **Lamb HM, Ormrod D, Scott LJ, and Figgitt DP**. 2002. Ceftriaxone: an update of its use in the management of community-acquired and nosocomial infections. Drugs. **62**(7): p. 1041-89.

124. **Tragesser LA, Wittum TE, Funk JA, Winokur PL, and Rajala-Schultz PJ**. 2006. Association between ceftiofur use and isolation of *Escherichia coli* with reduced susceptibility to ceftriaxone from fecal samples of dairy cows. American Journal of Veterinary Research. **67**(10): p. 1696-700.

125. **Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, and Walker RD**. 2001. Identification and expression of cephamycinase $bla_{(CMY)}$ genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrobial Agents and Chemotherapy. **45**(12): p. 3647-50.

126. **Dunne EF, Fey PD, Kludt P, Reporter R, Mostashari F, Shillam P, Wicklund J, Miller C, Holland B, Stamey K, Barrett TJ, Rasheed JK, Tenover FC, Ribot EM, and Angulo FJ**. 2000. Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC beta-lactamase. The Journal of the American Medical Association. **284**(24): p. 3151-6.

127. **U. S. Food and Drug Administration**. 2012. FDA to protect important class of antimicrobial drugs for treating human illness. Available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm285704.htm, accessed October 24, 2012.

128. **Hayes DJ and Jensen HH**. 2003. Lessons from the Danish ban on feed-grade antibiotics. Iowa State University, Department of Economics, Staff General Research Papers.

129. **Kaye KS, Cosgrove S, Harris A, Eliopoulos GM, and Carmeli Y**. 2001. Risk factors for emergence of resistance to broad-spectrum cephalosporins among *Enterobacter* spp. Antimicrobial Agents and Chemotherapy. **45**(9): p. 2628-30.

130. **Schwaber MJ, Cosgrove SE, Gold HS, Kaye KS, and Carmeli Y**. 2004. Fluoroquinolones protective against cephalosporin resistance in gram-negative nosocomial pathogens. Emerging Infectious Diseases. **10**(1): p. 94-9.

131. **Kanwar N, Scott HM, Norby B, Loneragan GH, Vinasco J, McGowan M, Cottell JL, Chengappa MM, Bai J, and Boerlin P**. 2013. Effects of ceftiofur and chlortetracycline treatment strategies on antimicrobial susceptibility profiles and their relationship with *tet*(A), *tet*(B) and bla_{CMY2} genes present among *E. coli* isolated from beef cattle feces (submitted).

132. **Cottell JL, Scott HM, Kanwar N, Norby B, Loneragan GH and Boerlin P**. 2013. Differential dynamics between resistance genes, plasmids and *E. coli* strains isolated from beef cattle exposed to antimicrobials. Submitted.

133. **Cottell JL, Kanwar N, Castillo-Courtade L, Chalmers G, Scott HM, Norby B, Loneragan GH, and Boerlin P**. 2013. $bla_{CTX-M-32}$ on an IncN plasmid in *Escherichia coli* from beef cattle in the United States. Antimicrobial Agents and Chemotherapy. **57**(2): p. 1096-1097.

134. **Boerlin P, Travis R, Gyles CL, Reid-Smith R, Heather Lim NJ, Nicholson V, McEwen SA, Friendship R, and Archambault M**. 2005. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. Applied and Environmental Microbiology. **71**(11): p. 6753-6761.

135. **Schrag SJ and Perrot V**. 1996. Reducing antibiotic resistance. Nature. **381**(6578): p. 120-1.

136. **Cottell JL, Webber MA, and Piddock LJ**. 2012. Persistence of transferable extended-spectrum-beta-lactamase resistance in the absence of antibiotic pressure. Antimicrobial Agents and Chemotherapy. **56**(9): p. 4703-6.

137. **Lipsitch M, Bergstrom CT, and Levin BR**. 2000. The epidemiology of antibiotic resistance in hospitals: paradoxes and prescriptions. Proceedings of the National Academy of Sciences of the United States of America. **97**(4): p. 1938-43.

138. **Fan W, Hamilton T, Webster-Sesay S, Nikolich MP, and Lindler LE**. 2007. Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. Molecular and Cellular Probes. **21**(4): p. 245-256.

139. **Odeh R, Kelkar S, Hujer AM, Bonomo RA, Schreckenberger PC, and Quinn JP**. 2002. Broad resistance due to plasmid-mediated AmpC β-Lactamases in clinical isolates of *Escherichia coli*. Clinical Infectious Diseases. **35**(2): p. 140-145.

140. **Sterne JAC, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, Wood AM, and Carpenter JR**. 2009. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. British Medical Journal. **339**(7713): p. 157.

141. **Burton A, Billingham LJ, and Bryan S**. 2007. Cost-effectiveness in clinical trials: using multiple imputation to deal with incomplete cost data. Clinical Trials. **4**(2): p. 154-61.

142. **Horton NJ and Lipsitz SR**. 2001. Multiple imputation in practice: Comparison of software packages for regression models with missing variables. The American Statistician. **55**(3): p. 244-254.

143. **Boyer TC and Singer RS**. 2012. Quantitative Measurement of *bla*$_{(CMY-2)}$ in a Longitudinal Observational Study of Dairy Cattle Treated with Ceftiofur. Foodborne pathogens and disease. **17**: p. 17.

144. **Fogel GB, Collins CR, Li J, and Brunk CF**. 1999. Prokaryotic Genome Size and SSU rDNA Copy Number: Estimation of Microbial Relative Abundance from a Mixed Population. Microbial Ecology. **38**(2): p. 93-113.

145. **Greenland S and Finkle WD**. 1995. A critical look at methods for handling missing covariates in epidemiologic regression analyses. American journal of epidemiology. **142**(12): p. 1255-1264.

146. **Peters SAE, Bots ML, den Ruijter HM, Palmer MK, Grobbee DE, Crouse JR, O'Leary DH, Evans GW, Raichlen JS, Moons KGM, and Koffijberg H**. 2012. Multiple imputation of missing repeated outcome measurements did not add to linear mixed-effects models. Journal of Clinical Epidemiology. **65**(6): p. 686-95.

147. **Steinman CR, Muralidhar B, Nuovo GJ, Rumore PM, Yu D, and Mukai M**. 1997. Domain-directed polymerase chain reaction capable of distinguishing bacterial from host DNA at the single-cell level: characterization of a systematic method to investigate putative bacterial infection in idiopathic disease. Analytical Biochemistry. **244**(2): p. 328-39.

148. **Ng LK, Martin I, Alfa M, and Mulvey M**. 2001. Multiplex PCR for the detection of tetracycline resistant genes. Molecular and Cellular Probes. **15**(4): p. 209-15.

149. **Levy SB and Marshall B**. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature Medicine. **10**(12 Suppl): p. S122-9.

150. **World Health Organisation**. 2001. WHO Global strategy for containment of antimicrobial resistance. http://www.who.int/drugresistance/guidance/en/index.html.

151. **Van den Bogaard AE and Stobberingh EE**. 1999. Antibiotic usage in animals: impact on bacterial resistance and public health. Drugs. **58**(4): p. 589-607.

152. **Grave K, Jensen VF, Odensvik K, Wierup M, and Bangen M**. 2006. Usage of veterinary therapeutic antimicrobials in Denmark, Norway and Sweden following termination of antimicrobial growth promoter use. Preventive Veterinary Medicine. **75**(1-2): p. 123-132.

153. **Wierup M**. 2001. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microbial Drug Resistance. **7**(2): p. 183-90.

154. **U. S. Food and drug Administration**. 2005. Withdrawal of approval of the new animal drug application for enrofloxacin in poultry. U.S. Department of Health and Human Services: Washington D.C.

155. **National Academy of science**. 1999. The use of drugs in food animals; benefits and risks. National Academy Press: Washington D.C.

156. **Oliver SP, Murinda SE, and Jayarao BM**. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathogens and Disease. **8**(3): p. 337-55.

157. **Levin BR, Lipsitch M, and Bonhoeffer S**. 1999. Population biology, evolution, and infectious disease: convergence and synthesis. Science. **283**(5403): p. 806-9.

158. **Alcaine SD, Sukhnanand SS, Warnick LD, Su WL, McGann P, McDonough P, and Wiedmann M**. 2005. Ceftiofur-resistant *Salmonella* strains isolated from dairy farms

represent multiple widely distributed subtypes that evolved by independent horizontal gene transfer. Antimicrobial Agents and Chemotherapy. **49**(10): p. 4061-7.

159. **National Committee for Clinical Laboratory Standards**. 2000. Methods for dilution antimicrobial susceptibiltiy tests for bacteria that grow aerobically. Vol 19(1): Wayne, PA.

160. **Clinical and Laboratory Standards Institute**. 2008. Performance standards for antimicrobial susceptibility testing: Eighteenth informational supplement. CLSI document M7–A7: Wayne, PA.

161. **National Antimicrobial Resistance Monitoring System**. 2009. National Antimicrobial Resistance Monitoring System: Animal Arm Annual Report: Georgi, Athens.

162. **Clinical and Laboratory Standards Institute**. 2011. Performance standards for antimicrobial susceptibility testing: Twenty first Informational supplement. CLSI document M100–S21: Wayne, PA.

163. **Harvey R, Funk J, Wittum TE, and Hoet AE**. 2009. A metagenomic approach for determining prevalence of tetracycline resistance genes in the fecal flora of conventionally raised feedlot steers and feedlot steers raised without antimicrobials. American Journal of Veterinary Research. **70**(2): p. 198-202.

164. **Hesketh SR and Skrondal A**, 2008.Multilevel and longitudinal modeling using Stata. Second ed., College Station, Texas: STATA. 331-372.

165. **Norby B, Scott HM, Loneragan GH, Moore S, Kanwar N, and Harvey R**. 2011. The effect of two intervention strategies on ceftiofur resistance in *E. coli*., abstr O03. 4th symposium on Antimicrobial Resistance in Animal and the Environment: Tours, France. p. 31.

166. **Akwar HT, Poppe C, Wilson J, Reid-Smith RJ, Dyck M, Waddington J, Shang D, and McEwen SA**. 2008. Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia. Canadian Journal of Veterinary Research. **72**(2): p. 202-10.

167. **O'Connor AM, Poppe C, and McEwen SA**. 2002. Changes in the prevalence of resistant *Escherichia coli* in cattle receiving subcutaneously injectable oxytetracycline in addition to in-feed chlortetracycline compared with cattle receiving only in-feed chlortetracycline. Canadian Journal of Veterinary Research. **66**(3): p. 145-50.

168. **Jones CS, Osborne DJ, and Stanley J**. 1992. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. Molecular and Cellular Probes. **6**(4): p. 313-7.

169. **Carattoli A, Tosini F, Giles WP, Rupp ME, Hinrichs SH, Angulo FJ, Barrett TJ, and Fey PD**. 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrobial Agents and Chemotherapy. **46**(5): p. 1269-72.

170. **Stegeman JA, Vernooij JCM, Khalifa OA, Van den Broek J, and Mevius DJ**. 2006. Establishing the change in antibiotic resistance of *Enterococcus faecium* strains isolated from Dutch broilers by logistic regression and survival analysis. Preventive Veterinary Medicine. **74**(1): p. 56-66.

171. **Hidalgo A, Carvajal A, Vester B, Pringle M, Naharro G, and Rubio P**. 2011. Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in Spain. Antimicrobial Agents and Chemotherapy. **55**(7): p. 3330-7.

172. **Hidalgo Á, Carvajal A, García-Feliz C, Osorio J, and Rubio P**. 2009. Antimicrobial susceptibility testing of Spanish field isolates of *Brachyspira hyodysenteriae*. Research in Veterinary Science. **87**(1): p. 7-12.

173. **Willett JB and Singer JD**. 1993. Investigating onset, cessation, relapse, and recovery: Why you should, and how you can, use discrete-time survival analysis to examine event occurrence. Journal of Consulting and Clinical Psychology. **61**(6): p. 952-965.

174. **Hosmer DW and Lemeshow S**, 2000.Applied Logistic Regression. 2nd ed: John Wiley & Sons Inc. 91-142.

175. **Rollo SN, Norby B, Bartlett PC, Scott HM, Wilson DL, Fajt VR, Linz JE, Bunner CE, Kaneene JB, and Huber JC**. 2010. Prevalence and patterns of antimicrobial resistance in *Campylobacter* spp isolated from pigs reared under antimicrobial-free and conventional production methods in eight states in the Midwestern United States. Journal of the American Veterinary Medical Association. **236**(2): p. 201-210.