THE EPIDEMIOLOGY OF TETRACYCLINE AND CEFTIOFUR RESISTANCE IN COMMENSAL ESCHERICHIA COLI

by

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Abstract

The modern phenomenon of increasing prevalence of antibiotic resistance in clinically relevant bacteria threatens humanity’s ability to use antibiotics to treat infection in both humans and animals. Despite the marked complexity of bacterial evolution, there is tremendous importance in unfolding the process by which antibiotic resistance genes emerge, disperse, and persist in the natural world. This thesis investigates certain aspects of this process in two experimental studies that differ primarily by scale but also by methodology.

The first study examined the long-term annual prevalence of ceftiofur and tetracycline resistance in Canadian beef cattle from 2002 to 2011 at both phenotypic and genotypic levels. Ceftiofur was present at a very low prevalence (<4%) that did not statistically increase over the decade (p<0.05). Relative proportions of tetracycline genes tet(A), tet(B), and tet(C) also did not significantly change over the observation period. However, it was surprising that almost 20% of isolates recovered from nonselective agar harbored tet(C) given that current literature generally indicates that tet(C) is significantly less prevalent than tet(A) or tet(B). The usage of historical samples in addition to parallel selective plating using agar supplemented with antibiotics provided insight into systemic bias present in common microbial approaches. Long-term sample freezing significantly diminished the recoverability of E. coli over time. Additionally the usage of selective MacConkey agar containing tetracycline biased the proportions of tetracycline genes to over-represent the tet(B) gene in commensal E. coli compared to nonselective MacConkey agar.

The second study attempted to explain the short-term selection effects of antibiotic treatment on the overall ecological fitness of commensal E. coli using bacterial growth parameters estimated from spectrophotometric growth curves as a simple surrogate of general
fitness. Treating cattle with either tetracycline or ceftiofur was found to not only select in favor of tetracycline resistant bacteria, but also increased the overall fitness among the tetracycline resistant population. However, growth curves were unable able to explain why transiently selected resistant bacteria were eventually replaced by susceptible bacteria once the selection pressure was removed.
# Table of Contents

List of Figures ................................................................................................................... vii
List of Tables ...................................................................................................................... ix
Acknowledgements ......................................................................................................... x
Dedication .......................................................................................................................... xi

Chapter 1 - Overview of tetracycline and ceftiofur resistance ....................................... 1
  Objective .......................................................................................................................... 1
  Purpose ............................................................................................................................. 1
  Tetracycline Resistance ................................................................................................. 2
  Ceftiofur resistance ....................................................................................................... 5
  Antibiotic Resistance in Beef Cattle .............................................................................. 7
  Bridging the Research Gap ............................................................................................ 12

Chapter 2 - Surveillance of tetracycline and ceftiofur resistance in Canadian beef cattle 2002-2011 ......................................................................................................................... 14
  Materials and Methods ................................................................................................... 16
    Sample Collection ......................................................................................................... 16
    Original Surveillance Testing ........................................................................................ 17
    Bacterial Re-isolation .................................................................................................... 17
    Antimicrobial Susceptibility Testing ............................................................................. 18
    Tetracycline Resistance Gene Determination .............................................................. 18
    Analysis .......................................................................................................................... 19

Results ............................................................................................................................... 21
  Freezing Effect ............................................................................................................... 21
  Prevalence of tetracycline and ceftiofur resistance ......................................................... 21
  MIC distribution ............................................................................................................. 22
  Multidrug Resistance ..................................................................................................... 23
  Tetracycline gene distribution ........................................................................................ 23
  Relationships between tet genes and multidrug resistance ........................................... 24
  Discussion ....................................................................................................................... 25
Tetracycline and Ceftiofur prevalence ................................................................. 25
Freezing effect .................................................................................................. 27
Multidrug resistance ......................................................................................... 28
Distribution of tetracycline resistance genes ..................................................... 28
Agar selective effect ......................................................................................... 31
Tetracycline co-resistance ................................................................................. 32
Conclusion ....................................................................................................... 32

Chapter 3 - Short-term population selection effects of chlortetracycline and ceftiofur treatment
on growth parameters of commensal E. coli ...................................................... 53
Introduction ..................................................................................................... 53
Materials and Methods ................................................................................... 54
Previous studies ............................................................................................... 54
Field trial .......................................................................................................... 54
Samples subjected to isolation ......................................................................... 55
Isolation of commensal E. coli ......................................................................... 55
MIC determination ............................................................................................ 56
Determining bacterial fitness .......................................................................... 56
Measurement of bacterial growth curves ......................................................... 59
Qualitative detection of resistance genes ......................................................... 60
Statistical analysis ............................................................................................. 60
Results .............................................................................................................. 62
MIC distributions ............................................................................................. 62
Tetracycline resistance gene selection ............................................................. 62
Growth curve parameter values ...................................................................... 63
Treatment effect on growth parameters .......................................................... 63
Growth parameters based on tetracycline resistance genotype ....................... 64
Discussion ....................................................................................................... 65

Chapter 4 - Final Conclusions and Implications .............................................. 92
Conclusions .................................................................................................... 92
References ...................................................................................................... 95
List of Figures

Figure 2-1  Bacterial isolation pipeline demonstrating how multiple bacterial isolates were recovered from a single caecal sample ................................................................. 34
Figure 2-2  Custom NARMS plate CMV2AGNF ................................................................. 35
Figure 2-3  Model predictions for freezing effect.................................................................. 36
Figure 2-4  Estimated True Prevalence of ceftiofur resistance............................................ 38
Figure 2-5  Multiresistance Distribution of isolates recovered from plain MAC .................. 39
The distribution of the multiresistance number calculated as the sum of drugs with an MIC above breakpoint values for all isolates recovered from plain MAC Figure 2-6 Multiresistance distribution of isolates recovered from plain MAC ......................................................... 39
The distribution of multiresistance calculated as the sum of drugs with an MIC above breakpoint values for all isolates recovered from plain MAC not including pan-susceptible isolates
Figure 2-7  Multiresistance Distribution of isolates recovered from MAC+tetracycline ......... 40
Figure 2-8  Proportional differences of multiresistance categories between agar types ........... 42
The proportional difference for each multiresistance category between plain MAC and MAC+tet distributions. For example, singly resistant isolates were roughly 50% of the resistant population recovered on plain MAC but were only 30% of the resistant population recovered on MAC+tet. Figure 2-9  Tetracycline genotype proportions by decade and agar 42
Proportions of tetracycline genotype are stratified by agar and decade. Figure 2-10 Proportional Venn diagrams of joint tetracycline genotype distributions .............................................. 43
Figure 2-11  Multiresistance distribution of tet(A) positive isolates (n=130) to the 14 drugs on the NARMS CMV2AGNF MIC plate ........................................................................... 45
Figure 2-12  Multiresistance distribution of tet(B) positive isolates (n=320) to the 14 drugs on the NARMS CMV2AGNF MIC plate ........................................................................... 46
Figure 2-13  Probability of tetra-resistance (multiresistance ≥4) ........................................... 47
Figure 2-14  Bivariate marginal predictions of tetA and tetB based on multiresistance and agar 48
Figure 3-1  Schematic diagram of the study design adapted from Kanwar et. al. 2013 .......... 70
Figure 3-2  Antibiotic treatment dosing timeline adapted from Kanwar et. al. 2013 ............ 71
Figure 3-3 Custom NARMS plate CMV1AGNF ................................................................. 72
Figure 3-4 Simplified Monoauxic Growth Curve .............................................................. 73
Figure 3-5 Simplified Diauxic Growth Curve ................................................................. 74
Figure 3-6 Growth curves of ATCC strains EC25922 and EC35218 .................................. 75
Figure 3-7 Sample growth curve with estimated growth parameters ............................... 76
Figure 3-8 Prevalence of tetracycline-resistant *E. coli* isolates, modeled as marginal predicted
probabilities, over days adapted from Kanwar et. al. 2013 ............................................. 77
Figure 3-9 Histogram of the global difference in OD stratified by broth ............................ 78
Figure 3-10 Histogram of the maximum slope stratified by broth .................................... 79
Figure 3-11 Marginal predictions of ΔOD based on animal treatment .............................. 80
Figure 3-12 Marginal predictions of maximum slope based on animal treatment ............ 82
Figure 3-13 Marginal predictions of maximum slope of resistant isolates grown in MAC+tet
based on animal treatment (enlarged) ............................................................................. 83
Figure 3-14 Marginal predictions of ΔOD based on tetracycline genotype ....................... 84
Figure 3-15 Marginal predictions of maximum slope based on tetracycline genotype ....... 85
Figure 3-16 Marginal predictions of ΔOD based on tetracycline genotype (only phenotypically
resistant isolates) ........................................................................................................... 86
Figure 3-17 Marginal predictions of maximum slope based on tetracycline genotype (only
phenotypically resistant isolates) .................................................................................... 87
Figure 3-18 Unpublished Growth curves of isolates recovered from Platt et. al. 2008 when grown
in plain MAC broth (provided by Dr. Javier Vinasco, Kansas State University) ............ 88
Figure 3-19 Unpublished Growth curves of isolates recovered from Platt et. al. 2008 when grown
in MAC broth containing tetracycline (16µg/ml) (provided by Dr. Javier Vinasco, Kansas
State University) ........................................................................................................... 89
List of Tables

Table 2-1 Tabulated annual caecal samples positive for growth..................................................... 49
Table 2-2 Tabulated annual prevalence of positive growth adjusted for freezing effects ............ 50
Table 2-3 MIC distribution of isolates recovered from plain MAC ................................................. 51
Table 2-4 MIC distribution of isolates recovered from MAC+tetracycline (16µg/ml) ............. 52
Table 3-1 PCR primers used for PCR reactions .............................................................................. 90
Table 3-2 Distributions of minimum inhibitory concentrations of 1,050 E. coli isolates to

    NARMS antibiotic panel CMV1AGNF......................................................................................... 91
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Dedication

To my mother and father who shared with me their thirst for knowledge and appreciation of science.

To my wife Jessy for sharing in all the kite-flying, kayak-paddling, mountain-conquering adventures that make life totally worth it!
Chapter 1 - Overview of tetracycline and ceftiofur resistance

Objective

This thesis is a combination of two studies designed to investigate epidemiological properties of both tetracycline and ceftiofur resistant commensal *E. coli* present in the intestines of cattle.

Purpose

The widespread usage of antimicrobials in animal agriculture for the treatment, control, and prevention of disease as well as growth promotion is generally considered to provide evolutionary selective pressure that contributes to the emergence, spread, and persistence of antimicrobial resistant bacteria. This research focuses on antimicrobial resistance (AMR) in beef cattle as one significant production system that regularly employs antibiotics and is being negatively impacted by antibiotic resistance. Among the many different antimicrobials used in treating cattle, ceftiofur and tetracycline are focused on here not only because of rising concern over the spread of extended-spectrum beta lactamases, but also because both drugs provide two distinct and potentially generalizable situations regarding the dynamics of antibiotic resistance.

On one hand, tetracycline is a very old antibiotic with an extended history and variety of uses in cattle. In addition to treatment use, the drug is also used for growth-promotion, prevention and control, and thus is administered at a wide range of dosages and regimens. The most common mechanism of resistance is by drug efflux, but there are other mechanisms such as ribosomal protection and, rarely, enzymatic inactivation. Tetracycline resistance is not only well established and commonly present in cattle commensal bacteria, many different tetracycline resistance genes often co-reside within local bacterial populations. Additionally, tetracycline is a
common denominator in nearly all multidrug resistance phenotypes that have emerged in the past decade. Even though tetracycline resistance itself emerged and spread in cattle decades ago, there is evidence to suggest that not all tetracycline resistance genes are created equal in their ability to persist in bacterial communities, spread to new bacterial species, and associate with other drug resistances. We looked at tetracycline resistance not as a primary emerging threat, but instead as serving a potential supporting role on the global stage of resistance. It was hypothesized that differences between tetracycline resistance determinants are competitively changing their distributions in bacterial populations and unevenly contributing to the emergence of multidrug resistance in beef cattle.

On the other hand, ceftiofur represents a more recently introduced antibiotic. There are relatively few recorded mechanisms of resistance to ceftiofur in North American agriculture and nearly all rely on enzymatic inactivation of the drug itself. The drug is parenterally administered at treatment dosages and while resistance has been detected in cattle, the prevalence is generally low in cattle not undergoing treatment. Considering a growing concern over a generally increasing prevalence of extended-spectrum beta-lactamases among Gram-negative enteric bacteria, ceftiofur resistance is looked at in this study as a resistance that has already emerged, is not yet widely established, but may currently be in the process of spreading in cattle populations. This study hypothesizes that ceftiofur resistance is in the initial stages of spreading, but may not yet be prevalent above the detection threshold of already established resistance surveillance programs.

**Tetracycline Resistance**

Since its discovery in 1945, tetracycline and its derivatives have played key roles in the antimicrobial arena because of its broad-spectrum activity, low toxicity, and low production
While remaining a widely used antibiotic class across the globe, their long term usefulness as a therapeutic agent in treating disease is becoming limited because of the increasing prevalence, spread, and persistence of a variety of tetracycline resistance determinants in microbial communities.

The presence of widespread tetracycline resistance raises particular concern in animal agricultural production systems. In addition to its therapeutic use for control and prevention of disease (generally doses greater than 70 mg/hd per day in beef cattle for chlortetracycline), tetracycline is also administered at sub-therapeutic levels in livestock as feed additives for improvement in feed efficiency and rate of gain (generally anything ≤70 mg/hd per day in growing cattle over 400 lbs) (3). The benefit of higher weight gain and from this use creates a strong incentive for farmers to use tetracycline as a feed additive. Consequently, they are regularly used except in countries or production systems that explicitly ban their use. The justification for using tetracycline or any antimicrobial as a growth promoter is increasingly contentious because there is growing unease that the constant use of antimicrobials at low dosages may significantly impact the emergence, selection, and spread of antibiotic resistance (4).

The antimicrobial mechanism of tetracycline relies on entering bacterial cells through passive diffusion as well as energy-dependent active transport (6). The drug accumulates in the cell and reversibly binds to the bacterial 30S ribosomal subunit. This binding causes a conformational change in the ribosome and prevents the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Protein synthesis is shut down after tetracycline accumulates in sufficient quantity to saturate the bacterial ribosomes. Tetracycline is often categorized as a bacteriostatic agent because it does not directly kill the cell.
The growing body of research regarding tetracycline resistance reveals that there is relatively high diversity among determinants compared to other antimicrobial classes (7). As of 2009, there were 35 different identified genes that confer resistance to tetracyclines (8). The mechanistic actions of these genes divide into three categories. The largest group confers resistance through energy-dependent efflux proteins that export tetracycline out of the cell before the drug can accumulate to concentrations required to inhibit synthesis. The second most common group codes for proteins that interact with the ribosome to interrupt the drug from halting translation. The current model for this process is that the protection proteins interact with a ribosome bound with tetracycline and cause a conformational change in the ribosome which results in tetracycline being released from the ribosome, allowing protein synthesis to continue (9). The third group confers resistance by enzymatic inactivation of the antibiotic. However, this third type of resistance has only been found in the obligate anaerobe Bacteroides, which ironically requires oxygen to work, and also is not clinically relevant (10).

Tetracycline resistance genes have spread to a widely diverse number of bacterial genera. Efflux and ribosomal protection genes have been found in both Gram-positive and Gram-negative microbes present in the environment and animals (11, 12). However, efflux genes have been reported more in Gram-negative genera and ribosomal protection genes more in Gram-positive genera (13). The extensive reach of these genes is largely attributed to their location on conjugative plasmids in the case of efflux genes and on conjugative transposons in the case of ribosomal protection genes (8). Incompatibility between the conjugation vectors and certain genera may explain the uneven distribution of resistance genes. One example is the absence of ribosomal protection genes tet(M) and tet(O) among Escherichia coli. Indeed, the conjugative transposons carrying tet(M) and tet(O) likely originate from Bacteroides and have been shown
to be unable to mobilize into *E. coli* (14). This incompatibility is not only restricted to ribosomal protection genes. Indeed, some efflux genes have been found to be associated with specific plasmid types (15). However, there has been speculation that the current understanding of the resistance distribution is distorted by unequal screening for genes in particular genera (16). A meta-analysis addressing this has yet to be published.

**Ceftiofur resistance**

Ceftiofur is classified as a third-generation cephalosporin. First commercially released in 1987, it has become a core antimicrobial for treating respiratory disease in ruminants, swine, and horses (17). Ceftiofur’s irreversible bactericidal mechanism of action is provided by a β-lactam ring in its molecular structure (18). This ring forms an analogue of the terminal amino acid residues on precursor subunits of the peptidoglycan layer. Because of this, the drug binds to penicillin-binding proteins (PBPs) which normally facilitate the final transpeptidation step in synthesizing the peptidoglycan layer of bacterial cell walls. The irreversible binding of beta-lactams to the PBPs prevents the final crosslinking of peptidoglycan and disrupts cell wall synthesis. With the cell wall compromised, the bacterial cell then fails to divide and dies. Drugs containing a β-lactam are classified based on other molecules attached to the ring in their molecular structure. Cephams, the group of β-lactams including cephalosporins, have the β-lactam ring fused to an unsaturated six-membered thazine ring.

Resistance to cephalosporins primarily occurs by the production of enzymes that hydrolyze the β-lactam ring and deactivate the drug before it can kill the bacterial cell. While nearly all confer resistance to penicillins, some provide activity against other β-lactams as well (19). The term extended-spectrum β-lactamases (ESBL) usually applies to enzymes also capable of hydrolyzing third-generation cephalosporins (e.g., ceftazidime, ceftriaxone, and
ceftiofur) but are not effective against cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g. meropenem or imipenem)(20). Carbapenemases and cephamycinases are β-lactamases with activity against carbapenems and cephamycins, respectively, in addition to extended-spectrum β-lactams. The most common classification system for these enzymes is the Ambler classification. This system divides β-lactamases into four classes A through D(21) based on their amino acid sequences.

Class A β-lactamases are the most commonly encountered. Enzymes in this class have a conserved serine residue at their active-site(22). Among class A enzymes, TEM-1 is the most commonly encountered and accounts for most ampicillin resistance in E. coli. Another class A enzyme, SHV-1, is often found in K. pneumonia and is highly homologous to TEM-1. Both of these confer resistance to penicillins such as ampicillin, but not to expanded-spectrum cepahlosporins, carbapenems, or cephamycins. The more distantly related CTX-M is noted for its greater activity against oxyimino-beta-lactam antibiotics including ceftiofur. The class A KPC enzyme is the most common form of carbapenem resistance in Klebsiella sp.

Class B enzymes are called metallo-β-lactamases because the bivalent metal ion, Zn2+, is required for activity(23). In addition to providing resistance to penicillins and cephalosporins, they also confer resistance to carbapenemases and are a rising threat to public health. The two most common families of class B β-lactamases are IMP and VIM. These were identified in Pseudomonas and Acinetobacter are not commonly found in Enterobacteriacea. However, the class B enzyme NDM-1 is becoming a growing threat because of its wider presence in other bacteria such as Enterobacteriacea(24).

Class C and D enzymes are like class A enzymes in that they also have a serine residue at their active-site. However, they have their own distinct amino acid sequences and do not share
homology with each other. Class C enzymes are also called AmpC β-lactamases because the
gene coding for these enzyme was originally identified from a stepwise mutation experiment
investigating the genetics of ampicillin resistant E. coli(25). In addition to ampicillin and other
penicillins, some AmpC enzymes confer resistance to cephalosporins and cephamycins but not
carbapenems. This is especially the case among plasmid-mediated AmpC β-lactamases. The
CMY-2 AmpC is the most widespread plasmid-mediated Class C enzyme and is an important
cause of resistance in E. coli and Salmonella strains(26). The most significant class D group
enzymes are the OXA β-lactamases which exhibit carbapenemase activity(27). In addition, they
are highly hydrolytic against oxacillin and cloxacillin and are poorly inhibited by clavulanic acid.
These are usually found in Acinetobacter, but have also been found in Klebsiella and rarely in
other Enterobacteriaceae(28-30).

Antibiotic Resistance in Beef Cattle

The use of antimicrobials in animal agriculture and their role in promoting AMR have
become a significant concern since the 1960s. Numerous organizations have published reports
that identify antimicrobial use, or sometimes phrased “abuse”, in animal agriculture as a
potential risk to human health. In particular, the use of antimicrobials as growth promoters is
often the most commonly identified practice of “abuse” that greatly contributes to the burden of
AMR. For example, in the WHO Global Principles for the Containment of Antimicrobial
Resistance in Animals Intended for Food, several principles were laid out as a framework of
recommendations to prevent the misuse of antimicrobials. Among these principles was the
specific recommendation that the usage of antimicrobial growth promoters that belong to
antimicrobial classes also used for treatment should be stopped in the absence of risk-based
evaluations.
Beef cattle production is one sector of animal agriculture that regularly uses antimicrobials for both treatment and growth promotion. The extent of this use is generally considered to be widespread in beef cattle. However, aside from professional opinion, there are very few quantitative estimates of the quantity and patterns of use (1). Ironically, despite the specific concern over the impact of growth promoters on AMR and the regular use of growth promoters in beef cattle, relatively little research has been conducted regarding AMR in beef cattle when compared to swine, poultry, and dairy cattle production systems (31). The current understanding of AMR in beef cattle is primarily based on testing from national surveillance programs. The US National Antimicrobial Surveillance System (NARMS) regularly tests commercial pork, ground beef, and poultry samples for foodborne pathogens as well as AMR patterns of found pathogens. Annual reports have been released since testing began in 2002 (32). The two most significant bacteria found contaminating beef were *Escherichia coli* and *Enterococcus*. *Campylobacter* was very rarely recovered from retail meat samples (<1% for all years) and *Salmonella* was also rarely recovered (<2%). This contrasted strongly with poultry where *Campylobacter* and *Salmonella* were regularly recovered from meat samples (>30% and >9% respectively from retail chicken samples). The lower prevalence of foodborne pathogens in beef samples may partially explain why swine and poultry are often focused on instead of cattle when investigating AMR.

Among commensal *Enterococcus* isolates recovered, AMR levels were found to be much lower in beef cattle compared to pork and poultry. Tetracycline resistance was common in both *Enterococcus* and *E. coli* (roughly 20-30% of isolates) and aminoglycoside resistance was also found in both organisms but only rarely (<10%). Among commensal *E. coli*, uncommon resistances (<10%) found were ampicillin, cepham, and phenicol resistances. While *E. coli*
isolates recovered from poultry displayed an increasing trend for resistance to cephalosporins from 2002 to 2011 (7.1% to 12.3% for ceftiofur), this trend was not observed in ground beef samples. However, low level cephalosporin resistance was detected in ground beef from 2003 onwards (0.3-0.9%). Multidrug resistance in *E. coli* was relatively low in ground beef with roughly 70-80% of isolates recovered being pan-susceptible, 10% of isolates being resistant to ≥3 antimicrobial classes, and 2-6% of isolates being resistant to ≥4 antimicrobial classes.

Aside from NARMS, there are few large scale reports on antimicrobial resistance, particularly in beef cattle. However, there are smaller studies that have incorporated beef cattle sampling in cross species investigations. Sayah et al. 2005 (33) looked at antimicrobial resistance in *E. coli* isolated from multiple sources such as domestic and wild animals as well as human septage and surface water. A total of 89 beef cattle from 7 farms were included in their sampling. Unfortunately, despite finding that beef and dairy cattle had different antimicrobial exposures through treatment, they ended up grouping all isolates as “cattle” in their analysis. Because dairy cattle tended to be much more likely to be treated with ceftiofur (20%) compared to beef cattle (1%), the reported percentage and odds ratio for finding antibiotic resistances in *E. coli*, especially cephalosporin resistance (21.87%, OR=1.13), may be significantly biased toward dairy cattle. However, their analysis on resistance presence in different sub-environments within beef cattle farms does give an indication on the general presence of resistance on beef farms. They found that common resistances such as tetracycline, ampicillin, and streptomycin were found in isolates recovered from cattle fecal samples as well as the farm environment and septage. However there were some exceptions to this in that neomycin, gentamicin, and sulfamethoxazole-trimethoprim resistance was only found in either the fecal, farm environment,
or septage. This finding differed from what they observed on dairy and poultry farms. However, they did not indicate which of these three categories the resistance was found.

Alexander et al. 2008 (34) investigated the effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic resistance in *E. coli* shed from feedlot cattle. They tested the five most common substances that are administered subtherapeutically in the industry for their treatment groups. These treatments were chlortetracycline, chlortetracycline plus sulfamethazine, virginiamycin, monensin, and tylosin. Surprisingly, they found that subtherapeutic treatment with tetracycline alone did not increase the prevalence of tetracycline resistance in *E. coli*. Rather, the prevalence of tetracycline resistance actually decreased over the first 40 days in unison with the control group. However, when treated with sulfamethazine in addition to tetracycline, the prevalence of tetracycline and ampicillin resistance was drastically higher than the control group. Other treatments did not impact either tetracycline or ampicillin resistance prevalence. Over the course of sampling, the cattle were switched from a silage based diet over to a grain based diet. Unexpectedly, switching to a grain based diet increased the prevalence of both tetracycline and ampicillin resistance as much as the tetracycline-sulfamethazine treatment. Ampicillin resistant isolates were further investigated by pulsed-field gel electrophoresis. They found that when the prevalence of ampicillin resistance increased in a pen, resistant isolates were clonal and tended to be shed in surges by most of the animals in the pen. However, isolates were not clonal from one surge to another. This may indicate some form of cross-colonization among animals through common contact or environment. However, without any environmental sampling this suggestion is conjecture.

There are also some studies that investigated the effects of therapeutic treatment with antibiotics on the gut flora of beef cattle. Lowrance et al. 2007 (35) treated cattle with several
different treatment regimens of ceftiofur and observed that treatment resulted in a drastic increase in ceftiofur resistance from less than 10% to over 60% of the E. coli population shortly after administration. It is important to note that the total E. coli population also decreased following treatment meaning that the observed increase in resistant fraction of bacteria was part expansion of that fraction, and part contraction of the susceptible fraction. Ceftiofur resistant isolates were also resistant to other antibiotics. These were tetracycline, ampicillin, sulfisoxazole, chloramphenicol, streptomycin, cefoxitin, and amoxicillin-clavulanic acid. The increase in ceftiofur resistance was transient and within two weeks ceftiofur resistance dropped back to pre-treatment levels. Singer et al. 2008 (36) reported similar findings that the drastic spike in ceftiofur resistance following treatment resulted in an unstable population that returned to pre-treatment levels in a short time span. On the other hand, Platt et al. 2008 (37) explored whether treating cattle with chlortetracycline at therapeutic dosages impacted the antimicrobial susceptibility of E. coli. Tetracycline exhibited a similar pattern to ceftiofur in that treatment with tetracycline resulted in a significant increase in tetracycline resistance. Unlike ceftiofur, treatment with tetracycline did not appear to select for highly multidrug resistant strains. While there was some co-selection for resistance to sulfisoxazole, streptomycin, and ampicillin, treatment paradoxically resulted in a significantly lower proportion of isolates resistant to ceftiofur. This paradox has yet to be fully explained, but one speculation is that strains possessing fewer drug resistances, in addition to tetracycline, may have a competitive advantage considering that there are many different resistance mechanisms to tetracycline while ceftiofur has relatively few resistance mechanisms.

While there are relatively few reports of AMR in beef cattle, there are even fewer studies that have attempted to go beyond exploring the phenotypic state of antimicrobial resistance in
beef cattle and actually look at the genes that are harbored in resistant bacteria. This is at least partly due to a lack of standardization in the identification and nomenclature of distinct resistance mechanisms. Historically, tetracycline resistance mechanisms were only defined by their relative genetic relationships based on their ability to hybridize with each other. However, the advent of accessible gene sequencing, PCR testing, and openly accessible repositories of gene data has been closing many of the communication gaps and has resulted in increasingly consistent nomenclature for resistance genes (38). Alexander et al. 2009 (39) followed up their early research into sub-therapeutic effects of antimicrobials on resistance by characterizing many of the recovered isolates. This was done by testing isolates for a large number of resistance genes. Of the determinants screened, *tet(A)*, *tet(B)*, *tet(C)*, *blaTEM1*, *sul1*, and *sul2* were the most frequently found.

**Bridging the Research Gap**

No doubt, the dynamics of antibiotic resistance in commensal organisms requires further study across the entire spectrum of food animals as well as environments surrounding them. However, the common multipurpose use of antimicrobials in beef cattle coupled with the fact that there is relatively little research regarding resistance in beef cattle highlights the specific need for more studies further investigating the impact of antimicrobial use on commensal flora in cattle as well as further characterizing the patterns of resistance present in beef cattle systems. While most of our current understanding of AMR in cattle is based on phenotypic data and has a crude view of what general patterns of resistance are currently present, more in depth studies are needed that investigate resistance at the molecular level for any hope of explaining why and how these patterns have emerged in cattle and may change in the future. The limited body of research regarding AMR in cattle has found some consistent patterns, but requires more in depth
investigation into the origins and genetic nature of these patterns to better explain natural AMR phenomena. First, NARMS found several types of resistance were commonly present in cattle populations in the past decade but have not been increasing in prevalence. Tetracycline resistance is the primary example of this, but sulfisoxazole and streptomycin resistances could also be considered to be following this pattern. NARMS also reported on some forms of resistance that have been increasing in prevalence in other animal production systems, but have yet to increase in cattle despite resistance being at least rarely present in cattle. Ampicillin and ceftiofur resistance are two examples of this pattern. Secondly, short-term treatment with antimicrobials appears to transiently select for resistant isolates among commensal flora but that populations tend to return to a pre-treatment “baseline” state over time. This suggests that the relative fitness of resistant bacteria compared to susceptible bacteria may play some role in whether a particular form of resistance persists. Despite the fact that there are some bacteria that possess resistance in cattle, there may be environmental selection pressures capable of preventing them from dominating the commensal flora in cattle unless an animal is actively being treated with antimicrobials. The two experimental studies in this thesis approach this notion that environmental selection pressures may create some form of ”baseline” that unstable commensal populations return to between antibiotic treatment events, at least in the relatively short term (days and weeks). Over the longer term (months to years), upward trends suggest that the return to baseline provides as false sense of security.
Chapter 2 - Surveillance of tetracycline and ceftiofur resistance in Canadian beef cattle 2002-2011

This intent of this study was to evaluate using selective media to determine whether the sample-level and isolate-level prevalence of antimicrobial resistance (AMR) to ceftiofur and tetracycline increased from 2002 to 2013 among commensal *E. coli* residing in cattle entering the Canadian food supply.

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) was established in 2002 as an integrated national surveillance program with the purpose of documenting the extent and variation of antibiotic resistance occurring geographically and temporally in both human and animal populations. Sampling and AMR testing methods were designed to generate AMR results that were both representative of the systems investigated as well as comparable to data gathered from other national surveillance counterparts such as the United States’ National Antimicrobial Resistance Monitoring System (NARMS). More importantly, the program was designed with expectation that the changing landscape of scientific knowledge regarding AMR would benefit from being able to revisit archived samples as well as isolates. This is unique in that most isolate-based surveillance systems (such as NARMS) retain only the bacterial isolate and not the sample from which it arose. With this mindset, CIPARS produces a continual report (40) on AMR in Canadian systems as well as an ever growing sample bank that broadly represents multiple systems where AMR is a growing concern.

Several recent studies have demonstrated that the prevalence of bacteria harboring genes coding for cephalosporin resistance has generally increased in several systems over the last decade (28, 41-43). The presence of these genes is cause for concern because they are capable of
enhancing resistance to third-generation cephalosporins. These drugs have historically been useful in treating hospital-acquired infections, but are fast becoming ineffective due to increasing levels of resistance. ESBL genes as well as class C β-lactamase genes have been isolated from numerous sources ranging from hospital infections to community water supplies (44, 45). Identifying environmental reservoirs is important in developing any future intervention strategies to reduce further dissemination. Cattle have been suggested as a reservoir for resistance to cephapycin and cephalosporin (41). Multiple β-lactamase genes have been found from bovine sources and *E. coli* is a common species identified as harboring containing AmpC determinants, namely CMY-2 (46-48). Often, the use of selective media containing a cephalosporin is required to isolate ESBLs from cattle, which suggests that bacteria harboring these resistances may comprise only a very small proportion of the total commensal bacteria population in healthy animals. Because the general screening methodology employed by the CIPARS program does not use selective media, the prevalence of ESBL-producing bacteria in Canadian cattle may have been under-reported because resistance was not present in sufficient quantities above the detection threshold. This study re-tested the CIPARS collection of cattle samples from 2002-2012 using a selective media containing ceftiofur. The purpose of this was to test whether there were samples with populations of ESBL producing *E. coli* that were not detected using the original nonselective approach and to compare the sample versus isolate prevalence of ceftiofur resistance.

In addition to ceftiofur resistance, tetracycline resistance was also further examined in detail beyond the capabilities of the original CIPARS surveillance methods. Samples were re-tested using selective media containing tetracycline to see whether tetracycline resistance also occurred at levels below original detection methods. Although tetracycline resistance is already
known to be well established in cattle, there is little reporting on the relative abundance of particular tetracycline genes in commensal flora. Therefore, *E. coli* isolates recovered from caecal samples were also tested for four tetracycline genes likely to be present in commensal *E. coli*. Furthermore, the relationship of any tested genes to particular resistance phenotypes was also examined. As for ceftiofur, the sample versus isolate prevalence of resistance was also compared.

**Materials and Methods**

**Sample Collection**

A two-stage sample design was used to obtain a representation of Canadian slaughter cattle, which comprised both fed beef and dairy beef in unknown proportions. In the first stage, abattoirs were randomly selected from a list of federally inspected slaughter plants with the probability of being selected proportional to its annual production volume. Abattoirs on this list account for over 90% of beef production in Canada. CIPARS began sampling in 2002 and has continued up until 2013. In this study, only samples from 2002 to 2011 were used.

The second stage was a systematic selection of animals on the slaughter line in each plant. The number of animals sampled in each abattoir was proportional to its production volume. For each abattoir, an annual target number of samples to be collected was divided by five with the result determining the number of annual sampling periods. Within 5 days in each collection period, five animals were sampled by taking a small portion of the caecal contents from discarded offal. These animals were from different lots to minimize lot clustering bias. Collection periods were uniformly distributed over each year. The sampling process for each plant was based on the same protocol, but differences in production line setup required slight modification for each plant. Samples were collected by industry employees under the oversight
of the Canadian Food Inspection Agency (CFIA) Veterinarian-in-Charge. Following collection, each sample was placed in Brucella Broth containing 15% glycerol and frozen at -80°C.

**Original Surveillance Testing**

The original surveillance testing protocols used similar methodology as those in this study in regards to bacterial isolation and MIC determination. However no selective media was used in the isolation step and there was no further investigation into tetracycline gene presence. In brief, caecal samples were plated on MacConkey agar and a single *E. coli* colony was isolated. MIC values were determined for each isolate using the TREK Sensititre® NARMS commercial panel of 15 different antibiotics (Figure 2-2).

**Bacterial Re-isolation**

Starting in 2012, caecal samples originally positive for *E. coli* growth were re-plated, producing a new set of bacterial isolates for downstream analysis. Two selective agar plates containing tetracycline or ceftiofur were used in addition to a nonselective MacConkey plate. This was done to potentially select for resistant bacteria populations at too low of a concentration to be isolated using the original nonselective methods, and also to better estimate the sample-level prevalence of resistance. Caecal samples were removed from storage and thawed. 1ml was inoculated into three MacConkey broth (Difco) tubes containing either no supplement, tetracycline (16ug/ml), or ceftiofur (8ug/ml). Then 10ul of each enriched sample was plated onto a MacConkey agar plate containing the same supplement. Not all caecal samples resulted in growth on all three plates. When growth occurred, one colony was selected from each of the three selective plates and spread on a Trypticase Soy Agar plate (Difco) as a purification step. Indole and citrate tests were performed to ensure that purified isolates were actually *E. coli*. 
Confirmed *E. coli* isolates were then stored in 1ml BB+15% glycerol for further analysis. A general outline of this procedure is presented in Figure 2-1.

All *E. coli* isolation and antimicrobial susceptibility testing was conducted at the Laboratory for Foodborne Zoonoses, St. Hyacinthe, Quebec. Because downstream genotyping of tetracycline resistance genes was done at another institution, the caecal samples were not processed in the serial order that they were originally obtained. Instead, samples were processed from the fringe sampling years stepwise towards the median year.

**Antimicrobial Susceptibility Testing**

Antimicrobial susceptibility testing was performed using the Sensititre (Trek Diagnostic Systems, Cleveland, Ohio, USA) automated broth microdilution system, as per CIPARS 2008 (40). The custom NARMS panel CMV2AGNF (Figure 2-2) was used with *E. coli* ATCC 25922 and ATCC 35218 as quality control organisms.

Bacterial isolates were tested to obtain their minimum inhibitory concentration for the 15 antibiotics included in the panel using breakpoints determined by the Clinical and Laboratory Standard Institute. For antibiotics without a CLSI breakpoint, NARMS consensus breakpoints were used. To ease further analysis, antibiotics that include an intermediate classification between resistant and susceptible were collapsed into binary classification with intermediate isolates being recorded as susceptible.

**Tetracycline Resistance Gene Determination**

Samples collected from years 2002, 2003, 2010, and 2011 were further investigated to determine tetracycline resistance genotype. These years were chosen because they are at the fringes of the sampling timeframe available. Tetracycline resistance conferring genes were detected in isolates using a polymerase chain reaction based (PCR) approach. Total DNA was
extracted from each isolate via boiling lysis. DNA was then screened for tetracycline resistance genes \textit{tet(A)}, \textit{tet(B)}, \textit{tet(C)}, and \textit{tet(E)} as described by Ng et al. (49) using referent strains provided by Dr. Marilyn Roberts (University of Washington). PCR assays were optimized using QIAGEN HotStarTaq® master mix. The Qiagility® benchtop robot was used for PCR setup, and the Qiaxcel® microcapillary electrophoresis system was used to identify the weights of the amplified PCR products. All genotyping was conducted at the Molecular Epidemiology/Microbial Ecology Laboratory (ME$^2$), Kansas State University, Manhattan, KS.

\textbf{Analysis}

With the samples having been frozen and stored since the original CIPARS surveillance study, it was important to determine whether the recoverability of \textit{E. coli} using historical samples was affected by the freezing time. This was done using a simple logistic regression with the dependent variable being the retested detection of \textit{E. coli} on the plain MacConkey plate with the number of years frozen as the independent variable. Because only originally positive \textit{E. coli} samples were retested, all of them should have tested positive had they been unaffected by freezing. Assuming no other differences between the two tests, there were no other measured variables that could have reasonably have affected the second test outcome other than the length of time they were frozen.

Logistic predictions of the freezing effect were used to estimate the reduced detection sensitivity for each year frozen using the original test result as a gold standard. While specificity could not be estimated since only originally positive samples were followed up in this study, it seems implausible that freezing would cause samples to produce \textit{E. coli} colonies when they originally did not. Therefore, the specificity was fixed at one. These testing parameters were then used to estimate the annual true prevalence of tetracycline and ceftiofur resistance at the sub-
population level during the sampling period. The annual number of caecal samples that produced colonies on plain MacConkey was compared to the number producing colonies on selective agar containing the antibiotics. True prevalence was estimated from these counts by adjusting for the freezing effect using the publicly available Epitools software package (50).

The crude isolate counts of all possible log2 MICs for each antibiotic included in the Sensititre panel were used to generate a distribution table across the entire sampling period. These counts were stratified by the agar type generating a distribution for all NTS E. coli at the within caecum population level (N=1,268), and a more specific distribution for phenotypically tetracycline resistant NTS E. coli recovered from MacConkey containing tetracycline at the within caecum population level (N=916). A distribution table was not calculated for isolates recovered from MacConkey agar containing ceftiofur because there were so few isolates recovered (n=26).

For the analysis of tetracycline gene detection results, the year variable was collapsed into a binary decadal variable because only isolates from 2002, 2003, 2010, and 2011 were used. Frequency distributions for all three genes were tabulated based on decade and agar type. These distributions were used to determine whether recovered E. coli populations changed over the first millennial decade as well as any differences seen between populations recovered from nonselective and selective methods. The relationship between harbored tet genes and resistance to antibiotics other than tetracycline was also evaluated. A multidrug resistance variable was calculated for each isolate by taking the total number of antibiotics on the panel that had an MIC above the established breakpoints and were classified as “resistant”. Antibiotics that utilize a three tier classification system were collapsed into a binary variable with “intermediate” isolates being coded as “susceptible”. Based on histograms of multiresistance number for either tet(A) or
tet(B), a cutpoint was established splitting isolates with any phenotypic resistance into drug categories of ‘1-3 drug resistances’, and ‘>4 resistances’. This was then used to logistically model whether agar type (i.e., selective versus non-selective) and the harboring of either tet(A) or tet(B) impact the likelihood of it having >3 drug resistances. Because there were no tet(C) isolates with >3 drug resistances that did not also harbor tet(A) or tet(B), tet(C) was not included in the model. This full-factorial model included tet(A), tet(B), and broth type as covariates to predict multiresistance as an outcome. A second bivariate logistic model was run using both tet(A) and tet(B) as dual outcomes which included multiresistance coded as an ordinal variable and broth for covariates. All statistical analyses were carried out using STATA® SE Release version 12.1.

**Results**

**Freezing Effect**

Because archived samples had been frozen for multiple years, the freezing time was tested as a potential confounder in that if older samples had overall reduced bacterial recovery, prevalence estimates would also appear lower. The freezing effect was logistically modeled using *E. coli* recovery as the dependent variable with time frozen as an integer coded independent variable. Indeed, freezer storage time did have a significant impact on the recovery of *E. coli* from the caecal samples (P<0.05). Samples frozen for shorter periods of time resulted in higher recovery than older samples stored for extended lengths. Each year of storage time resulted roughly in a 6% reduction in recoverability (Figure 2-3).

**Prevalence of tetracycline and ceftiofur resistance**

After cross-tabulating each apparent prevalence and adjusting for the associated reduction in test sensitivity, the true prevalence of caecal samples exhibiting any growth on media
containing tetracycline ranged from 74% in 2002 to 90% in 2011 (Figure 2-4). This adjustment inherently assumed that freezing was equally detrimental to all *E. coli* both susceptibles and resists alike. Likewise the estimated true prevalence of any growth on MAC containing ceftiofur was 2.5% in 2002 and 3.8% in 2011 (Figure 2-5). Because there were so few isolates recovered from agar supplemented with ceftiofur (n=26), this agar category was not included in downstream analysis when stratifying by agar type.

**MIC distribution**

Of 1,268 isolates recovered from nonselective MacConkey agar, 481 (37%) were phenotypically resistant to at least one antibiotic in the Sensititre panel (Table 2-3). The most common drug resistances were tetracycline (n=361 [28%]), sulfisoxazole (n=164 [13%]), streptomycin (n=164 [12%]), ampicillin (n=37 [3%]), and chloramphenicol (n=33 [3%]). Ciprofloxacin was the only drug with no isolate MIC recorded above the CLSI resistance breakpoint.

The MIC distribution pattern of isolates recovered from MacConkey containing tetracycline was largely in agreement with the plain MacConkey distribution even though the relative proportions were different (Table 2-4). In total, 70% (n=641) of the 916 isolates were phenotypically resistant to at least one other drug in addition to tetracycline. As with isolates recovered from nonselective agar; sulfisoxazole (n=477 [52%]), streptomycin (n=449 [49%]), ampicillin (n=97 [11%]), and chloramphenicol (n=79 [9%]) were the most common resistances. In this case, every antibiotic had at least 3 isolates with an MIC above its respective CLSI or NARMS breakpoint; however, ciprofloxacin resistance was again the rarest phenotype recorded.
**Multidrug Resistance**

The majority of isolates recovered from plain MacConkey were pan-susceptible (62%) (Figure 2-5). Unsurprisingly, isolates with a single resistance phenotype comprised the next largest category (20%). This single resistance phenotype was almost always tetracycline resistance. Notably, there was little difference in the proportion of isolates with 2 or 3 resistances (7.2% and 7.9% respectively). After removing the pan-susceptible isolates, the proportions of single, double, and triple resistances among isolates recovered from plain MacConkey were 47%, 22%, and 22% respectively (Figure 2-6). The multidrug resistance distribution of isolates recovered on MacConkey containing tetracycline differed greatly from those recovered on nonselective agar with the single, double, and triple resistance proportions being 31%, 27%, and 30% respectively (Figure 2-7). Aside from the obvious agar selection pressure eliminating recovery of pan-susceptible isolates (i.e., all isolates were necessarily resistant to tetracycline in the media at CLSI breakpoint of 16 µg/ml); the relative ratios indicate a selection bias against singly resistant bacteria in favor of higher order multi-resistance numbers. This can be better seen by plotting the proportional difference for each multiresistance category (Figure 2-8).

**Tetracycline gene distribution**

Of the four tetracycline genes tested, only tet(A), tet(B), and tet(C) were found. The tet(E) gene was not found in any of the isolates. Despite there being significant differences between agar types, prevalence did not significantly change over the decade for any gene (Figure 2-9). Among all *E. coli* isolates recovered from plain MacConkey agar, isolates harboring only tet(A), tet(B), or tet(C) were 9%, 13%, and 19% respectively. This was unexpected since tet(B) is the most commonly reported tetracycline gene and is considered to be the most widely disseminated. However, the MIC distributions for each gene differed in that most tet(C) isolates had an MIC
ranging from 4 to 16 ug/ml while \textit{tet}(A) isolates MICs were usually =32 or >32, \textit{tet}(B) isolates always had a MIC of >32 µg/ml. Therefore, while nearly all \textit{tet}(A) and \textit{tet}(B) isolates were phenotypically resistant, many isolates harboring \textit{tet}(C) were actually phenotypically susceptible to tetracycline at breakpoint values (or intermediate before collapse of categories). When comparing the relative proportions of only phenotypically resistant isolates, the \textit{tet}(A), \textit{tet}(B), and \textit{tet}(C) proportions were 27%, 40%, and 23% respectively (Figure 2-10). Among isolates recovered from MacConkey supplemented with tetracycline, the proportion of \textit{tet}(A), \textit{tet}(B), and \textit{tet}(C) isolates were 17%, 67%, and 2% respectively. There were several isolates that harbored multiple tetracycline genes, especially among isolates recovered from MacConkey containing tetracycline. Multi-gene isolates were predominantly either AC or BC. However the AB combination was rare. Only two isolates were found to harbor all three tetracycline genes.

**Relationships between tet genes and multidrug resistance**

The distribution of multiresistance in \textit{tet}(A) containing isolates was different enough from those harboring \textit{tet}(B) to suggest that one may be more associated with higher orders of multiresistance. The bimodal phenotypic resistance distribution of \textit{tet}(A) isolates showed that a majority of these were singly resistant, but also there were some isolates with 9-12 resistances (Figure 2-11). The distribution of \textit{tet}(B) differed in that single, double, and triple resistant isolates were roughly equal in proportion (Figure 2-12) and represented a predominant mode to the left of the scale from 0-15 resistances. Furthermore, the bimodal hump in the 9-12 resistances category was not as pronounced as the \textit{tet}(A) distribution. The full-factorial logistic regression of the association of tetracycline genes with high varieties of drug resistance (≥4 drug resistances) confirmed that \textit{tet}(A) isolates were much more likely to harbor high numbers of phenotypic resistance (Figure 2-13). In addition, the use of selective agar appeared to further increase the
probability of a \( \text{tet}(A) \) isolate being multiresistant. This was further confirmed by the ordered logistic model which demonstrated that while singly resistant isolates were equally as likely to be of \( \text{tet}(A) \) or \( \text{tet}(B) \) genotype, double and triple resistant isolates were likely \( \text{tet}(B) \) and multiresistance greater than 3 were more likely to be \( \text{tet}(A) \) (Figure 2-14).

**Discussion**

**Tetracycline and Ceftiofur prevalence**

There were two distinct estimates of antibiotic resistance prevalence for both tetracycline and ceftiofur. The first is an isolate-level estimate of resistance among \( E. \text{coli} \) populations in a given caecal sample and this is what was determined and published in the annual CIPARS reports. This is based off the MIC value of the single isolate taken from growth on nonselective agar. When considering the case that the entire \( E. \text{coli} \) population in a caecal sample will be a mixture of both sensitive and resistant bacteria, the most dominant phenotypes are likely to be selected when taking one colony from all colonies that grow on nonselective agar. Therefore, an isolate with a MIC above the breakpoint suggests that resistant bacteria are in the caecal sample at high enough proportions to be selected from the total bacteria population including susceptibles. The second estimate is an animal/sample-level estimate of resistance. By using media containing an antibiotic at the breakpoint value, the presence of growth indicates that a caecal sample harbors at least one resistant bacterium regardless of whether it is only a very small proportion of the total \( E. \text{coli} \) in a caecal sample. In effect, the first predicts the prevalence of resistance \( E. \text{coli} \) among populations in caecal samples with seemingly high levels of resistance (i.e., above some threshold) while the second predicts the prevalence of any resistant \( E. \text{coli} \) in samples with any levels of resistance.
In the case of tetracycline resistance, the animal/sample-level prevalence was much higher than the crude bacterial-level estimates. Even though 916 out of 1,368 (72%) *E. coli* positive caecal samples grew on Mac supplemented with tetracycline, only 361 out of 1,268 (28%) of isolates recovered from plain Mac exhibited an MIC above the breakpoint. This suggests that tetracycline resistance was often present at low levels and went undetected in the isolate-level screening. Further, while estimated animal-level resistance did not significantly change, the bacterial-level prevalence did steadily increase between 2009 and 2011.

The confirmed low and stable prevalence of ceftiofur resistance is encouraging from a human health perspective, and also of some comfort to beef cattle sectors of agriculture (including fed beef and dairy beef) in Canada. The hypothesis that ceftiofur resistance was increasing in prevalence, but was occurring at a level below the detection threshold of the original CIPARS methodology did not hold true. This is perplexing given that it suggests that when present, it is above the threshold of detection for surveillance using plain agar, and when apparently absent using plain agar, it really is absent and not simply below limit of detection. While indeed the sample-level prevalence of ceftiofur resistance was higher than the bacterial population level prevalence, both were quite low and neither indicated any increase in resistance over the decade. While 26 out of 1,368 (2%) of *E. coli* positive caecal samples grew on Mac containing ceftiofur, only 3 out of 1,264 (0.24%) isolates collected from nonselective agar had an MIC above the breakpoint. The population level prevalence was effectively negligible and there was no increase over the decade. The sample-level prevalence also relatively very low and did not change over the decade.
**Freezing effect**

Before estimating resistance prevalence, it was important to account for any loss in test sensitivity due to the samples having been frozen for many years. The effect of freezing on caecal tissue samples was testable because results from the original surveillance tests could be compared to post-freezing tests performed in this study.

There were 100 out of 1,368 samples in which no *E. coli* was recovered, even though all caecal samples used in this study originally had growth on nonselective Mac before being frozen in a sample bank. Importantly, these were not evenly distributed across the sampling years. Instead, older samples were more likely to have degraded to the point where *E. coli* could not be recovered at all. Had this effect not been quantified and treated as a reduction in sensitivity, prevalence predictions for earlier years would have been underestimated and annual changes would have artificially appeared to increase over time. Because the original testing only used nonselective agar, it was not possible to determine whether the 6% sample loss per-year-of-freezing is the same for nonselective media. However, a conservative assumption would be that it is approximately the same. Nevertheless, there is a strong possibility that antibiotic pressures on growth of free-compromised bacteria in caecal samples might further limit sensitivity and this remains unknown.

It is quite uncommon for studies to account for the effects of freezing when using historical samples. This is mostly because the historical unit of concern is usually the bacterial isolate and not the sample matrix the isolate was recovered from; therefore the possibility of determining reduced recoverability is not usually feasible. Even though there was sufficient historical data regarding the samples in this study to estimate a freezing effect, most historical sample databases probably do not have enough background data to do this. It is uncertain how generalizable these intrinsically determined freezing effect estimates are given that no extensive
study has been done on the long-term viability of *E. coli* in frozen faecal specimens. There is research that demonstrates that by using a buffered glycerol-saline additive, viability is drastically increased in frozen fecal samples (51). Larger volumes of sample likely also help; the CIPARS samples were very small (1 ml) and so there is little protective effect of the sample for bacteria in the core of the sample. In addition, freeze/thaw effects are more likely when freezer doors are left open with small samples. Perhaps if other caecal samples were collected and stored in a similar manner, the effect may be comparable. However, other materials such as tissue or swabs would be affected very differently by freezing. Regardless, it is evident that storing and freezing samples has clear potential to bias results, at least diagnostic sensitivity, and should always be addressed and compensated for when possible.

**Multidrug resistance**

The top three drug resistance phenotypes found were tetracycline, sulfisoxazole, and streptomycin. These drugs have been in use in animal agriculture for decades, and *E. coli* is already known to commonly be resistant to them. Likewise, their co-resistance with each other is well established. It was perhaps unexpected that the levels of resistance were relatively low when compared to other studies looking at resistance in commensal bacteria (52). Nonetheless, beef cattle and dairy cattle *E. coli* isolates are almost uniformly of lower resistance prevalence around the world, especially among slaughter-age animals (31).

**Distribution of tetracycline resistance genes**

Though few, there are earlier studies that have also examined the relative distributions of tetracycline resistance genes in various animal sources including cattle. Bryan et. al. (2004) (53) conducted a study looking at tetracycline resistance in *E. coli* isolated from different sources including humans, pigs, chickens, turkeys, sheep, cows, and goats using a nonselective approach.
to see what tetracycline resistance genes were present in nonclinical environments. They found that roughly 30% of *E. coli* isolated from cattle were resistant to tetracycline using a population-level approach. This closely agrees with the 28% population-level estimate found in this study. When they looked at genotype distributions, they found both *tet(A)* and *tet(B)* in cattle derived isolates at a ratio of roughly 1:2. Even though *tet(C)* was not found in any of the cattle isolates, it was found to be significant in horses, dogs, humans, pigs, and sheep. Unfortunately, because the authors only tested isolates that had a very high tetracycline MIC (≥93 μg/ml), the gene distributions were likely biased, given *tet(C)* appears to have MIC values much lower than their exclusion cut-point. They were also not able to detect *tet(E)* in any of their isolates. Therefore, it seems that *tet(E)* is probably extremely rare in *E. coli* and does not contribute significantly to tetracycline resistance levels, though its MIC distribution could also have precluded its identification in this study.

The high occurrence of *tet(C)* isolates is interesting because despite having a lower MIC, it seems able to consistently persist in the commensal bacteria in cattle. The relatively low MIC associated with *tet(C)* does not seem to be restricted to only cattle and has been shown to be potentially a widespread phenomenon for this specific resistance gene (54). It is peculiar that a tetracycline resistance gene that often fails to actually provide resistance would be maintained in the intestinal flora when there are many other tetracycline genes that provide resistance to higher concentrations of tetracycline. One possible explanation for this is that while *tet(C)* may not have a selective advantage in the intestinal environment and may even have a marked disadvantage when the animal is treated with tetracycline, and it may especially have an advantage in soil. Most enteric bacteria spend a considerable amount of time between hosts in the ambient environment. When Schmitt et. al. (2006) (55) looked at tetracycline resistance present in
agricultural soils fertilized with animal manure, they found that not all tetracycline resistance genes present in animal manure were able to persist in the soil microbiome. In particular, despite both tet(B) and tet(C) being clearly present in pig manure, only tet(C) was found in the soil two hours after applying manure to the landscape. While speculative, it may be that the ability of tet(C) to persist in a soil or soil-like environment after being excreted may have something to do with its relatively high prevalence in cattle. Volkova et. al. (2012) (56) comprehensively modeled the dynamics of ceftiofur-resistance in commensal E. coli living in the intestines of cattle. In addition to variables such as bacterial population size, growth rate in vivo, plasmid fitness cost, and the concentration of antibiotic metabolites, they included an “in-flow” variable that represented bacteria ingested by the cattle. Ultimately they found that this “in-flow” component was an important factor in determining the burden of resistance in the commensal E. coli. Admittedly their model primarily focused on ceftiofur resistance but their conclusions also may apply to tetracycline resistance. Due to the limited variety of ceftiofur resistance determinants, they only were really concerned with the frequency of resistance and not the diversity of resistance as an output. Nevertheless, this “in flow” effect may also have a similar significant impact on the diversity of resistance exhibited when there are many possible determinants such as the case of tetracycline resistance.

The notion that bacteria ingested by cattle significantly affect the intestinal flora may go beyond explaining why tet(C) was found at such a high prevalence and also help explain why the relative proportion of tetracycline genes were stable over the course of a decade. Even without data regarding the levels and diversity of tetracycline resistance present in the environments the cattle in this study were exposed to, it is not unreasonable to assume that these environments are considerably more stable in regards to the tetracycline resistance levels than the intestinal
environment in cattle. Numerous studies have shown that bacterial communities in soil are very resilient to the selective effects of antibiotics. Hund-Rinke et. al. (2004) (57) investigated this by spiking soil with bacterial resistance laden pig manure and then treating the soil with tetracycline at varying concentrations to see whether strong selection can help establish resistance genes that are present in the feces. They concluded that even very high concentrations (50mg/kg) of tetracycline had little effect on the soil communities and that the environmental change from manure to soil had a much larger impact on whether a particular resistance gene would persist in the soil.

**Agar selective effect**

The extreme difference in tetracycline resistance gene distributions based on which agar the isolates were recovered from clearly demonstrates the dangers of introducing bias into a study aiming to quantify the relative abundance of tetracycline genes in a bacterial population. When grown on selective Mac containing tetracycline, very few isolates harbored only *tet(C)* despite it actually being the most common gene in isolates obtained nonselectively. In addition, isolates that did harbor *tet(C)* were more likely to also harbor *tet(A)* or *tet(B)*. However, co-resident *tet(A)* and *tet(B)* in any isolates was very uncommon which suggests an incompatibility between the two genes or their associated plasmids. Comparing gene proportions between nonselective and selective agars indicates that MacConkey agar with tetracycline strongly favors isolates harboring *tet(B)*. Perhaps even more importantly, selective agar also was biased towards recovering multidrug resistant bacteria. There was roughly a 30% reduction in isolates possessing a single drug resistance while the two-, three-, and four-antibiotic categories were respectively enriched by 10%, 9%, and 7%. There was also a slight enrichment for isolates with >4 drug resistances, though these bacteria rarely harbored *tet(B)*. This finding certainly
demonstrates that isolates obtained from selective agar are not representative of the general resistant population and should probably not be used as inference to the population as a whole.

**Tetracycline co-resistance**

When testing associations with resistances other than tetracycline, \(\text{tet}(A)\) and \(\text{tet}(B)\) were both found to be highly associated with sulfisoxazole and streptomycin, while \(\text{tet}(C)\) was not as strongly associated with other resistances. Multiresistance histograms for \(\text{tet}(A)\) and \(\text{tet}(B)\) indicate that \(\text{tet}(A)\) may have a stronger association with phenotypes exhibiting >3 drug resistances. When modeling the multiresistance variable using a cutpoint of >3 drugs, \(\text{tet}(A)\) was significantly more positively associated with MDR than \(\text{tet}(B)\). The most common 4-drug phenotype was streptomycin, sulfisoxazole, tetracycline, and chloramphenicol. Out of all isolates genotyped that were phenotypically resistant to chloramphenicol, 48 out of 55 of them harbored \(\text{tet}(A)\) while only 15 harbored \(\text{tet}(B)\). This does seem to suggest an underlying correlation between \(\text{tet}(A)\) and chloramphenicol resistance that further molecular analysis would be able to discern as to chromosomal or plasmid based biological genetic linkage. The common \(\text{cmlA}\) chloramphenicol gene and tetracycline and sulfamethoxazole resistance has already been determined to be co-localized on transferable plasmids(58). However, particular associated tetracycline genes were not identified. Given that \(\text{tet}(A)\) and chloramphenicol are both plasmid based, their correlation suggests that they may be localized on the same plasmid. The potential for chloramphenicol resistance plasmids to preferably also harbor the \(\text{tet}(A)\) gene specifically has yet to be studied and warrants further inquiry.

**Conclusion**

Historical samples obtained through the CIPARS program provided the opportunity to re-evaluate antimicrobial resistance occurring in Canadian beef cattle over the past decade. The
core hypothesis was that ceftiofur and tetracycline resistances in Canadian beef cattle were changing in both prevalence and diversity, albeit at levels below the detection threshold of the original testing methods. Ceftiofur resistance did not appear to increase in prevalence over the decade while tetracycline resistance at the sub-population level did increase from 2010 to 2011, but has remained stable at the bacterial population level for the past decade. While multiple tetracycline resistance determinants were detected, they were not evenly distributed. The unexplained persistent success of \textit{tet(C)}, despite having a lower MIC, and the possible association between \textit{tet(A)} and chloramphenicol and other antibiotic resistances should be further investigated because of their potential to explain these uneven distributions and perhaps take advantage of such features to manage bacterial populations and select against multiple resistance strains.
Figure 2-1 Bacterial isolation pipeline demonstrating how multiple bacterial isolates were recovered from a single caecal sample

The original CIPARS surveillance testing consisted of plating a caecal sample on plain MAC agar. If the sample exhibited growth, the MIC for one E. coli isolate selected from the plate was tested via Sensititre. Caecal samples were then frozen and stored in a sample bank. When re-evaluated, only caecal samples originally testing positive for E. coli growth were used. These samples were parallel plated on MAC containing no antibiotic, tetracycline, or ceftiofur. If there was growth on a plate, one isolate was selected and tested for resistance.
The custom NARMS plate CMV1AGNF tests for the minimum inhibitory concentration to 15 different antibiotics. Each well contains a pre-determined concentration of antibiotic in µg/ml. An appropriate range of log₂ dilution concentrations are used for each antibiotic.
The impact of freezing on recovery of *E. coli* was logistically modeled. Because samples were non-sequentially processed, there were no samples that were frozen for 3 years.
The animal level prevalence (expressed as a proportion) of tetracycline resistance was calculated after accounting for a varied reduction in test sensitivity due to freezing time.
The animal level prevalence of ceftiofur resistance was calculated after accounting for a varied reduction in test sensitivity due to freezing time.
The distribution of the multiresistance number calculated as the sum of drugs with an MIC above breakpoint values for all isolates recovered from plain MAC.
Figure 2-6 Multiresistance distribution of isolates recovered from plain MAC
(without pan-susceptible isolates)

The distribution of multiresistance calculated as the sum of drugs with an MIC above breakpoint values for all isolates recovered from plain MAC not including pan-susceptible isolates
The distribution of the multiresistance number calculated as the sum of drugs with an MIC above breakpoint values for all isolates recovered from MAC containing tetracycline.
The proportional difference for each multiresistance category between plain MAC and MAC+tet distributions. For example, singly resistant isolates were roughly 50% of the resistant population recovered on plain MAC but were only 30% of the resistant population recovered on MAC+tet.
Figure 2-9 Tetracycline genotype proportions by decade and agar

Proportions of tetracycline genotype are stratified by agar and decade.
Proportional venn diagrams demonstrate the difference in tetracycline gene distributions between non-selective and selective MAC.
Figure 2-11 Multiresistance distribution of tet(A) positive isolates (n=130) to the 14 drugs on the NARMS CMV2AGNF MIC plate
Figure 2-12 Multiresistance distribution of $tet(B)$ positive isolates (n=320) to the 14 drugs on the NARMS CMV2AGNF MIC plate
Figure 2-13 Probability of tetra-resistance (multiresistance ≥4)
Figure 2-14 Bivariate marginal predictions of tetA and tetB based on multiresistance and agar
<table>
<thead>
<tr>
<th>Year Sampled</th>
<th>No. samples used in sensitivity calculation</th>
<th>No. samples growth positive on MAC+cef</th>
<th>No. samples growth positive on MAC+tet</th>
<th>No. samples per year</th>
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<tr>
<td>2002</td>
<td>93</td>
<td>3</td>
<td>61</td>
<td>93</td>
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<td>2003</td>
<td>308</td>
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<td>141</td>
</tr>
<tr>
<td>2004</td>
<td>308</td>
<td>3</td>
<td>118</td>
<td>167</td>
</tr>
<tr>
<td>2005</td>
<td>122</td>
<td>2</td>
<td>74</td>
<td>122</td>
</tr>
<tr>
<td>2006</td>
<td>150</td>
<td>1</td>
<td>85</td>
<td>150</td>
</tr>
<tr>
<td>2007</td>
<td>188</td>
<td>0</td>
<td>121</td>
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<tr>
<td>2008</td>
<td>172</td>
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<td>2009</td>
<td>119</td>
<td>4</td>
<td>72</td>
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</tr>
<tr>
<td>2010</td>
<td>77</td>
<td>2</td>
<td>54</td>
<td>77</td>
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<tr>
<td>2011</td>
<td>139</td>
<td>5</td>
<td>121</td>
<td>139</td>
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Table 2-2 Tabulated annual prevalence of positive growth adjusted for freezing effects

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<tr>
<th>Year</th>
<th>Sensitivity</th>
<th>Total annual samples</th>
<th>Samples that grew on MAC+cef</th>
<th>Samples that grew on MAC+tet</th>
<th>Prevalence of positive growth on MAC+cef</th>
<th>95% CI</th>
<th>Prevalence of positive growth on MAC+tet</th>
<th>95% CI</th>
</tr>
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<tr>
<td>2002</td>
<td>0.892</td>
<td>93</td>
<td>3</td>
<td>61</td>
<td>0.025</td>
<td>0</td>
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<tr>
<td>2003</td>
<td>0.903</td>
<td>141</td>
<td>5</td>
<td>101</td>
<td>0.039</td>
<td>0.016</td>
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<td>0.794</td>
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<tr>
<td>2004</td>
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<td>3</td>
<td>118</td>
<td>0.02</td>
<td>0.005</td>
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<tr>
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<td>Antimicrobial</td>
<td>% Resistant</td>
<td>95% CI</td>
<td>Distribution of MICs in µg/ml (%)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>&lt;0.015</td>
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<td>0.17-1.02</td>
<td>4.3</td>
<td>26</td>
<td>59.9</td>
<td>9.1</td>
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<td>0.3</td>
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<td>/Clavulanic Acid</td>
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<td></td>
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<td>0.5</td>
<td>0.31</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0.02-0.57</td>
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<td>0</td>
<td>0.1</td>
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<td>0.2</td>
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<td>0</td>
<td>0.2</td>
<td>0.59</td>
<td>0.1</td>
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<td>0</td>
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<td>2.6</td>
<td>1.80-3.64</td>
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<td>0</td>
<td>0.36</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Ciprofloxacin</td>
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<td>0.00-0.44</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>Nalidixic Acid</td>
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<td>0.05-0.69</td>
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<td>Streptomycin</td>
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<td>10.18-13.82</td>
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<td>0</td>
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<td>0.91</td>
<td>0.1</td>
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<td>Sulfisoxazole</td>
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<td>11.13-14.91</td>
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<td>0</td>
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<td>0</td>
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<td>Tetracycline</td>
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<td>26.00-31.04</td>
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<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
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<td>0.49-1.65</td>
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Table 2.3 MIC distribution of isolates recovered from plain MAC
<table>
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<tr>
<th>NARMS Code</th>
<th>Antimicrobial</th>
<th>% Resistant</th>
<th>95% CI</th>
<th>Distribution of MICs in ug/ml (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Aug</td>
<td>Amoxicillin/Clavulanic Acid*</td>
<td>0.87</td>
<td>0.38-1.71</td>
<td>6.3 25 54 12.8 1 0.7 0.2</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
<td>10.59</td>
<td>8.67-12.77</td>
<td>0.1 0.5 0.29 0.01 0 0 0.1</td>
</tr>
<tr>
<td>AZI</td>
<td>Azithromycin</td>
<td>0.33</td>
<td>0.07-0.95</td>
<td>0 0 0 0 0.1 0.7 0.16 0 0 0</td>
</tr>
<tr>
<td>FOX</td>
<td>Cefoxitin</td>
<td>1.31</td>
<td>0.68-2.28</td>
<td>0 0 0.2 0.56 0.17 0 0 0</td>
</tr>
<tr>
<td>XNL</td>
<td>Ceftiofur</td>
<td>0.55</td>
<td>0.18-12.69</td>
<td>0.04 0.4 0.5 0 0 0 0 0</td>
</tr>
<tr>
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<td>Ceftriaxone</td>
<td>0.55</td>
<td>0.18-1.27</td>
<td>0.99 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>CHL</td>
<td>Chloramphenicol</td>
<td>8.62</td>
<td>6.89-10.63</td>
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</tr>
<tr>
<td>CIP</td>
<td>Ciprofloxacin</td>
<td>0.33</td>
<td>0.07-0.95</td>
<td>0.94 0 0 0.01 0 0 0 0 0</td>
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</tr>
<tr>
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<td>Kanamycin</td>
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</tr>
<tr>
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<td>Streptomycin</td>
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<td>45.73-52.31</td>
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<tr>
<td>SXT/SXT</td>
<td>Trimethoprim/Sulfamethoxazole**</td>
<td>2.95</td>
<td>1.95-4.26</td>
<td>85.3 11.6 0.2 0 0 0 2.95</td>
</tr>
</tbody>
</table>

Table 2.4: MIC distribution of isolates recovered from MAC+tetracycline (16µg/ml)
Chapter 3 - Short-term population selection effects of chlortetracycline and ceftiofur treatment on growth parameters of commensal *E. coli*

**Introduction**

Despite evolutionary models growing increasingly complex, “survival of the fittest” remains the core axiom of evolutionary theory. In a most basic sense, antimicrobials seem capable of providing the motive force to drive evolution. The presence of both natural antibiotic producers and antibiotic resistance genes in natural environments does suggest this has been in existence for epochs of both bacterial and higher order life form existence (59). There is no doubt that in an environment containing an antimicrobial, possessing resistance is massively beneficial to the survival of a bacterium. However, what are the costs of having resistance genes when that antimicrobial is not present? The synthesis of superfluous nucleic acids and proteins theoretically should result in some reduction in growth potential. Yet, there is experimental evidence suggesting that certain resistance determinants can persist in environments even after antibiotics are removed, and for extended periods of time (60). At least some of these determinants have been found to possess compensatory mutations or regulatory pathways that could significantly alleviate fitness costs (61, 62). Even more surprising, multiple different resistance determinants to the same antibiotic have been seen to co-exist in both antibiotic pressured and naïve bacterial communities, such as is the case for tetracycline efflux genes (53).

Even though all three detected tetracycline genes in the CIPARS study maintained stable prevalence proportions at the isolate-level, the strong selective effect of the agar as well as the drastic difference in MIC distribution between the three genes seemed to indicate that short-term
selective pressure may differentially select for particular tetracycline genes at the animal-level. This was not testable in the CIPARS isolates because none of the animals sampled would have been undergoing antibiotic treatment due to a regulated withdrawal period before slaughter and pre-withdrawal treatment history was unknown.

The primary objective of this second study was to investigate whether a strong short-term selection pressure from antibiotic treatment with tetracycline or ceftiofur resulted in a differential selection of gut flora in favor of bacteria with relatively higher fitness associated with the antibiotic pressure. Further, the bacteria arising from different selection pressures were tested to see whether particular resistance genes or phenotypic multi-drug resistance patterns were associated with higher fitness values.

**Materials and Methods**

**Previous studies**

The *E. coli* isolates used in this study are the same isolates used in Kanwar et al. (2013) (63). That previous study was designed to test whether co-administering chlortetracycline to cattle following ceftiofur treatment would impact the levels of ceftiofur and tetracycline resistance among commensal *E. coli*. The researchers’ findings and how they relate to the isolates’ relative fitness will be presented in the discussion section.

**Field trial**

88 steers were evenly distributed among eight pens. This process was a blocked randomization procedure so that all pens had similar average weights. Two potential intervention strategies were employed in a full two-way factorial design for a total of four treatment groups. Pens were randomly assigned to one of four treatment groups (Figure 3-1). The four treatment groups are as follows; all steers in pens 1 and 2 were treated with ceftiofur first and then
chlortetracycline, one steer in pens 3 and 4 was treated with ceftiofur and then all steers in pens 3 and 4 were treated with chlortetracycline, steers in pens 5 and 6 followed that of pens 1 and 2, steers in pens 7 and 8 followed that of pens 3 and 4. Ceftiofur crystalline-free acid (6.6mg/kg) was administered via a single subcutaneous injection on day 0 according to label recommendations. Chlortetracycline (22mg/kg) was top-dressed on feed in three 5-day periods starting on days 4, 10, and 16, with a one day break on days 9 and 15. Cattle were followed up for 26 days with fecal samples being collected per rectum every other day starting on day 0 (figure 3-2). These fresh samples were mixed 1:1 with glycerol, placed in 5ml cryo-vials, and stored at -70°C.

**Samples subjected to isolation**

Due to time and labor constraints, not all fecal samples underwent further bacterial isolation. Only samples from days 0, 4, 12, and 26 were used. These days were chosen based on the antibiotic dosing timeline. On day 0, neither antibiotic would have affected the gut flora yet. Ceftiofur was administered starting on day 0 and would have exhibited full effect by day 4, when the chlortetracycline was added to the feed, though no tetracycline effects was expected until day 12. Both antibiotics would have taken effect by day 12 and left the system by day 26.

**Isolation of commensal E. coli**

Selected fecal samples were thawed and 200 milligrams of each fecal sample were diluted in 1.8 milliliters of buffered peptone water. Fifty microliter aliquots of the suspensions were surface plated onto 10cm plates containing MacConkey agar (BL Difco™) using a spiral plater (Eddy Jet; IUL Instruments). Colony forming unit counts were converted based on the spiral plater settings to determine original CFU concentration. Three separate E. coli colonies were picked from the original fecal sample spiral plate and further plated onto fresh MAC plates.
The purified *E. coli* isolates were then plated one more time on a trypticase™ soy agar plate (BL Difco™) before further testing.

**MIC determination**

Antimicrobial susceptibility testing of pure isolates was performed using the Sensititre (Trek Diagnostic Systems, Cleveland, Ohio, USA) automated broth microdilution system. The custom National Antimicrobial Resistance Monitoring System (NARMS) panel CMV1AGNF was used. Briefly, isolate colonies were taken from a trypticase soy agar plate and diluted to a 0.5 McFarland standard. This was added with Mueller Hinton broth to the 96-well plate containing a panel of 15 antibiotics (Figure 3-3). These plates were incubated for 24 hours at 37°C and then read using the Sensititre ARIS® and Vizion™ systems (Trek Diagnostic Systems, Cleveland, Ohio, USA). Antibiotic resistance was determined by first using CLSI guideline breakpoints; however, not all antibiotics on the panes had established breakpoints. For these, consensus breakpoints for enteric bacteria established by NARMS were used. Using described breakpoints, isolates were coded as either resistant, intermediate, or susceptible. This three tier system was later collapse into a binary classification to simplify downstream analysis. Isolates that tested as having an intermediate resistance were recoded as susceptible. To ensure quality control, the following strains were tested with each batch of Sensititre plates: *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212.

**Determining bacterial fitness**

To obtain a general measurement of fitness cost incurred by bacteria harboring resistance genes, a methodology was developed that allowed the testing of a large number of bacterial isolates that was both cost and labor efficient.
Numerous studies have attempted to quantify the fitness cost associated with antibiotic resistance (62, 64-66). The most common approach to assessing fitness cost is to use a competitive growth assay. This process consists of inoculating a medium with an experimental strain and a reference strain. The ratio of one strain to another is then measured over a specified length of time. However, this method usually requires insertion of a resistance gene into a laboratory adapted strain with some form of reporter to allow detection. Because of this, strains used typically are not very representative of naturally resistant strains. Therefore, the results of these experiments have very limited external validity.

While uncommon in antimicrobial resistance research, the use of growth curves is very common in food microbiology (67, 68). The bacterial growth curve is characterized by a succession of phases (Figure 3-4). After being inoculated into a broth or onto an agar plate, bacteria will not divide for a brief time. This period is usually termed the lag phase. Once division begins to occur, the rate of division slowly increases. This is usually called the acceleration phase. Once maximum growth rate is reached, division continues at the constant maximum rate for a period called the exponential phase. Then the growth rate starts decreasing during the retardation phase until the bacterial population no longer increases and stabilizes. This period is called stationary phase. Eventually, bacteria start to die off and the population decreases during the death phase. No doubt, this generalized process is not without variation. Often, it is difficult to identify when one phase ends and another begins. This is not overly surprising given the vast number of physiological processes that culminate in biological growth. Furthermore, complex growth cycles can occur, resulting in multi-modal curves (Figure 3-5). Many of these phenomena can be explained by the selective metabolization of energy sources when there are
multiple sources in the environment, or as nutrient resources of certain types begin to dwindle (69).

Historically, the gold standard for growth curve measurement is the change in colony forming units (CFU) over time. However, manual counting is labor intensive and not conducive to large sample sizes and high-throughput studies. Instead of CFU counting, spectrophotometric values in the form of optic densities (OD) are used as a replacement or surrogate form of measurement. Historically, there have been several reservations to using OD values instead of CFU values. The primary concern is that a maximum growth rate determined from CFU values can be clearly defined as the biological doubling rate while the maximum rate of increase in optic density is not as directly linked to a biological rate. To address this concern, there has been considerable effort to determine whether there is a clear correlation between growth rates determined via CFU counts and growth rates determined via OD measurements. Due to the complex shape of the growth curve, multiple differential equation models have been used to generate values that allow CFU based curves to be compared to OD curves (70, 71). Ironically, while the two rates are generally correlated, there is just as much disagreement about which model equations are best as whether it is even beneficial to correlate the two rates for conversion. There are several theoretical explanations as to why the two might differ, but the most likely reasons are the impact of clumping and variability in cell size. Fortunately, using spectrophotometric growth curves for the purposes of assessing fitness cost associated with antibiotic resistance does not necessarily require a clear biologically defined growth rate. Instead of requiring absolute growth parameters, relative differences in growth parameters may be just as helpful in investigating different resistance determinants. Having ready access to an automated spectrophotometer, several simple experiments were performed to assess the possibility of using
growth curves measured via OD to assess the relative fitness of antibiotic resistant bacteria
grown under different conditions.

Because minimum inhibitory concentration (MIC) determination is commonplace in
antibiotic resistance research, there was great value in identifying a way to incorporate growth
curve measurement into the general MIC protocol to allow for efficient parallel processing of
isolates. Shortly before inoculating a microbroth plate to determine MIC values, bacterial isolates
were diluted to a 0.5 McFarland standard. This standardized dilution step was identified as a
potential point for parallel processing because at this point, all isolates would be diluted to the
same concentration. To test whether this dilution step would provide reproducible results, eight
0.5 McFarland standards were prepared using ATCC laboratory control strains EC25922 and
EC35218. These dilutions were then successively diluted 1:2 eight times. An optical plate was
inoculated with these dilutions and McConkey broth was added. OD measurements were taken at
10 minute intervals for 24 hours using the Bioscreen C™ (Growth Curves USA, Piscataway, NJ)
automated spectrophotometer. Growth curves followed a simple monoauxic growth pattern and
did not exhibit any complex growth patterns such as diauxic curves. The replicate effect was
non-significant to the point that OD readings were essentially on top of each other from one
replicate to the next (Figure 3-6). Furthermore, the 2:1 dilutions were spaced at regular intervals.
Most importantly, it was easy to distinguish one strain from another.

Measurement of bacterial growth curves

The same bacterial isolates used to inoculate Sensititre plates were also used to inoculate
100-well optical plates. Isolates diluted to a 0.5 McFarland standard were added to plain
MacConkey broth in a 1:9 ratio for a final concentration of roughly 1.5*10^7 CFU/ml. In parallel
fashion, isolates were also added to MacConkey broth (BL Difco™) supplemented with
tetracycline at a concentration of 16 µg/ml, using the same dilution ratio. Optical plates were incubated at 37°C and optic density was measured at 10 minute intervals with the plates being shaken between measurements. Both incubation and spectrophotometric tasks were done using the Bioscreen C™ (Growth Curves USA) automated microbiology growth curve analysis system. Optic Density (OD) was measured using a wideband filter (420-480nm) to minimize sensitivity to color changes in broth.

**Qualitative detection of resistance genes**

Previously described *E. coli* isolates were tested for the presence of multiple different resistance genes that confer resistance to either tetracycline or ceftiofur. DNA from bacterial isolates was prepared by boiling multiple pure colonies suspended in nuclease-free water. Tetracycline resistance genes *tet(A)*, *tet(B)*, and *tet(C)* were detected using PCR methods previously described (11). The third generation cephalosporin resistance gene *blaCMY-2* was also detected via PCR. Primers for all PCR reactions are listed in Table 3-1. The PCR processing pipeline consisted of multiple automated laboratory machines. Plate preparation was carried out using the QIAgility™ (Qiagen, Venlo, Netherlands) benchtop robot. Reactions were facilitated using the Eppendorf Mastercycler® gradient thermal cyclers (USA Scientific, Inc., Ocala, FL). Resulting PCR product was detected using the QIAxcel™ (Qiagen, Venlo, Netherlands) microcapillary electrophoresis system. DNA extracted from the following strains was used for quality control: *E. coli* ATCC 47042 (known to harbor *tet(B)*), *E. coli* XL1-Blue (known to harbor *tet(A)*), and *E. coli* M1 (known to harbor *blaCMY-2*).

**Statistical analysis**
Because the bacterial growth curves were simply a set of OD measurements taken at specified intervals, it was important to extract specific growth parameters that could be used in downstream analysis. Based on initial tests with ATCC quality control strains EC25922 and EC35218, two aspects of the growth curves were identified as being important for differentiating one strain from another. These were the maximum growth rate during exponential phase and the upper asymptotic OD reached at stationary phase (and its difference from the baseline OD value at time = 0). Values appeared to be conserved for a particular strain over multiple replicates and did not appear to be affected by the starting concentration of bacteria (Figure 3-6). To calculate the maximum growth rate, the difference in OD between each interval was calculated and the maximum increase was recorded as the max ΔOD/10 minutes. The stationary OD was calculated by recording the maximum OD reached throughout the entire growth curve. To ease later analysis, the starting OD was then subtracted from the maximum OD to give the parameter a base of zero and can be thought of as the global ΔOD from lag phase to stationary phase. A sample growth curve with estimated parameters is illustrated in Figure 3-7.

After the maximum slope and global ΔOD were determined for each isolate based on their growth curve, these two parameters were studied first by assessing whether the animal level treatment affected the overall distribution of growth parameters of the isolates. Multivariate linear regression analysis was done using both growth parameters as dependent variables and using the administration of ceftiofur and tetracycline, the selective media (tetracycline versus no tetracycline), as well as day of collection as independent variables. All three covariates were included as a full-factorial model. Additionally, both growth parameters were tested to see whether any particular tet genes could be identified as having significantly different growth
parameters. Neither treatment nor day of collection was included in this latter model. All statistical analysis was carried out using STATA® SE Release version 12.1.

**Results**

**MIC distributions**

The MIC distributions of all 1,050 isolates to each of 15 antibiotics included in the NARMS panel are presented in Table 4-2. Of all isolates, at least 1 antimicrobial resistance was detected in 710 (67.62%) isolates; 340 (32.38%) isolates were found to be susceptible to all 15 antimicrobials. Tetracycline was the most common drug resistance (61.14%) with ampicillin (37.24%), sulfisoxazole (31.33%), and streptomycin (29.33%) also being fairly common.

**Tetracycline resistance gene selection**

As reported in Kanwar et al. (2013), a generalized estimating equation (GEE) logistic model was run to test whether treatments preferentially selected for tet(A) or tet(B) (Figure 3-8). The likelihood of recovering isolates harboring tet(A) or tet(B) increased with ceftiofur treatment (Figure 3-8A). Cattle in the treatment group that received ceftiofur, but no chlortetracycline saw an increase in tetracycline resistance prevalence for both tet(A) and tet(B) between day 0 and day 4 (5% to 25% for tet(A) and 5% to 35% for tet(B). The increased prevalence of tetracycline resistance did not persist past day 4 and by day 26, prevalence was roughly at the same value as day 0. Cattle receiving only chlortetracycline, but no ceftiofur saw an increase in tetracycline resistance prevalence for both tet(A) and tet(B) between day 4 and day 12 (20% to 35% for tet(A) and 15% to 60% for tet(B). Combined treatment with ceftiofur and chlortetracyclineresulted in an increase in resistance prevalence for both tet(A) and tet(B) between day 0 and day 4, as well as a second increase between day 4 and day 12 (30% to 50% to 60% for tet(A), 10% to 25% to
30% for $tet(B)$) (Figure 3-8C). While ceftiofur treatment more favorably selected for $tet(A)$ over $tet(B)$, whether or not chlortetracycline was co-administered, treatment with chlortetracycline alone favorably selected for $tet(B)$ over $tet(A)$ (Figure 3-8D).

**Growth curve parameter values**

In total, growth parameters for 1,056 bacterial isolates were determined from their growth curves. Because each isolate was grown in both plain MAC broth and MAC broth containing tetracycline, two sets of parameters were calculated for each isolate. Overall histograms of growth parameters for each broth are presented in Figures 3-9 and 3-10. While all isolates were capable of growing in plain MAC broth, not all isolates grew in MAC broth containing tetracycline. This resulted in both maximum slope and global $\Delta$OD distributions for the MAC+tet broth to have a strong zero inflation characteristic. However, aside from this inflation, growth parameters appeared reasonably normally distributed conditioned on them being capable of growing in the broth (Figure 3-10).

**Treatment effect on growth parameters**

While the treatments did not impact the $\Delta$OD parameter of sensitive and resistant isolates when grown in plain MAC broth (all had $\Delta$ODs around 1.5 with 95% CI’s completely overlapping), all treatments resulted in increased $\Delta$ODs for resistant isolates when grown in MAC broth containing tetracycline (Figure 3-11). As expected, the $\Delta$ODs for susceptible isolates when grown in MAC+tet broth were zero because the antibiotic inhibited any growth. The predicted increase in $\Delta$OD for resistant isolates grown in MAC+tet broth closely matches the dosing timeline with there being little overlap in 95% confidence intervals when a treatment effect was observed. *E. coli* recovered from cattle treated with ceftiofur on day 0 (0.48 [0.29,0.68]) exhibited an increase in $\Delta$OD on day 4 when grown in MAC+tet broth (1.23
E. coli recovered from cattle treated only with chlortetracycline exhibited an increase in ΔOD between day 4 (0.834 [0.70-0.97]) and day 12 (1.32 [1.22-1.43]). Administration of ceftiofur on day 0 and then chlortetracycline on day 4 resulted in a ΔOD pattern similar to cattle only treated with ceftiofur and the additional treatment with chlortetracycline did not further increase ΔOD values (day 0: 1.13 [1.00,1.25], day 4: 1.36 [1.25,1.46], day 12: 1.42 [1.32,1.52]).

Marginal predictions for the maximum growth rate followed a pattern similar to predictions for ΔOD (Figure 3-12 & Figure 3-13). Phenotypically resistant isolates recovered after animals were treated with antibiotics had a higher growth rates in broth containing tetracycline than those recovered pre-treatment. Like ΔOD, both tetracycline and ceftiofur treatment selected for isolates with higher growth rates, but dual administration with ceftiofur and tetracycline did not result in increased growth rates beyond day 4 of treatment with ceftiofur alone.

**Growth parameters based on tetracycline resistance genotype**

When collapsed across days and treatment, growth parameters differed based on which tetracycline genes were present (Figures 3-14 & 3-15). Most significantly, tet(C) exhibited a drastically reduced ΔOD and maximum growth rate (ΔOD: 0.16 [0.10,0.23]) compared to tet(A) (ΔOD: 1.34 [1.29,1.38]) and tet(B) (ΔOD: 1.40 [1.35,1.45]) with there being no overlap in 95% CIs. However, isolates harboring tet(C) and either tet(A) or tet(B) did not have reduced growth parameters compared to those with only tet(A) or tet(B). However, because isolates containing multiple tet genes were so rare, 95% confidence intervals were much wider for these genotypes. Interestingly, isolates harboring only tet(B) had higher maximum growth rate values than tet(A) when grown in MAC+tet (tet(B)= 0.23 [0.21,0.24], tet(A)=0.19 [0.18,0.20]). The
diminished growth parameter estimates for $tet(C)$ were partly due to a lower MIC compared to $tet(A)$ an $stet(B)$, but $tet(C)$ still had lower growth parameters even when phenotypically susceptible isolates were excluded from the model with there being no overlap in 95% confidence intervals (Figures 3-16 & 3-17).

**Discussion**

The primary goal of this project was to investigate whether treating an animal with either chlortetracycline or ceftiofur affected the relative fitness of commensal *E. coli* recovered throughout the treatment. Further, the growth parameters of isolates carrying any combination of $tet(A)$, $tet(B)$, or $tet(C)$ were compared. This aim of quantifying the effect of short-term antibiotic use on the ecological fitness of commensal bacteria using growth curves is unprecedented. However, before observed differences in growth parameters can be properly discussed, it is important to explore a few prior studies that gave rise to the hypotheses tested in this study.

Platt et al. (2008) (37) found that the selective effects of treating cattle with chlortetracycline on commensal *E. coli* resulted in a significantly increased likelihood to recover tetracycline resistant isolates from cattle treated with chlortetracycline (37). This increase was temporary and by 33 days post-treatment, the ratio of recovered sensitive and resistant bacteria returned to pre-treatment levels. Importantly, the levels of ceftiofur resistance were also measured and chlortetracycline treatment was shown to drastically reduce the likelihood of recovering ceftiofur resistant *E. coli* during the treatment period. Given that ceftiofur resistance is seldom found without a co-resistance to tetracycline, the paradoxical selection against ceftiofur resistance during chlortetracycline treatment suggested that the possession of ceftiofur resistance may result in a lower overall relative fitness compared to tetracycline resistant strains without ceftiofur resistance; that is, when grown in the presence of tetracycline.
In an unpublished follow-up study, simple growth curves were generated for all previously obtained isolates. Even without a more in-depth analysis by calculating specific growth parameters, there were several observations that allowed comment regarding the impact of chlortetracycline treatment on growth curves of commensal *E. coli*. Growth curves of isolates recovered post-treatment appeared to be roughly similar to pre-treatment isolates when grown in plain MacConkey broth (Figure 3-18). However, when grown in MacConkey broth containing tetracycline, growth curves of post-treatment isolates had a much tighter grouping pattern than pre-treatment and control group isolates (Figure 3-19). A reasonable hypothesis for these observations is that a variety of tetracycline resistance determinants in *E. coli* populations have relatively equivalent fitness in non-selective environments but may not be equally as fit to grow in environments containing tetracycline. When the cattle were treated with tetracycline, the most fit tetracycline genes may have dominated both susceptible isolates and resistant isolates that had lower fitness. This may explain the paradoxically reduced recovery of ceftiofur resistant bacteria from cattle treated with chlortetracycline despite ceftiofur resistance being strongly linked to tetracycline resistance.

Kanwar et al. (2013) explored whether chlortetracycline’s seemingly paradoxical negative selective potential against ceftiofur resistance could be used to reduce the selective effect of treatment with ceftiofur. The researchers ultimately found that chlortetracycline did not have the same effect found by Platt et al. (2008). Ultimately, further administering chlortetracycline following ceftiofur treatment only seemed to further select for ceftiofur resistant bacteria and further delayed the temporary window that ceftiofur resistance dominated the commensal *E. coli*. 
The growth curves from the Platt et. al. (2008) trial provided a framework to generate a general hypothesis regarding the selective effects of chlortetracycline treatment on the population dynamics of commensal *E. coli*. First, the initial passage of antibiotic through the gut would not only result in an expansion of resistant bacteria, but would also preferentially select for the fittest bacteria among the latent resistant populations present before selection. However, as the antibiotic leaves the animal, the fitness advantage of these dominating bacteria wanes and the population returns to its original equilibrium.

Under the assumption that the parameters estimated in this study are a surrogate of bacterial fitness, our results confirm this, at least in part. Indeed, both the global ΔOD and the maximum growth rate increased among the recovered resistant populations of *E. coli* following antibiotic treatment when grown in broth containing tetracycline. This increase closely corresponded to the dosing timeline and was followed by a slow return towards pre-treatment values. This rise and fall pattern was not specific to chlortetracycline treatment and also occurred with ceftiofur treatment as well.

Generalized estimating equation models of tet(A) and tet(B) prevalence following treatment presented in Kanwar et. al. 2013 suggested chlortetracycline does have favorably select for tet(B). This observation is in agreement with what was observed in Platt et. al. (2008) even though this favorable selection was unable to provide sufficient counter-selection against ceftiofur resistance in the Kanwar trial. However, both genes seemed to be equally capable of growing in MAC+tet broth with tet(B) only having a slightly higher maximum growth rate than tet(A). While the drastically lower growth parameters for tet(C) was no surprise, this resistance gene also was the only gene to have significantly reduced fitness in MAC broth without tetracycline.
The lack of any difference in growth parameters between resistant and susceptible isolates when grown in plain MAC broth may have an actual biological basis. Given that tetracycline efflux pump genes generally have tightly controlled regulators (6, 62), it is possible that the carriage of these genes do not carry a high fitness cost. Nguyen et. al. (1989) (62) actually attempted to test the fitness effect of Tn10 tetracycline-resistance operons by artificially introducing different elements into *E. coli* K12. They found that while induced and constitutive expression of the resistance protein did incur a fitness reduction, carriage of the plasmid itself had only a minimal impact on fitness. However, this fails to explain why resistant bacteria only transiently dominate the commensal community following a strong drug selection and after the pressure is removed, susceptible bacteria return. Instead, the lack of differences in growth parameters may be because of how the growth curves were generated. Keep in mind that the isolates were grown in a nutrient rich aerobic environment. While the growth parameters provide a surrogate measure of fitness, they are not all-encompassing. Given that energy production and conservation is much more important in anaerobic growth conditions, planktonic aerobic growth curves may not be an accurate representation of anaerobic fitness. In the case of using plain MAC broth, relative fitness differences (or lack thereof) seem especially non-representative in a biological sense. Therefore it doesn’t really make sense to equate growth in plain MAC to an intestinal environment without any antibiotic selection pressure. Ironically though, the observation that all isolates behaved very similarly in plain MAC broth may actually give more credence to the differences seen when grown in MAC+tet. This is because the differences can be more attributed to the antibiotic effect and not the broth itself. Examining growth curves of these isolates when grown in anaerobic conditions may reveal fitness differences to explain why
susceptible bacteria repopulate after a short-term antibiotic treatment in the case of ceftiofur and tetracycline resistance.

In conclusion, there does in fact seem to be some selection favored toward particular tetracycline genes at least when either tetracycline or ceftiofur is present. While the selection advantage of \textit{tet}(B) over \textit{tet}(A) is arguable, both of these genes clearly have a huge advantage over \textit{tet}(C). Suprisingly, ceftiofur is just as capable of selecting bacteria with a high fitness when grown in media containing tetracycline. Ultimately, the use of simple growth curves is unable to explain why the highly fit resistant bacteria cannot persist at high numbers and are ultimately replaced by susceptible bacteria as soon as the selection pressure is removed. This may be due to the methodology not being sensitive to smaller differences in fitness. Nevertheless, it is clear that a much better understanding of bacterial fitness will be required to fully explain the transient selection effect of antibiotic treatment.
Figure 3-1 Schematic diagram of the study design adapted from Kanwar et. al. 2013

88 steers were randomly allocated to one of four treatment groups divided into eight pens. The four treatment groups are as follows; all steers in pens 1 and 2 were treated with both ceftiofur and chlortetracycline, all steers in pens 3 and 4 were treated with ceftiofur and none received chlortetracycline, one steer in pens 5 and 6 were treated with ceftiofur and all steers were treated with chlortetracycline, one steer in pens 7 and 8 were treated with ceftiofur and none received chlortetracycline.
Ceftiofur treatment consisted of a 6.6mg/kg dose of Excede ceftiofur crystalline free acid subcutaneously on day 0. Chlortetracycline was top-dressed on feed (22mg/kg) in three 5-day periods starting on days 4, 10, and 16. Chlortetracycline treatment periods are shown in dark grey.
The custom TREK® NARMS plate tests for minimum inhibitory concentration values for 14 different antibiotics. Antibiotic concentration ranges (in µg/ml) encompass current CLSI breakpoints.
This is a simplified example of a monoauxic growth curve of bacteria grown in broth measuring the optic density over time. This illustrates the basic phases of the bacterial growth curve: lag phase, acceleration phase, exponential growth phase, stationary phase, and death phase.
This is a example of a more complicated diauxic growth curve in which bacteria goes through two cycles of phases. This is usually due to multiple sources of energy being present in the growth medium, but can also be due to many other factors.
Two different ATCC quality control strains were grown in plain MacConkey agar using a scheme of successive 1:2 serial dilutions. Each dilution was done in seven replicates. Each growth curve was fit using a modified Gompertz equation (compliments of Stephan Guillossou) for illustration purposes. Replicates had very little variation to the point that the curves stacked on top of each other. A change in inoculation concentration (dilution) affected neither the maximum slope nor the maximum OD reached but did increase the lag time.
Simple growth parameters were estimated from the entire bacterial growth curve. The maximum slope was estimated by recording the maximum increase in optic density over a single 10 minute interval. The global change in optic density (ΔOD) was estimated by taking the difference between the maximum and minimum optic densities recorded throughout the entire 24 hours observed.
Figure 3-8 Prevalence of tetracycline-resistant *E. coli* isolates, modeled as marginal predicted probabilities, over days adapted from Kanwar et al. 2013

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 4 treatment groups are: (A) CCFA administered to all steers within pens without subsequent CTC administration at pen level; (B) CCFA administered to 1 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 1 out of 11 steers within pens followed by CTC administered at pen level.

Results provided compliments of Neena Kanwar, Kansas State University
Histograms of the global difference in OD when stratified by broth demonstrate that overall, isolates capable of growing in the broth have a reasonably normal distribution. However, isolates susceptible to tetracycline create a strong zero inflation when grown in MAC+tet.
Histograms of the maximum slope when stratified by broth demonstrate that overall, isolates capable of growing in the broth have a reasonably normal distribution. However, isolates susceptible to tetracycline create a strong zero inflation when grown in MAC+tet.
The four sub-graphs represent modeled marginal predictions of the mean global change in optic density factored on treatment, broth, tetracycline resistance, and day. The two left graphs provide a comparison of fitness between susceptible and resistant isolates when grown in plain MAC. Both susceptible and resistant groups had a ΔOD around 1.5 with 95% CIs heavily overlapping when grown in plain MAC. The two right graphs provide a comparison of fitness between susceptible and resistant isolates when grown in MAC containing tetracycline. The upper right graph demonstrates that all phenotypically susceptible isolates had no change in ΔOD which accounts for the negative inflation seen in Figure 3-9. In the bottom right graph, the selective effect of ceftiofur and chlortetracycline appear to select for isolates with higher ΔOD. From day 0 to day 4, the mean ΔOD of resistant *E. coli* in cattle receiving only ceftiofur
increased from 0.48 [0.29, 0.68] to 1.23 [1.12, 1.34]. This increase between day 0 and 4 was not observed in cattle not receiving ceftiofur with no overlap in 95% confidence intervals. From day 4 to day 12, the mean ΔOD of resistant *E. coli* in cattle receiving only chlortetracycline increased from 0.83 [0.70, 0.97] to 1.32 [1.21, 1.43].
Figure 3-12 Marginal predictions of maximum slope based on animal treatment

The four sub-graphs represent modeled marginal predictions of the maximum slope factored on treatment, broth, tetracycline resistance, and day. The two left graphs provide a comparison of fitness between susceptible and resistant isolates when grown in plain MAC. The two right graphs provide a comparison of fitness between susceptible and resistant isolates when grown in MAC containing tetracycline. The upper right graph demonstrates that all phenotypically susceptible isolates had no change in OD which accounts for the negative inflation seen in Figure 3-10. The bottom right graph is enlarged and presented more in-depth in Figure 3-13.
Marginal predictions of the maximum slope factored on treatment, broth, tetracycline resistance, and day for phenotypically tetracycline resistant isolates have been enlarged to better show the treatment effect on resistant isolates as well as the clear separation in 95% confidence intervals. Marked increases in mean maximum slope correspond closely to the treatment timeline. Ceftiofur injections were given on day 0 and *E. coli* recovered from cattle receiving ceftiofur had significantly higher maximum slopes on day 4 when ceftiofur would have had full effect (p<0.05). Likewise, chlortetracycline treatment began on day 4 and *E. coli* recovered from cattle receiving chlortetracycline had significantly higher maximum slopes on day 12 when chlortetracycline would have had full effect (p<0.05).
Figure 3-14 Marginal predictions of ΔOD based on tetracycline genotype

Modeled marginal predictions of the global change in optic density were factored by tetracycline genotype and broth. All isolates were included in this analysis including phenotypically sensitive isolates. However, not all isolates that tested positive for a tetracycline resistance gene also tested phenotypically resistant to tetracycline (MIC≥16µg/ml).
Figure 3-15 Marginal predictions of maximum slope based on tetracycline genotype

Modeled marginal predictions of the maximum slope were factored by tetracycline genotype and broth. All isolates were included in this analysis including phenotypically sensitive isolates. However, not all isolates that tested positive for a tetracycline resistance gene also tested phenotypically resistant to tetracycline (MIC≥16µg/ml).
Figure 3-16 Marginal predictions of ΔOD based on tetracycline genotype (only phenotypically resistant isolates)

Modeled marginal predictions of the global change in optic density were factored by tetracycline genotype and broth. Only phenotypically resistant isolates (MIC≥16µg/ml) were included in this analysis.
Modeled marginal predictions of the maximum slope were factored by tetracycline genotype and broth. Only phenotypically resistant isolates (MIC≥16µg/ml) were included in this analysis.
Figure 3-18 Unpublished Growth curves of isolates recovered from Platt et. al. 2008 when grown in plain MAC broth (provided by Dr. Javier Vinasco, Kansas State University)

Analysis provided compliments of Javier Vinasco, Kansas State University
Figure 3-19 Unpublished Growth curves of isolates recovered from Platt et. al. 2008 when grown in MAC broth containing tetracycline (16µg/ml) (provided by Dr. Javier Vinasco, Kansas State University)

Analysis provided compliments of Javier Vinasco, Kansas State University
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Expected Product Size (bp)</th>
<th>GenBank Accession no.*</th>
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<td><em>bla</em>CMY-2</td>
<td>585F</td>
<td>5’- CAG ACG CGT CCT GCA ACC ATT AAA -3’</td>
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<td>AB212086</td>
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<td>1038R</td>
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<td>tet(B)(F)</td>
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<sup>a</sup>Primer set used is from Alali et. al. (2009) (72)

<sup>b</sup>Primer set used is from Ng et. al. (2001) (49)

*Sequence used for primer design
### Table 3-2 Distributions of minimum inhibitory concentrations of 1,050 *E. coli* isolates to NARMS antibiotic panel

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<th>NARMS Code</th>
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<th>% Resistant</th>
<th>95% CI</th>
<th>Distribution of MICs in µg/ml (%)</th>
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<td></td>
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<td>2.17-4.39</td>
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</tr>
<tr>
<td>STR</td>
<td>Streptomycin</td>
<td>61.14</td>
<td>58.12-64.10</td>
<td>61.14 1.05 5.33 0.48 0.10 0.00 0.10 3.05</td>
</tr>
<tr>
<td>TET</td>
<td>Tetracycline</td>
<td>22.95</td>
<td>20.44-25.62</td>
<td>4.86 18.38 47.43 2.10 1.14 3.14 9.43</td>
</tr>
</tbody>
</table>

* A indicates that the distribution is not statistically different from 0%

** Sulfamethoxazole** indicates that the distribution is not statistically different from 0%
Chapter 4 - Final Conclusions and Implications

Conclusions

The core objective of this thesis were to explore and identify epidemiological properties of tetracycline and ceftiofur resistant commensal *E. coli* present in the intestines of cattle. Both ceftiofur and tetracycline were focused on not only because of their medical importance, but also because both drugs were hypothesized to be undergoing evolutionary patterns that could be identified in the two experiments presented. Cattle were focused on partially to fill in the research gap regarding AMR in cattle when compared to more heavily researched animal agricultural systems such as pork and poultry, but also because tetracycline and ceftiofur resistance are not currently as widespread in cattle.

In the first experiment presented, the aim was to determine basic epidemiological properties of tetracycline and ceftiofur resistance in the Canadian beef production system beyond what the current CIPARS surveillance program is capable of. This was done by using selective media containing tetracycline and ceftiofur. This allowed for the estimation of both an animal and isolate level prevalence for both ceftiofur and tetracycline resistance. The hypothesis was that ceftiofur resistance was present in cattle at levels below the detection threshold of the isolate level surveillance being done by CIPARS. Ultimately, ceftiofur resistance was not found to be clandestinely present below current detection limits and had a low animal level prevalence that did not change from 2002 to 2011 (<4% of caecal samples). Tetracycline resistance however did have a higher animal level prevalence compared to the isolate level prevalence (74%-90% for the animal level prevalence and 28% for the isolate level prevalence). Furthermore, while the isolate level prevalence did not increase over the decade, the animal level prevalence rose between 2009 and 2011.
In addition to investigating phenotypic resistance, the first study dove deeper and looked at the genotypic variation in tetracycline genes by testing all isolates for four resistance genes commonly found in *E. coli* (*tet(A)*, *tet(B)*, *tet(C)*, and *tet(E)*). While *tet(A)*, *tet(B)*, and *tet(C)* were found, *tet(E)* was not detected in any of the isolates. When the relative abundance of tetracycline genes was tested to see whether there was change over the decade, it was found that the relative proportions of genes did not change. However, the usage of selective agar was found to bias genes recovered and result in much higher proportions of isolates harboring *tet(B)*. When *tet(A)* and *tet(B)* were tested for correlation to multidrug resistance, *tet(A)* was found to have a higher association with multidrug resistance phenotypes. In particular, *tet(A)* was specifically associated with chloramphenicol resistance which warrants further molecular investigation.

In the second study presented, the aim was to see whether the growth curves of *E. coli* recovered from cattle undergoing antimicrobial treatment would explain the transient rise and fall of AMR observed in commensal bacteria during an antimicrobial treatment regimen. Two core hypotheses to explain this were that resistant isolates were more fit in the presence of antimicrobials, but had reduced fitness when the antimicrobial was absent. A supporting hypothesis was that even among different tetracycline genes, particular genes incurred lower fitness costs than others.

While it was not surprising that tetracycline resistant bacteria had growth parameters that were higher than susceptible bacteria when grown in the presence of tetracycline, it was interesting that treating cattle with tetracycline or ceftiofur resulted in selecting among resistant bacteria for those with higher growth parameters when grown in the presence of tetracycline. More importantly, when looking at the mean growth parameters of different tetracycline resistance genotypes, *tet(B)* was found to be more fit than *tet(A)* and both were more fit than
This finding is in agreement with how tetracycline genes appeared to be selected for in vivo when cattle were treated with chlortetracycline in Platt et. al. 2008 as well as Kanwar et. al. 2013. Furthermore, this was also in agreement with the agar bias effect observed in the first study.

There are several unexplained findings from these experiments that should be further explored. First, the strong association between tet(A) and chloramphenicol resistance has yet to be described in literature. Given that chloramphenicol is not used in veterinary medicine, the link between the two is particularly perplexing. Also, it was not clear why multiple tetracycline genes maintained stable relative proportions over the decade studied, especially given that the genes were determined to not have equal fitness values. One potential realm of explanation may lie in environmental pressures that ultimately play a much larger role in the selection of resistance in the long-term rather than the short-term selection provided by antimicrobial treatment.

Nevertheless, the results from both experiments have served to further current knowledge of AMR in cattle and provide mortar for the founding of new hypotheses to continue the ever ongoing scientific process.
References


32. **System NARM.** 2011. NARMS retail meat annual report (Online).


