The Impact of Ceftiofur Removal on Recovery of Salmonella enterica and Escherichia coli Resistant to Third Generation Cephalosporins

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Katie Elizabeth Kleinhenz, B.S., D.V.M.

Graduate Program in Veterinary Preventive Medicine

The Ohio State University

2012

Master's Examination Committee:

Dr. Thomas Wittum, Advisor

Dr. Armando Hoet

Dr. Päivi Rajala-Schultz
Abstract

Ceftiofur use in animal agriculture has been disputed due to the concern over the impact it may be having on the spread of resistance genes among foodborne pathogens. Several attempts to restrict the extra label use of ceftiofur have resulted in failure until the recent FDA ban. No study has been performed to see what impact ceftiofur removal would have on the reduction of third generation cephalosporin resistance mediated by the β-lactamase gene, \( \text{bla}_{\text{CMY-2}} \), among \textit{Salmonella enterica} and \textit{Escherichia coli} organisms.

The purpose of our study was to establish a baseline prevalence of third generation cephalosporin resistance among commensal \emph{E. coli} organisms as well as the prevalence of \textit{Salmonella enterica} among livestock populations and their represented meat products. Following the baseline establishment, ceftiofur use was discontinued to simulate a ban on the drug use, and the organisms of interest were monitored to determine the impact on the resistance genes among the enteric microflora. Monthly fecal samples were taking from select groups of livestock, and weekly ground beef and pork samples were collected.

At the conclusion of the study, the removal of ceftiofur did not impede our ability to culture third generation cephalosporin resistant \emph{E. coli} organisms from the studied livestock populations. With regards to the fresh ground meat products, organisms
phenotypically resistant to third generation cephalosporins infrequently contaminate these products.
Dedicated to my dear husband, Michael D. Kleinhenz, D.V.M.
Acknowledgments

I wish to thank my husband, Mike Kleinhenz, for his unwavering support and encouragement through this whole process. Mike, you never questioned if I was going to finish this degree, and you were always there cheer me on when I felt like quitting. Thank you and I love you!

I want to thank my parents. My mother always promoted education and pushed me to achieve academic success. My father taught me that I could do anything I want to do if I worked at it. You guys showed me the value of hard work and an education. I hope to pass these values on to my children.

I would like to thank my daughter, Harriet. You came into my life and made me realize that there is more to life than myself. I hope you learn to handle what life hands you and always finish what you start.

To my advisor, Dr. Tom Wittum, I appreciate your patience and constant reinforcement that this was not the worst graduate work in your vast experience. It meant a lot to me that you were willing to help me finish something that was so difficult for me. And to the
remainder of my committee members, Drs. Armando Hoet and Päivi Rajala-Schultz, I appreciate your time and assistance with my thesis and defense.

Many thanks go to my good friend and partner in crime, Dixie Mollenkopf. We shared many adventures at the Ohio Department of Rehabilitation and Corrections farms collecting data for this study. But most of all, you always bailed me out of whatever jam I was in with regards to posters, powerpoints, and editing. You always made my presentations look like a million bucks. Thanks!

Luke Heider, you were always there to help with sample collection and processing. My favorite was the fact that you always laughed at my jokes and ridiculous views and opinions.

Finally, to my sister, Lisa, it was your clever use of reverse psychology that truly fueled me to finish. Your attempt to make me believe that you didn’t think I could finish made me realize that it was time. I will never forget the kindness you have shown me throughout the years.
Vita

May 6, 1980 ...................................................Born – Saint Marys, Ohio

2002.................................................................B.S. Food, Agriculture and Environmental
Science, The Ohio State University

2002-2005 ........................................................Herdsman, Canal View Holstein Farm, Saint
Marys, Ohio

2005-2008 ........................................................Research Assistant, Dept. of Veterinary
Preventative Medicine, The Ohio State
University

2012.................................................................D.V.M, The Ohio State University

Publications

Mollenkopf DF, Kleinhenz KE, Funk JA, Gebreyes WA, Wittum TE. Salmonella
enterica and Escherichia coli Harboring bla CMY in Retail Beef and Pork
Products. Foodborne Pathogens and Disease 2011;8:333-6.

Fields of Study

Major Field: Veterinary Preventive Medicine
# Table of Contents

Abstract ......................................................................................................................................... ii

Dedication .................................................................................................................................... iv

Acknowledgments .......................................................................................................................... v

Vita ............................................................................................................................................... vii

List of Tables ................................................................................................................................. vii

List of Figures ................................................................................................................................. xi

Chapter 1: Review of the Literature Relate of β-Lactamase Mediated Antimicrobial Resistance Throughout the Food Chain .............................................................................................................. 1

  Cephalosporins .......................................................................................................................... 4

  Ceftiofur Use in Livestock ......................................................................................................... 7

  Enzymatic Inactivation of Cephalosporins ............................................................................ 9

  AmpC ......................................................................................................................................... 10

  Extended Spectrum β-lactamase .............................................................................................. 12

  Differences Between AmpC and ESBL .................................................................................. 13
List of Tables

Table 1. The proportion of fecal and ground meat samples positive for *Salmonella* spp. and *E. coli* resistant to third generation cephalosporins recovered from two farms in Ohio................................................................................................................................... 53

Table 2. The average number of dairy cows treated with a particular antibiotic per month per herd during the three phases of antimicrobial usage on two farms in Ohio. ............... 54

Table 3. The 8 biotypes of *E. coli* isolates recovered from coarse ground meat and livestock fecal samples collected from two farms in Ohio. ....................................................... 55

Table 4. Number of antimicrobial treatments administered to beef, dairy, or swine populations during the each phase of the study on two farms in Ohio. ............................... 55
List of Figures

Figure 1. Dairy cattle *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio.......................................................... 56

Figure 2. Dairy cattle *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio......................................................................................... 57

Figure 3. Beef cattle *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio.......................................................... 58

Figure 4. Beef cattle *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio......................................................................................... 59

Figure 5. Swine *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio.............................. 60

Figure 6. Swine *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio................................................................................................. 61
Chapter 1: Review of the Literature Relate of β-Lactamase Mediated Antimicrobial Resistance Throughout the Food Chain

Antimicrobial drug use in food animals has raised concern regarding the emergence of antimicrobial resistance among foodborne pathogens.\textsuperscript{1} Research has shown an association between antimicrobial drug use and the selection and dissemination of bacterial organisms harboring resistant genes, chromosomal or acquired, within livestock populations.\textsuperscript{2,3} The Food and Drug Administration (FDA) is responsible for publishing guidelines for antimicrobial drug use in food animals as well as publishing mandatory slaughter withdrawal periods for each drug to ensure tissue residue levels fall below FDA published safety levels. While the tissue residues may fall below the acceptable residue levels, the effect of antimicrobial drug treatment could result in the shedding of resistant enteric bacteria, which may result in the spreading of resistant pathogens from the culled carrier to the other animals coming into contact with the carrier. During harvest, carcasses have the potential to become contaminated with pathogens via evisceration or hide removal as well as airborne due to the plant’s airflow patterns.\textsuperscript{4,5}

Since the discovery of antibiotics in the early twentieth century, the benefits of antimicrobial drug use in both human and veterinary medicine have resulted in a decrease
for both morbidity and mortality associated with diseases. Despite these advantages antimicrobials afforded the populations in which they are used, there were certain drawbacks associated with their use with the emergence of resistant organisms leading to a loss of treatment efficacy, and researchers have concluded antimicrobial resistant bacterial infections have contributed significantly to both increased medical costs and societal costs as well as an increase in the duration of affected individuals’ hospital stay.

The emergence of resistant organisms within livestock populations has been attributed to the presence of bacteria naturally carrying genetic elements, which allow them to survive exposure to antimicrobial drugs. Mechanisms of antimicrobial resistance have been found in most species of bacteria prior to the antibiotic era in the form of heavy metal efflux pumps. Retrospective studies have shown that bacteria were harboring antibiotic resistance genes prior to the therapeutic use of antibiotics in both human and veterinary medicine. Studies have shown these resistance genes from the pre-antibiotic era to be both conjugatively and non-conjugatively spread to other bacteria. Certain interest groups believe the use of antibiotics in agricultural settings is the main contributing factor to the issues surrounding antimicrobial resistance in human health today. Regardless of using antimicrobial either therapeutically or subtherapeutically, selection pressure is placed on the microbial population of the treated individual resulting in the surfacing of antimicrobial drug resistance. Once treatment is administered to an animal, the microbial population of its intestinal tract may be altered, and the susceptible organisms
will be reduced while resistant organisms thrive. In the absence of antimicrobial agents, the susceptible organisms flourish and out-compete the resistant organisms due to the higher metabolic cost associated with maintenance of large resistance genes.

During the 1990’s, the European Union (EU) used avoparcin, a glycopeptide antibiotic, as a growth promoting feed additive in cows, pigs, chickens, turkeys, sheep, and goats. European scientists were concerned avoparcin use in livestock was resulting in an increase in vancomycin-resistant *Enterococci* (VRE) isolates found among human asymptomatic carriers.\textsuperscript{15} This association between the food chain and human resistance was only a theory and no proof has been found to actually link the two together. Public pressure resulted in the discontinuation of avoparcin for growth promotion rather than scientific evidence.\textsuperscript{15} Witte et al. suggested animal agriculture’s use of avoparcin as being responsible for the dissemination of the vancomycin resistance gene, vanA, among the individuals who consumed meat products from treated animals.\textsuperscript{16} This finding led to the EU’s 1997 decision to ban avoparcin use in food animal populations. Prevalence data for VRE in humans went from 12\% in 1994 to only 3\% in 1997 following the avoparcin ban.\textsuperscript{17} These data seemed to suggest the ban of avoparcin was responsible for the sharp decline in prevalence of VRE in humans; however, Klare et al. also reported the prevalence for 1996 to be 6\%, which suggests a decline may have been occurring before the ban was in effect. Despite the removal of avoparcin from animal feeds, VRE still persist in poultry populations that were never feed avoparcin but were reared in houses where avoparcin was previously used.\textsuperscript{18} In addition to its use in the EU, avoparcin was
also used as a feed additive in Costa Rica,\textsuperscript{19} Korea,\textsuperscript{20} Taiwan,\textsuperscript{21} and New Zealand.\textsuperscript{22} Countries where avoparcin was used as a growth promoting feed additive for livestock report VRE colonization of the intestinal flora of individuals living in those countries, although the incidence of serious VRE infections is low. However, in countries like the United States where avoparcin has never been approved for use as a livestock feed additive, VRE has not been reported to colonize the intestinal tract of individuals but has been associated more with serious nosocomial infections.\textsuperscript{23} While VRE infections occurring in countries that used avoparcin as a feed additive has been associated with animal agriculture, the VRE currently affecting US hospitalized patients likely arise in healthcare environments. There is a difference among the VRE organisms found in the USA and EU. The VRE found among humans in Germany and Denmark were reported as not being of clinical significance whereas the VRE commonly associated with the USA has been shown to have clinical significance, and the use of glycopeptide antibiotics in the USA hospitals is substantially more common than EU hospitals, which may contribute to the differences among VRE in the USA and EU.\textsuperscript{15}

Cephalosporins

The cephalosporin family of antimicrobial drugs is important in both human and veterinary medicine. In human medicine, cephalosporins are commonly prescribed for the treatment of infections associated with the respiratory tract, skin, gastrointestinal tract, as well as joint and bone infections. Ceftriaxone, a third generation cephalosporin, is an important drug for children 12 years old and under. Ceftriaxone is used for the
treatment of systemic *Salmonellosis* in children where fluoroquinolones, which are used in adults to treat systemic *Salmonellosis*, are not recommended for use in children under the age of 12 because of the toxic effects fluoroquinolones have on this age group.\(^{24,25}\)

Annually in the US, an average of 1.4 million non-typhoidal *Salmonellosis* cases occur with the vast majority of these cases originating from food,\(^{26}\) and children infected with *Salmonellosis* account for roughly one third of the all cases (http://www.cdc.gov/ncezid/dfwed/PDFs/SalmonellaAnnualSummaryTables2009.pdf).

In veterinary medicine, multiple cephalosporin drugs are approved for use in both companion and food animals. In food animals, cephalosporins are commonly used for the treatment of mastitis, respiratory tract infections, foot rot, and metritis in cattle and for respiratory infections in swine. Ceftiofur is the only third generation cephalosporin approved for use in food animals. There is growing concern regarding ceftiofur use in food animal populations leading to the foodborne transmission of third-generation cephalosporin resistant enteric pathogens.

Since the introduction of its use in food animal medicine, the prevalence of ceftiofur resistant organisms within livestock population has been increasing.\(^{27}\) Ceftiofur resistance is frequently the result of an AmpC \(\beta\)-lactamase gene that originated from *Citrobacter freundii*.\(^{28-30}\) A transposon transferred the chromosomal encoded resistant gene known as \(bla_{\text{CMY}-2}\) to a mobile plasmid, and this plasmid has been observed in Gram-negative bacteria including *E. coli* and *Salmonella* spp.\(^{31}\) In addition to ceftiofur resistance, \(bla_{\text{CMY}-2}\) confers resistance to ceftriaxone and other third-generation
cephalosporins, presenting an important public health concern. Fortunately, the frequency of ceftriaxone-resistant pathogens recovered in diagnostic laboratories is low.\textsuperscript{32}

Cephalosporin resistance encoded by the $bla_{CMY-2}$ gene facilitates the hyper production of a $\beta$-lactamase enzyme among microorganisms harboring this gene.\textsuperscript{24, 33} Over the past decade, the emergence of $Salmonella enterica$ strains carrying genes encoding for $\beta$-lactamase production, have raised concern in public health, especially to children under 12 years of age.\textsuperscript{25} The use of ceftiofur in livestock populations is hypothesized to be the culprit of this emergence of ceftriaxone resistance; however, there is no concrete evidence tying ceftiofur use to the emergence and dissemination of the ceftriaxone resistant $Salmonella$ spp. In 2000, case report was published documenting how a young child’s indirect exposure to a ceftriaxone resistant $Salmonella enteric$ serotype Typhimurium as a result of the child’s father treating calf diarrhea with ceftiofur.\textsuperscript{25} Scientists reported an association between ceftiofur use and the presence of the $bla_{CMY2}$ gene within a herd of dairy cattle but failed to find an individual affect on cows recently treated with ceftiofur.\textsuperscript{3} In contrast, Heider et al. found no association with ceftiofur use and the recovery of $E. coli$ harboring the $bla_{CMY2}$ gene at the herd level, but instead, found an association between recovering the $bla_{CMY2}$ gene and the recovery of $Salmonella$ spp. within a farm.\textsuperscript{34} This link could be a result of extra label drug use under the direction of a licensed veterinarian for the treatment of clinical $Salmonellosis$ in cattle.
Ceftiofur Use in Livestock

Ceftiofur is the only third generation cephalosporin approved for use in veterinary medicine, and it is the most commonly used in lactating dairy cattle. The parental formulas of the drug do not cross the blood-udder barrier, which reduces the risk of antimicrobial drug residues entering the milk supply, and ceftiofur is a broad spectrum antibiotic with good action against most Gram negative organisms as well as some Gram positive species. It is approved for the treatment of respiratory infections, metritis, mastitis, and foot rot. Research has shown treatment of cattle with ceftiofur can select for enteric *E. coli* resistant to third generation cephalosporins as well as stimulate rapid shedding and dissemination of these resistant *E. coli* organisms.\(^2\) Since the introduction of ceftiofur use in food animal medicine, ceftiofur-resistant organisms have been increasing over time in food animal populations. From 1999 to 2003, ceftiofur resistance among National Antimicrobial Resistance Monitoring System (NARMS) *Salmonella* spp. isolates went from 4% to 18.8%.\(^{27}\) If these resistant organisms enter the food supply, any resulting human infections treated with ceftriaxone would be at risk of treatment failure.

All *Salmonella* serotypes are considered pathogenic to mammals. While any serotype is capable of being multidrug resistant, two serotypes have consistently been associated with multidrug resistance, and those serotypes are *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Newport. Both serotypes have been shown to harbor the AmpC plasmid, which confers resistance to ceftriaxone,\(^{35}\) and recently, evidence has suggested *Salmonella enterica* serovar Typhimurium may also
have a chromosomal cephalosporin gene.\textsuperscript{36} \textit{Salmonella enterica} serovar Newport is thought to primarily inhabit cattle populations, and most human epidemics involving \textit{Salmonella enterica} serovar Newport have been associated with direct contact with dairy cattle or consumption of raw or undercooked animal products suggesting cattle to be the reservoir for human infections.\textsuperscript{37}

Over the past several years, an increase in multidrug resistant (MDR) \textit{Salmonella} Newport in the United States has served as an example of the rapid emergence and dissemination of third generation cephalosporin resistant organisms.\textsuperscript{38} \textit{Salmonella enterica} serovar Newport has been recovered from humans, cattle, swine, and chickens supporting the hypothesis that the use of ceftiofur in food animals could result in the zoonotic foodborne spread of ceftriaxone-resistant pathogens.\textsuperscript{39} Frye \textit{et al.} found 2.4\% of the \textit{Salmonella enterica} isolates recovered at slaughter resistant to ceftriaxone, and of those isolates, 58\% were S. Newport.\textsuperscript{27} This showed that multidrug resistant pathogens do enter the food chain, but the observed frequency of the pathogens resistant to ceftriaxone was relatively low.

In 2002, the Centers for Disease Control and Prevention (CDC) performed an investigation of a \textit{Salmonella} Newport outbreak in humans involving five states. At the same time, they observed a similar \textit{Salmonella} Newport outbreak among dairy cattle populations within the same geographic region. The investigation found afflicted individuals had direct contact with dairy farms, sick cattle, undercooked ground beef, and
unpasteurized dairy products. This outbreak was significant not only because of its epidemic nature but because ceftriaxone-resistant Salmonella were first reported in the US only four years earlier. The presence of MDR Salmonella spp. carrying mobile plasmids encoding for ceftriaxone resistance coupled with their epidemic spread over a four year period was alarming to public health professionals.

Enzymatic Inactivation of Cephalosporins

β-lactamase are enzymes capable of breaking the β-lactam ring, which inactivates β-lactam antibiotics such as penicillins, cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors like clavulanate. These enzymes can be induced either chromosomally or by a mobile plasmid. In general, Gram negative organisms including E. coli and Klebsiella spp. contain areas on their chromosomes encoding for β-lactamase production, but the use of β-lactam antibiotics do not result in increased production of these naturally occurring β-lactamase enzymes. Therefore, these organisms cannot produce enough β-lactamase enzyme to overcome the pharmacological effects of this class of antibiotic. Gram negative organisms require the incorporation of a mobile plasmid carrying additional genetic material encoding for mass β-lactamase production to be resistant. Applying selection pressure in the form of a broad spectrum β-lactam antibiotic to a population of microorganisms can select for organisms containing the gene which encodes for β-lactamase enzymes production. Organisms harboring β-lactamase enzymes have the potential to result in treatment failures if they are able to establish infections among humans.
AmpC

The AmpC β-lactamase genes encode for enzyme production and can be incorporated onto the bacteria’s chromosome by the acquisition of a mobile plasmid carrying this gene. Further study of plasmids have revealed several different types of AmpC β-lactamase genes which include ACC, ACT, CMY, DHA, FOX, LAT, MIR, and MOX, each of which can be further classified into gene subtypes. The largest family of the plasmid-encoded AmpC β-lactamase is the CMY family, which currently has 43 different recognized alleles. The CMY plasmid-borne genes were found to be related to the chromosomal AmpC β-lactamase. The plasmid-encoded AmpC β-lactamase CMY-2 (bla_{CMY-2}) is the most common CMY gene found globally. The bla_{CMY-2} gene was found to originate from the chromosomal-mediated AmpC β-lactamase on *Citrobacter freundii*. 

Third generation cephalosporins are effective at killing *E. coli* and *Klebsiella* spp. carrying a chromosomal AmpC β-lactamase gene because the organism cannot produce enough enzyme to counteract the effects of the drug. However, organisms harboring the bla_{CMY-2} gene on a mobile plasmid have the ability to produce enough enzymes to overcome the third generation cephalosporin because the gene encodes for the hyperproduction of β-lactamase. With the plasmid inducing hyperproduction, the bacterium can generate enough β-lactamase to inhibit β-lactam activity.
The origin of the mobile plasmid is from a *Citrobacter freundii* in which a transposable element was able to transfer the genetic material from the chromosome to the mobile plasmid. The mobile plasmid is then able to be spread via horizontal transfer from *Citrobacter* to other bacterial species, particularly Gram negative species. The $bla_{CMY-2}$ gene is commonly found among *E. coli* and *Salmonella* spp. including *Salmonella enteric* serovar Newport.

$CMY-2$. The $bla_{CMY-2}$ gene is currently the most prominent of the AmpC $\beta$-lactamase genes as well as the most widely distributed geographically. The $bla_{CMY-2}$ gene is located on specific transposon-like element, which facilitates the spread of the gene among Enterobacteriaceae. In ruminants, the transfer of the $bla_{CMY-2}$ gene among bacterial species has been shown to be facilitated by certain rumen protozoa.

Over the past decade, the $bla_{CMY-2}$ gene has been observed with increasing frequency among samples from a variety of animal species. In companion animals, dogs have been the source for the nosocomial spread of organisms harboring $bla_{CMY-2}$ in animal hospitals. In food animal populations, $bla_{CMY-2}$ is commonly found among *E. coli* and *Salmonella* spp. isolates from multiple species. Plasmids harboring $bla_{CMY-2}$ can be exchanged between *E. coli* and *Salmonella* spp. via conjugation. Winokur et al. (2001) provided evidence suggesting $bla_{CMY-2}$ could be passed from animals to humans. In addition, NARMS data shows an increase of $bla_{CMY-2}$ found among *Salmonella enterica* serovar Newport isolates. Recently, a Canadian study showed evidence of
plasmids with similar genetic fingerprints circulating in both cattle and humans, which supports the hypothesis that food animals can be a source of multidrug resistant pathogens for humans or vice versa.

As a consequence of the increase in third generation cephalosporin resistance among foodborne pathogens, the use of ceftiofur in food animal medicine has been challenged. The \( bla_{\text{CMY-2}} \) located on \( Salmonella enterica \) serovars Newport and Typhimurium confer multidrug resistance and are considered to be an important threat to the public health. Therefore, it is critical to fully understand the impact ceftiofur use in veterinary medicine has on the spread of the \( bla_{\text{CMY-2}} \) among pathogenic bacteria. This would allow for the development and implementation of appropriate intervention strategies to reduce the potential for zoonotic spread of resistant pathogens. The removal of ceftiofur use from livestock populations has been proposed as a potential food safety intervention to protect public health, and extra label use of ceftiofur has currently been modified. However, data describing the effectiveness of removing ceftiofur use from animal agriculture as an intervention for reducing the dissemination of resistant organisms and resistance genes are not available.

**Extended Spectrum \( \beta \)-lactamase**

Extended-spectrum \( \beta \)-lactamases (ESBL) are the result of a different plasmid-mediated resistance gene, which encodes for a different type of enzyme than the AmpC genes. The ESBLs confer varying degrees of resistance based on the specific family of genes. These
β-lactamase enzymes have β-lactam hydrolyzing capabilities, but the rate at which the enzymes are able to hydrolyze β-lactams varies. Slow hydrolyzing enzymes are not able of generate enough enzyme to effectively reverse the effects of the β-lactam antibiotics, but the faster hydrolyzing enzymes allow for quicker enzyme activity resulting in higher resistance. The most commonly studied ESBL families are the TEM, SHV, OXA, and CTX-M families. The TEM and SHV families are capable of hydrolyzing the group of β-lactams containing an oxyimino-aminothiazole compound, but unlike the AmpC bla<sub>CMY</sub>-2, these groups are also unable to hydrolyse both the alpha-methoxycephalosporin group and the imipenem groups. CTX-M enzymes are of particular importance because these enzymes are currently increasing among Salmonella spp.<sup>54</sup>,<sup>55</sup>

**Differences Between AmpC and ESBL**

There are four major areas that allow for the phenotypic differentiation between the AmpC enzymes and the ESBL enzymes: cephalosporin generation resistance, cephalosporin generation susceptibility, target populations, and the origin. AmpC enzymes are resistant to second and third generation cephalosporins as well as the β-lactam inhibitor, clavanic acid; however, AmpC enzymes are susceptible to fourth generation cephalosporins. Animals are commonly the source of the AmpC enzymes with the exception of Europe and Asia. The origin of the AmpC mobile plasmid was from a Citrobacter freundii.<sup>28</sup>
In contrast, ESBL enzymes are resistant to first, third and fourth generation cephalosporins but are typically susceptible to second generation cephalosporins. The most common source of ESBL enzymes is thought to be for humans through nosocomial infections; however, there is evidence of these enzymes starting to appear in livestock populations.54

Reservoir for CMY-2 Resistance

Commensal enteric organisms have the potential to act as a reservoir for the bla\textsubscript{CMY-2} gene due to the gene’s location on a mobile plasmid capable of spreading to other Gram negative organisms via horizontal transfer.56 Previous work done by researchers at Iowa State University found both \textit{E. coli} and \textit{Salmonella} isolates containing genetically identical resistance plasmids, which is potential evidence for commensal organisms acting as a reservoir for resistance to the intestinal microflora.42 Poppe \textit{et al}. (2005) demonstrated the ability of \textit{Salmonella enterica} serovar Newport to acquire multidrug resistant plasmids via conjugation from commensal intestinal \textit{E. coli} in turkey poults.57 Poults were inoculated with both a known antimicrobial susceptible \textit{Salmonella enterica} serovar Newport strain and an \textit{E. coli} harboring a multidrug resistant plasmid. The inoculant strain of \textit{E. coli} harboring the plasmid was able to share its plasmid not only with \textit{S. Newport} but also with other commensal \textit{E. coli} within the gastrointestinal tracts of the poults. From the results of this study, one could speculate the same transfer could occur in the gastrointestinal flora of humans.
Currently, no research has shown the use of antimicrobial drugs other than third gener

However, a study was published evaluating screening methods for MDR *Salmonella* spp. and MDR AmpC *Salmonella* spp., and the use of chloramphenicol in screening media was suggested to be effective at selecting for both types of *Salmonella* spp. including those resistant to ceftiofur. This research may help in providing information as to how ceftiofur resistant organisms are maintained in a microbial population once the therapeutic use of ceftiofur is removed. While the use of other antimicrobial drugs may not be actively selecting for the *bla*<sub>CMY-2</sub> gene, their use may be responsible for maintaining the plasmid when ceftiofur use is restricted.

**Epidemiology of CMY-2**

*E. coli* isolates resistant to third generation cephalosporins can be recovered from livestock populations regardless ceftiofur use; however, the recovery rates are much lower in cattle herds reporting no ceftiofur use as compared to herds reporting ceftiofur use. In general, *E. coli* isolates resistant to third generation cephalosporins are commonly recovered from dairy cattle herds regardless of ceftiofur use status. Associations with the recovery of resistant *E. coli* include the presence of *Salmonella* spp. on the farm and the individual farm’s level of milk production. As milk production increases, the odds of recovering resistant *E. coli* also increase.

A preliminary study performed in Dr. Thomas Wittum’s research laboratory looked at the effect of ceftiofur use on the intestinal microflora of both treated and untreated animals as
a group. Fifteen mature beef cows were penned together, and ten of the fifteen received a 2.2 mg/kg dose of ceftiofur. Prior to ceftiofur administration, fecal samples were collected from all fifteen animals and screened for *E. coli* using both non-selective media and selective media containing cefoxitin and ceftriaxone, which specifically selects for *bla*<sub>CMY-2</sub> resistant organisms. The cefoxitin, a second generation cephalosporin, eliminates the ESBL population. Bacterial counts were assessed in both the treated and untreated populations. Prior to treatment, *E. coli* was readily recovered from the non-selective media, but only one animal was culture positive for resistant *E. coli*. After the administration of ceftiofur, both susceptible and resistant *E. coli* counts were close to zero CFU/g 8 hours post treatment, and the susceptible *E. coli* population did not recover until 72 hours post treatment. Resistant *E. coli* isolate recovery was observed 24 hours post treatment in both the treated and untreated groups. By day 7 post treatment, the susceptible *E. coli* population was back to the baseline level, and the resistant *E. coli* in treated cattle decreased to 4%. The preliminary study showed there was an effect on enteric microflora resulting in the fecal shedding of *bla*<sub>CMY-2</sub> resistant organisms in both treated and untreated herd mates following administration of ceftiofur. A similar study involving feedlot cattle and the effect of using a long-acting ceftiofur product was published by researchers at West Texas A&M. The results of both studies correspond to another study where ceftiofur was reported to have a transient effect on the intestinal flora by reducing the susceptible commensal population allowing the resistant organisms to flourish due to the lack of competition as a result of the reduction susceptible commensal organisms. The previous studies helped establish the hypothesis that the
removal of ceftiofur use from a population would result in decreased recovery rates of third generation cephalosporin resistant *E. coli* from the fecal flora over time.

An important observation was made regarding the herd effect of ceftiofur use. Untreated animals were observed shedding third generation cephalosporin resistant *E. coli* after herd mates received a dose of Excenel, ceftiofur hydrochloride, a third generation cephalosporin approved for use in food animals. Even though the untreated cattle didn’t shed as high CFU/g of feces as the treated cattle did, their enteric flora appeared to be affected, making untreated herd mates of treated animals a potential source of resistant organisms upon entering the food chain.

MDR *Salmonella* spp. are monitored in retail meat products by NARMS. Pathogens like MDR *Salmonella* spp. pose an immediate risk to public health due to the morbidity and mortality associated with ingestion of these pathogens by individuals not following safe handling guidelines. *E. coli* antimicrobial resistance patterns are equally important to monitor because *E. coli* has the potential to be pathogenic to humans as well as having the ability of colonizing the gastrointestinal tract and potentially acting as a reservoir for multidrug resistance plasmids including those harboring the *bla*<sub>CMY-2</sub> gene. The prevalence of the *bla*<sub>CMY-2</sub> gene recovered from retail meat products is low. In a recent study evaluating retail meat *Salmonella* isolates, non-typhoidal *Salmonella* isolates were screened for multidrug resistant AmpC *Salmonella enterica* serovar Newport, and of the 689 isolates submitted for evaluation, 9 isolates were confirmed multidrug resistant
AmpC *Salmonella enterica* serovar Newport,\(^{50}\) which supports the previous research findings of a low frequency of *Salmonella* spp. resistant to third generation cephalosporins entering the food chain.\(^{31,43}\) Zhao *et al.* (2009) evaluated 344 *Salmonella* isolates, which was 12.7% of all *Salmonella* isolates collected from retail meat samples during 2002 to 2006 for a NARMS study.\(^{32}\) These 344 isolates where selected because they were confirmed carriers of the AmpR gene. The majority of these isolates were recovered from poultry products; however, 13 isolates came from beef and 11 from pork. Zhao *et al.* (2009) found 50% of the *Salmonella* isolates harbored the \(\text{bla}_{\text{CMY-2}}\) gene, and 9 of the \(\text{bla}_{\text{CMY-2}}\) carrier isolates also contained a \(\text{bla}_{\text{TEM-1}}\) as well. It was discovered that 50% of the total isolates were resistant to ceftiofur, and 2% of the isolates were found to be resistant to ceftriaxone proving ceftriaxone resistance is rare.

The majority of human infections from *Salmonella enterica* harboring the \(\text{bla}_{\text{CMY-2}}\) gene are associated with foodborne outbreaks.\(^{38,59}\) Investigation of an outbreak of *Salmonella enterica* serotype Newport in France concluded that the offending *S*. Newport was imported from another country, and upon further genetic analysis, the organism was hypothesized to have entered France from US imported of horse meat. This conclusion was based on the lack of evidence of the \(\text{bla}_{\text{CMY-2}}\) gene not being found in animal isolates from France. The majority of the isolates from the outbreak shared common genetic element suggesting the clonal dissemination of MDR *Salmonella* Newport.\(^{59}\) These specific examples provide evidence of food animals being a source of zoonotic transfer of organisms carrying genes conferring resistance to the third generation cephalosporins.
According to NARMS, the prevalence of third generation cephalosporin resistant organisms is on the rise. Prevalence reports of ceftiofur resistant *Salmonella* spp. in human isolates has increased from 0.2% in 1996 to 3.4% in 2004 (CDC, 2007). Children were cited as the most common age group affected by MDR *Salmonella* spp. and are more likely to experience systemic infections, higher mortality rates, and re-admittances to hospitals.\(^{60}\)

In a retrospective study performed by the University of Wisconsin, 286 patients infected with MDR *Salmonella enterica* serovar Newport had isolates submitted for antimicrobial susceptibility testing, and it was discovered that 4 of the isolates were resistant to ceftriaxone. These results were compared to the NARMS data available during the same time period. The Wisconsin isolates displayed higher resistance when compared to the US isolate pool.\(^{61}\) The elevated resistance associated with the Wisconsin isolates was attributed to the dense dairy population in the state. Reported risk factors associated with human infections with the resistant *S*. Newport include interactions with either cattle or petting zoos, as well as the consumption of raw dairy products. The findings indicated that the recovery of ceftriaxone resistant *S*. Newport was relatively low even though the level of ceftiofur resistance is increasing among human isolates. Risk factors of humans contracting a MDR *Salmonella* pathogen identified in this study related to occupation, unsanitary eating practices, and history of previous antimicrobial drug treatment.\(^{25,61}\) Individuals infected with MDR *Salmonella* Newport pose a potential health risk if
hospitalized because they could potentially be the source of nosocomial infection. This has not been reported in a MDR *Salmonella* case; however, other pathogens such as methicillin-resistant *Staphylococcus Aureus* (MRSA) and *Clostridium difficile* have been reported as nosocomial offenders.

The World Health Organization (WHO) estimates morbidity caused by non-typhoidal *Salmonella* infections to affect 1.4 million individuals in the US annually resulting in roughly 15,000 hospitalizations and 580 deaths (http://www.who.int/mediacentre/factsheets/fs139/en/). Prior to the 1990s, almost all human cases of *Salmonellosis* were susceptible to the antimicrobial drugs commonly used in human medicine. Physicians noticed their first-choice drug treatments were losing efficacy. Resistance to fluoroquinolones was noticed first followed by ceftriaxone. In 1998, the first US case of ceftriaxone resistance, a third generation cephalosporin commonly used in children, was documented in a child, which was the result of an indirect exposure to ill cattle treated with ceftiofur. The cephalosporin resistance has caused more anxiety to public health leaders than the fluoroquinolone resistance because unlike the fluoroquinolone resistance, which was the result of a bacterial genetic mutation, the cephalosporin resistance was produced by a mobile plasmid capable of being transferred between different species of bacteria.

The majority of *Salmonella* spp. infections occur as individual events and normally go unreported. Outbreaks happen at a much lower frequency but tend to be more publicized
due to the generalized public health risks associate with mass infections.26 Evaluation of MDR Salmonella outbreaks has allowed for the assessment of risk factors associated with these outbreaks. The initial case of ceftriaxone resistant Salmonella enterica serotype Typhimurium occurred in 1998 in a child indirectly exposed to ill cattle treated with ceftiofur.25 At the time of infection, the child had recently been treated with amoxicillin-clavulanate for a sinus infection, and 4 days following the last dose of amoxicillin-clavulanate, the child developed acute abdominal pain. Suspected of having appendicitis, ampicillin-sublactam was administered and an appendectomy was performed. Histological results revealed severe mucosal inflammation, and amoxicillin-clavulanate treatment was resumed 2 days post surgery. Salmonella enterica serotype Typhimurium was cultured from a bout of diarrhea, which developed immediately following the reinstatement of amoxicillin-clavulanate treatment. The Salmonella Typhimurium isolate was confirmed positive for the blaCMY-2 gene. This gene encoded for resistance to ampicillin, chloramphenicol, tetracycline, sulfisoxazole, kanamycin, streptomycin, broad-spectrum cephalosporins (cefoxitin), expanded-spectrum cephalosporins (ceftriaxone and ceftiofur), aztreonam, gentamicin, and tobramycin. The child’s father reported having treated calves for severe diarrhea, and upon further investigation, the calves were found to be shedding Salmonella enterica serotype Typhimurium. Laboratory analysis preformed on isolates from both the child and the calves provided evidence of the infected calves being the origin of the child’s infection.
Four years following the initial case of MDR *Salmonella enterica* serotype Typhimurium, a strain of MDR *Salmonella enterica* serotype Newport was responsible for triggering a five-state epidemic. Forty-seven individuals cultured positive for *Salmonella* Newport, and one individual was able to provide a frozen ground meat sample, which cultured positive for *Salmonella* Newport. Pulsed-field gel electrophoresis (PFGE) identified the isolates as *Salmonella enterica* serotype Newport.\(^{38}\) In 2009, Beef Packer, Inc, Fresno, California, issued a ground beef recall in response to an outbreak of *Salmonella* Newport that was occurring in Colorado, in which 21 individuals were reported sick (http://www.marlerblog.com/2009/08/articles/case-news/beef-packer-inc-aka-cargill-recalling-ground-beef-sold-to-retail-markets-due-to-salmonella-newport-illnesses-in-11-states/). Other researchers have documented outbreaks of *Salmonella* Newport in other countries including the United Kingdom and Finland,\(^{62}\) Canada,\(^{57}\) Denmark,\(^{63}\) and France\(^{59}\) to name a few. With NARMS data finding more isolates cultured from retail meat samples showing resistance to both ceftiofur and ceftriaxone, the food chain has been accused of being a reservoir for antimicrobial resistance genes entering the consumer population. However, there is no literature regarding how the use of ceftiofur on the farm is directly impacting the microflora of meat products from animals within these farming populations.

Case follow-up reports have contributed to risk assessment and allowed associations to be made between the afflicted individuals and outbreaks. Risks associated with individual cases of MDR *Salmonella* spp. include individual history of antimicrobial drug use,\(^{35}\)
exposure to ill cattle,\textsuperscript{25,64} consuming either raw or undercooked ground beef,\textsuperscript{38} or consuming unpasteurized dairy products.\textsuperscript{61} While the consumption of raw or undercooked foods can be reduce by the public practicing better hygiene and cooking techniques in their homes and restaurants, animal husbandry practices require a deeper evaluation on the practices that could be influencing these organisms from entering the food chain. Producers should be more conscientious about the animals they cull because sick animals are at an increased risk of harboring pathogens.\textsuperscript{65} These animals potentially should be euthanized on the farm instead of going to slaughter. Veterinarians should be establishing on-farm treatment protocols to foster a more prudent use of antimicrobial drugs. This particular research evaluating the impact of ceftiofur and its withdrawal will provide a critical piece of information by reporting the influence of drug use on animal population for the first time. These results will provide much needed information to allow a base for further study.

Conclusion

Since its introduction in the early 1990’s, ceftiofur has been widely accepted into the veterinary communities surrounding livestock production. Because of its use label for lactating dairy cattle, it is the most common antibiotic prescribed, but part of its usefulness is the zero milk withhold while the animal is being treated. The current extra label use restrictions placed on this class of drugs is the result of an ongoing dilemma over the perceived public health risks associated with the use of a third generation cephalosporin in food animals. If ceftiofur is truly the threat some parties say it is, then what types of interventions can be established to reduce the threat to society?
The purpose of work presented in the following chapter was to determine if the removal of ceftiofur from livestock populations would prove to be an appropriate strategy in the reduction of third generation cephalosporin resistant organisms inhabiting the gastrointestinal tracts of the livestock harboring these organisms. It is hypothesized that the removal of ceftiofur will result in an immediate reduction of the shedding of $\text{bla}_{CMY-2}$ positive $E. \ coli$. This statement is based on the preliminary work with the beef cows, where part of the group received a dose of ceftiofur, and the shedding of resistant $E. \ coli$ was dramatically reduced by day 7 post treatment. However, the discontinuation of avoparcin as a feed additive in the European Union did not result in an immediate reduction of VRE, and for many years following the ban, VRE continued to be cultured out of broilers not treated with avoparcin.

No study has been conducted so far to monitor the effects of ceftiofur removal a population of animals and the meat products they yield.
References


Chapter 2: The Impact of Ceftiofur Removal on Recovery of *Salmonella enterica* and *Escherichia coli* Resistant to Third Generation Cephalosporins

Introduction

Foodborne pathogens are responsible for an estimated 81 million illnesses among humans in the US annually in which 15,000 hospitalizations and 9,000 deaths result.\(^1\) Public health officials are concerned the emergence of antimicrobial drug resistance within foodborne pathogens could result in therapeutic treatment failures in systemically ill patients. *Salmonella* spp. are considered pathogenic to mammals, and the majority of cases go unreported. However, outbreaks do occur at relatively low frequencies\(^2\), and they are commonly foodborne in origin.\(^3\).\(^4\) Controversy regarding antimicrobial drug use in food animal production medicine has been increasing among public health officials due to the unknown risks of antimicrobial resistance among foodborne pathogens. Some authors have suggested antimicrobial drug use in food animal production is associated with human clinical cases of resistant foodborne pathogens.\(^4\)\(^6\) Although these studies have some evidence that the use of antimicrobial drugs have some impact on public health, the degree of impact is unknown. Risk factors associated with the development of antimicrobial resistant infections include the consumption of raw or
undercooked meat products or raw dairy products, or recent antimicrobial treatment for other bacterial diseases unrelated to a foodborne pathogen.\textsuperscript{7, 8}

Antimicrobial drugs are commonly used for both treatment and prevention of a variety of bacterial diseases in food animal production systems. Research groups have studied the effects of antimicrobial drug use at the herd level.\textsuperscript{7-10} Lowrance et al (2007) reported that the use of therapeutic doses of ceftiofur, a third generation cephalosporin, in a group of feedlot steers resulted in the shedding of ceftiofur resistant \textit{E. coli} in both treated and untreated animals and concluded the effect of ceftiofur use on the recovery of \textit{E. coli} harboring the $\text{bla}_{\text{CMY-2}}$ gene was seen at the herd-level rather than the individual-level.\textsuperscript{8} The $\text{bla}_{\text{CMY-2}}$ gene encodes for the hyperproduction of β-lactamase enzymes, and this gene is found on a mobile plasmid. The origin of the mobile plasmid is from a \textit{Citrobacter freundii} in which a transposable element was able to transfer the genetic material from the chromosome to the mobile plasmid.\textsuperscript{11} The mobile plasmid is then able to be spread via horizontal transfer from \textit{Citrobacter} to other bacterial species, particularly Gram negative species. The $\text{bla}_{\text{CMY-2}}$ gene is commonly found among \textit{E. coli} and \textit{Salmonella} spp.\textsuperscript{12, 13} including \textit{Salmonella enteric} serovar Newport.\textsuperscript{14} Antimicrobial drug use varies from farm to farm, and studies have shown the use of antimicrobial drugs influence the enteric microflora’s ability to harbor antimicrobial resistance genes.\textsuperscript{7-10} Avoparcin, a glycopeptide antibiotic, was once used as a feed additive for both poultry and swine in several European countries. The selection pressure placed on the intestinal flora of the animals receiving avoparcin led to an observed increase in vancomycin
resistant *Enterococci* spp. in poultry and swine production systems in Europe. These studies imply veterinary antimicrobial drug use is a potential threat to public health, but the implications are based on circumstantial evidence.

Cephalosporins are important group of antibiotics in both human and veterinary medicine targeting both Gram negative and Gram positive bacterial populations. Third generation cephalosporins are commonly used in both humans and animals to target Gram negative organisms as well as some Gram positive bacteria. Ceftiofur is the only approved third generation cephalosporin used in food animals for the treatment of respiratory infections, metritis, mastitis, and foot rot. The pharmacokinetics of ceftiofur allows the drug to be rapidly metabolized by the animal reducing the risk of tissue residues within the animal’s body. Additionally, parental treatments of ceftiofur results in very low drug residues entering the mammary system allowing for ceftiofur to be labeled for zero milk withhold and minimal slaughter withhold times. Similar in molecular structure to ceftiofur, ceftriaxone is used to treat Gram negative infections in humans and is critically important for the treatment of salmonellosis in children under the age of 12 years old where fluoroquinolone toxicity has adverse health implications.

Commensal organisms such as *E. coli* can potentially act as a reservoir for the *bla*<sub>CMY-2</sub> gene, which is located on a mobile plasmid capable of spreading to other Gram negative organisms. Poppe et al. (2005) demonstrated the ability of *Salmonella enterica* serovar
Newport to acquire multidrug resistant plasmids via conjugation from commensal intestinal *E. coli* in turkey poults.\textsuperscript{17}

The current study was designed to evaluate the impact of ceftiofur on intestinal microflora in cattle and pigs as well as the meat products produced by these animals. The third generation cephalosporin is of particular interest because both human and veterinary medicine have a drug of significant importance, ceftriaxone for humans and ceftiofur in animals. None of the current literature has evaluated how use of ceftiofur in veterinary medicine affects the resistance patterns found in the microflora of meat products. The current concern is that ceftiofur use in livestock is contributing to ceftriaxone resistance among humans. Since the introduction of ceftiofur use in food animal medicine, the prevalence of ceftiofur resistant organisms within livestock population has been increasing.\textsuperscript{18}

The objective of the study was to provide information on the use of ceftiofur in animal agriculture and determine how ceftiofur use impacts ceftriaxone resistance among *Escherichia coli* in ground meat products. To assess the level of resistance throughout the food chain, monthly animal samples and weekly meat samples were collected and evaluated for the presence of the *bla_{CMY-2}* gene. The ground meat samples originated from the same population of animals observed for the duration of the study.
Materials and Methods

*Animal Sampling.* The Ohio Department of Rehabilitation and Corrections (ODRC) possess 5 commercial type dairies, 5 commercial cow-calf herds, 8 feedlots, and 3 farrow to finish swine operations. The dairy herds ranged in size from 80 to 200 mature Holstein cows, and the housing included tie-stalls, freestalls, bedded packs or pasture. The cow-calf herds ranged in size from 40 to 200 and contained both purebred and crossbred stock. The feedlots were comprised of weaned ODRC beef calves, purchased calves from local sale barns, and all the Holstein bull calves. The feedlot capacity ranged from 100 to 300 head on feed. The sow herds were comprised of crossbred sows and ranged in size from 50 to 100 sows.

All the farms within the ODRC system participated in the study with regards to treatment protocols, but only two locations were selected for monthly sample collection. The two sites were chosen because they had all 3 animal production systems in close proximity, which made the logistics of sample collection possible. The Ohio State University College of Veterinary Medicine Department of Preventive Medicine was contracted by the ODRC to provide veterinary services for the livestock within its farming system. Farm A had a 100 cow dairy housed in sand freestalls, and its feedlot was capable of housing 200 head. Farm A was phasing out its farrowing operation and was receiving feeder pigs from the other 2 prison swine facilities. Farm B had a 150 cow dairy housed on a bedded pack with access to pasture during the day. Farm B’s feedlot was capable of
holding up to 200 head, and the sow herd was 75 head. The ODRC discontinued Farm B’s swine operation in July of 2006.

The study lasted for 15 months. During the first 6 months of the study, January 2006 through June 2006, monthly fecal and weekly coarse ground meat samples were screened to establish the prevalence of *E. coli* with reduced susceptibility to ceftriaxone and *Salmonella* spp. For the second 6 month period of the study, July 2006 through December 2006, ceftiofur was removed from the treatment protocols of dairy cattle, feedlot calves, and swine. The remaining 3 months of the study, January 2007 through March 2007, ceftiofur was re-introduced into the treatment protocols.

From January 2006 to March 2007, fresh fecal samples were collected monthly from beef cattle, dairy cattle, and finisher pigs at two independent operations containing all three animal populations. From each animal group, 50 samples were collected from different individuals unless there were fewer than 50 animals in the pen, in which all the animals were sampled. One of the farms stopped raising finisher pigs during August 2006. Both of the dairies were closed herds, and the beef calves on both farms were obtained from a variety of sources. The majority of finisher pigs came from either the operation itself or from a different operation associated with the system; however, during the final 4 months of the study, the operation purchased feeder pigs from an outside source. Samples were either collected directly from the animal or from a fresh fecal pat on the pen floor. The
samples were packed on ice and immediately transported back to the laboratory for microbiological analysis.

For the duration of the study, the antimicrobial usage information was attained for the six different animal populations studied. The purpose was to establish the frequency of antimicrobial treatment for each study group as well as to determine if antimicrobial drug use affected the ability to recover resistant organisms. The livestock sampling was divided into three phases based on antibiotic usage to examine the effect of ceftiofur use on the intestinal microflora of each animal population.

**Ground Meat Sampling.** From March 8, 2006 to April 4, 2007, weekly ground meat samples of both beef and pork were obtained from the ODRC’s packing plant. This plant harvested the majority of the finished animals as well as the cull animals from the system. Some weeks over the course of the study the plant would only harvest one species; therefore, only one species would be available for sampling. A single 10 pound package of coarse ground product was used to screen for *E. coli* and *Salmonella* spp. From each package, 20 aliquots were taken for microbiological analysis.

**Laboratory Analysis.** Microbial analysis included *E. coli* with reduced susceptibility to ceftriaxone and *Salmonella* spp. For *E. coli* isolation, a 2-phase selection process was used to try to identify presumptive positive *E. coli* isolates harboring the *bla*$_{\text{CMY-2}}$ gene. A 10 g fecal sample was aseptically mixed with 90 ml nutrient broth containing 4 µg/ml
cefoxitin and incubated at 37°C for 18-24 hours. On the following day, each sample was plated on to a MacConkey agar plate containing 8 µg/ml ceftriaxone (Mac-cef) and incubated at 37°C for 18-24 hours. Isolated pink lactose-fermenting colonies were placed into Tryptophan broth at 37°C for 18-24 hours. Following the incubation, Kovac’s reagent was added to the Tryptophan broth to confirm *E. coli*.

The isolation of *Salmonella* spp. from cattle and pigs differed only on the initial day of sample collection. For cattle, 4 g of feces was placed in a sterile 50 ml conical tube with 36 ml of Tetrathionate Broth (TTB) containing iodine, brilliant green, and Tergitol-7 and incubated at 37°C for 18-24 hours. For pigs, 10 g of feces was placed in a sterile specimen cup along with 90 ml of TTB containing iodine only and incubated at 37°C for 18-24 hours. The remaining steps were the same for both cattle and pigs. After incubating in TTB, each sample was vortexed and 0.10 ml of the TTB was incubated in 10 ml of Rappaport-Vassiliadis R10 Broth (RV) at 42°C for 18-24 hours. The following day, the RV was plated on Xylose-Lysine-Tergitol 4 agar (XLT-4) and incubated at 37°C for 18-24 hours. The XLT-4 plate was evaluated after 24 hours for presence of black colonies. An isolated black colony was plated on a MacConkey agar (Mac) plate at 37°C for 18-24 hours to determine if the organism fermented lactose or not. Non-lactose fermenting organisms were further tested by inoculating triple sugar iron slant (TSI) and urea broth with a colony from the Mac plate and incubated at 37°C for 18-24 hours. A polyvalent antisera test was conducted to confirm the isolates were *Salmonella* spp., with agglutination indicating a positive reaction.
Dairy Farm A had a history of multidrug resistant *Salmonella enterica* serovar Newport. All salmonella isolates collected from Farm A were plated on to MacConkey agar containing 16 µg/ml of ceftriaxone and incubated at 37°C for 18-24 hours. Isolates growing on the antibiotic plate were further tested with an antisera group associated with the Newport species, and a positive antisera test resulted in coagulation upon mixing dilute bacteria and antisera.

The ground meat products were also screened for both *E. coli* with reduced susceptibility to ceftriaxone and *Salmonella* spp. From the single 10 pound package of either beef or pork, a total of 20 samples weighing 10 g each were placed into sterile specimen cups. Ten of the samples were used for the *E. coli* screen and the remaining 10 for the *Salmonella* spp. For the *E. coli* protocol, 90 ml nutrient broth containing 4 µg/ml cefoxitin was added to 10 of the specimen cups containing either ground beef or pork. The rest of the protocol was identical to the *E. coli* screen in feces. As for the *Salmonella* spp. screen, 90 ml of buffered peptone water was added to the remaining specimen cups containing ground beef or pork, and the samples were incubated at 37°C for 18-24 hours. The remainder of the *Salmonella* spp. screening protocol for meat was identical to the *Salmonella* spp. screening protocol of feces for the second day forward.

**Biotyping.** Biotyping was performed on the *E. coli* isolates. It was thought the isolates could be grouped based on their ability to ferment six different types of sugars, D-
raffinose, L-sorbose, L-rhamnose, sucrose, dulcitol, and 2-deoxy-D-ribose. Each *E. coli* isolate was grown on a MacConkey agar plate and 3 colonies were picked and placed into sterile distilled water. The diluted colonies were then plated in triplicate on a 96 well plate, with each well containing media with one of the six sugars. Each of the *E. coli* isolate’s fermentation pattern was recorded, and the results were categorized dichotomously as either utilizing the sugar or not utilizing the sugar. For each sugar, the following reagents were combined to make the agar base: 500ml distilled water, 7.5g buffered peptone water, 0.01g Bromocresol Purple, and 7.5g Difco-Bacto agar. Once the reagents were dissolved, the media was autoclaved at 121°C for 15 minutes and cooled to 55°C. The appropriate amount of sugar was added to each of the agars, and the agar was dispensed in the 96 well plates. For D-raffinose, L-sorbose, L-rhamnose, and sucrose, 2.5g/500ml of each sugar was dispensed into one of the sterilized agars. For dulcitol and 2-deoxy-D-ribose, 5.0g/500ml of each was placed separately in the remaining two sterilized agars.

Each isolate was grown on a MacConkey agar (Mac) plate at 37°C for 18-24 hours. The following day, 1500 µl of physiological saline (NaCl 8.5g/L) was aseptically dispensed into each well of a high volume 96-well round bottom micro-dilution box. An isolated *E. coli* colony was used to inoculate each dilution box well, with a bacterial concentration of approximately 10^8 cfu/L. Dilutions were gently mixed for homogenatity and used to inoculate the prepared biotype plates in triplicate. The 96-well sugar plates were incubated at 37°C for 24-48 hours depending on the sugar. L-sorbose, 2-deoxy-D-ribose,
and L-rhamnose incubate for 24 hours, and D-raffinose, dulcitol, and sucrose incubate for 48 hours.

Results

From January 2006 through March 2007, a total of 4101 fecal samples were collected from beef cattle, dairy cattle, and pigs. In addition to fecal samples, both ground beef and ground pork were collected from these animal populations during March 2006 through April 2007 for a total of 89 ground beef and ground pork samples. In Table 1, an overview shows the proportion of both *E. coli* presumably harboring the \( \text{bla}_{\text{CMY-2}} \) gene and *Salmonella* spp. isolates recovered.

During the study, 1637 of the 4101 (40%) livestock samples and 11 of the 89 (12%) ground meat samples yielded third generation cephalosporin resistant *E. coli* isolates. As for the *Salmonella* spp. results, 312 isolates came from the 4101 livestock fecal samples (8%), and 4 isolates came from the ground meat samples (4%). Of the three livestock populations sampled, the beef cattle samples consistently produced the highest amount of *E. coli* isolates resistant to third generation cephalosporins, and the dairy cattle samples furnished most of the *Salmonella* spp. isolates. The swine samples provided minimal amounts of both *E. coli* and *Salmonella* spp. isolates; however, of the fresh meat products cultured for these two bacterial groups of interest, the ground pork provided nearly all the isolates of both *E. coli* and *Salmonella* spp.
The recovery of *E. coli* resistant to third generation cephalosporins from the dairy cattle populations was constant throughout the study, and the removal of ceftiofur from the treatment protocol on the dairies did not decrease the recovery of resistant organisms. Figure 1 represents the recovery rates of *E. coli* resistant to third generation cephalosporins, and figure 2 represents the *Salmonella* spp. recovery rates. Table 2 represents the average number of animals treated parentally per month per herd with the antibiotics available for use. Mastitis drugs are not included even though ceftiofur mastitis products were also withheld during the ceftiofur removal period. Ceftiofur treatment was replaced with penicillin and oxytetracycline.

Similar to the dairy results, both *Salmonella* spp. and *E. coli* resistant to third generation cephalosporins were continually recovered from the beef calves. Figure 3 represents the recovery rates of *E. coli* resistant to third generation cephalosporins, and figure 4 represents the *Salmonella* spp. recovery rates. After investigating antimicrobial treatment records, it was noted that no treatments of ceftiofur was used during the study. Treatments include florfenicol (NuFlor, Schering-Plough Animal Health), fluoroquinolone (Baytril, Bayer Animal Health), aminoglycosides (Draxxin, Pfizer; Micotil, Elanco).

Looking at the swine results, third generation cephalosporin resistant *E. coli* was cultured more frequently during the first part of the study and dropped off during the second half of the study. Figure 5 represents the recovery rates of *E. coli* resistant to third generation cephalosporins.
cephalosporins, and figure 6 represents the *Salmonella* spp. recovery rates. Although ceftiofur was used on Farm B prior to its removal during the study, no therapeutic antibiotics were used during the course of study on Farm A. Farm B phased out its swine production system in July of 2006, and their treatment records were lost and unavailable for use in this study.

Farm B had a known history of multidrug resistant *Salmonella enterica* serovar Newport within its cattle populations. A total of 117 *Salmonella* isolates were collected from farm B, and when plated on a MacConkay agar plate containing 16 µl/ml ceftriaxone, 88 of the 117 (75%) isolates grew. All of the 88 Salmonella isolates that grew on the antibiotic plate reacted positively to the polyvalent antisera test. The growth on the plate and agglutination of the antisera test indicated the *Salmonella* spp. recovered could potentially be multidrug resistant *Salmonella enterica* serovar Newport.

After looking at the biotyping results, there were 8 biotypes associated with the meat isolates. These meat biotypes were compared to the biotyping results from the animal isolates. Frequently, the animal biotyping patterns matched those from the meat isolates. Of the 1637 E. coli isolates recovered, 1577 (96%) were biotyped. In table 3, the different meat biotypes are shown along with the number of *E.coli* isolates collected from three different production systems that shared that particular pattern. Also, the most frequent biotypes associated with each production system are displayed.
In assessing the antimicrobial usage information for the different production systems, Table 4 shows the number of animals treated with the different antibiotics.

Discussion

The 6 month removal of ceftiofur did not impact our ability to recover *E. coli* resistant to third generation cephalosporins in the enteric microflora of the livestock populations during the non-use period. When looking at the percentage of *E. coli* recovered during the different phases of ceftiofur use, baseline was expected to have the highest recovery rates because ceftiofur was used placing selection pressure on the gastrointestinal microflora resulting in the selection for the *bla*<sub>CMY-2</sub> phenotype; however, the removal of ceftiofur did not affect our ability to recover organisms resistant to third generation cephalosporins. Daniels *et al.* (2009) found ceftiofur use did not impact the ability to recover third generation cephalosporin resistant organisms at the herd level. In Daniels’ study, the prevalence of resistant organisms in 2 of the 3 organic herds were found to be comparable to the prevalence found in conventional herds. This suggests there is some other mechanism allowing the *bla*<sub>CMY-2</sub> gene to be maintained in a group of animals when ceftiofur selection pressure is absent. In our study, the recovery rates of resistant *E. coli* from cattle populations were variable throughout the entire study period. Yet, for the pigs, the results of ceftiofur removal followed our expectations in that the baseline sampling period gave the most resistant *E. coli* and tapered off after ceftiofur was removed from treatment protocols. With the pigs, most of the resistant organisms were
cultured from Farm A, which reported no antimicrobial drug use during the study. Farm B phased out their swine production unit during July of 2006, and the treatment records associated with this facility were lost.

Others have reported a transient shedding pattern of *E. coli* resistant to third generation cephalosporins in cattle following treatment with ceftiofur. Others have reported a transient shedding pattern of *E. coli* resistant to third generation cephalosporins in cattle following treatment with ceftiofur.\(^8\) Preliminary data associated with this project followed a group of beef cows housed together to study the effect individual animal treatments had on the microflora within the whole population. Quantitative measures in the form of fecal counts were taken of *E. coli* isolates both susceptible and resistant to third generation cephalosporins. After a baseline of both *E. coli* populations was established, some the cows were given a therapeutic dose of Excenel, which is ceftiofur hydrochloride. Post treatment effects on the enteric microflora showed a rise in the shedding of *E. coli* resistant to third generation cephalosporins within 24 hours as well as a decline in the susceptible *E. coli* populations. Results of the study revealed a 7-day shedding pattern of resistant *E. coli*, and cows receiving no treatment shed resistant *E. coli* but at a lower quantity than their treated cohorts. Seven days following treatment, the enteric microflora showed a shift back towards the pretreatment *E. coli* populations. (Wittum *et al*, 2005, unpublished)

After evaluating the antimicrobial usage records, it was noted that ceftiofur was replaced with ampicillin and tetracycline. It has been shown that the plasmid containing the *bla\(_{CMY-2}\)* gene commonly confers resistance to other antimicrobial drugs such as
ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline.\textsuperscript{22} Tetracycline lacks the ability to crossover from the blood stream into the intestines, so an increase in the amount of tetracycline resistance in the intestinal flora would be highly unlikely to occur from parenteral. However, ampicillin can apply enough selection pressure to allow the resistance genes of interest to remain circulating in the intestinal flora. It is thought that July would be the point in time when occurrence of cattle diseases would start increasing due to the heat stress of the summer time. Higher temperatures can lead to a decrease in feed consumption and more standing time, which could result in increased foot lesions and a reduction in the needed nutrients for healthy immune function. These events could bring about an increased need for antimicrobial drugs during the late summer months, which would ultimately explain why the \textit{E. coli} harboring the \textit{bla}_{CMY-2} gene could be maintained by ampicillin and tetracycline. Another explanation for the recovery of the resistance \textit{E. coli} could be environmental selection pressures in which these animals were housed allowing the gene to remain in circulation. Given ceftiofur was removed during July and it was October before the first freeze, the lag time in the temperature could potentially allow the resistant organisms to be maintained in the environment. Biosecurity was not strictly observed on the farms, and equipment was shared between the dairy and beef cattle facilities on each location.

Even though resistant organisms were commonly cultured from the feces of the live animals, resistant organisms were rarely transferred into the meat products. The prevalence of phenotypic resistance to ceftriaxone among \textit{E. coli} was 2.1\% in ground
beef and 19.0% in ground pork. Schroeder et al. (2003) studied antimicrobial resistance among E. coli isolates cultured from ground beef and found resistance to both ceftiofur to be 4% and ceftriaxone to be 1%, which agrees with our 2.1% finding.23 The original instructions for the processing plant were to collect 10 independent course ground meat samples for both beef and pork at different grindings sessions during the week. However, this method was never observed by the plant, and single, 10 pound package of ground product was used to screen for organisms with phenotypic resistant to third generation cephalosporins. Receiving a single bulk sample of ground beef and pork per week was not the ideal sampling the project originally designed, and several attempts were made by lab personnel to ensure the meat samples were correctly being collected. However, the meat plant did not comply with the study design. Another issue with the meat component, the meat plant was processing cattle and hogs from sources other than the farms participating in the study, and it was not possible to know if the meat samples we received came from the study animals or purchased animals.

Only one resistant E. coli was recovered from the ground beef samples, and the isolate was cultured during the second week of sample collection. Because the recovery rate for resistant E. coli was low, it is hard to assess if the removal of third generation cephalosporins really impacted the resistance genes associated with antimicrobial use was reduced; however, the low prevalence of the E. coli resistant to third generation was consistent with previous work looking at the resistance patterns from commesal E. coli
isolates collected from grocery store samples from both Washington D.C. and Nova Scotia.\textsuperscript{23, 24}

As for the ground pork, 42 samples were collected, and \textit{E. coli} expressing phenotypic resistance to third generation cephalosporins was cultured from eight different samples. All the isolates were cultured during the period of time when no third generation cephalosporin products were being used on the farms. On two occasions, multiple \textit{E. coli} isolates were cultured from the same sample, and the \textit{E. coli} expressed different biotype results. It is impossible to know if the animals from which the positive samples came from were from the system under study or from an outside source.

After looking at the results from the biotyping information collected from all the isolates, it was clear there were differences in the \textit{E. coli} isolates recovered. Biotyping was used as a way to group the \textit{E. coli} recovered for the different sample populations. The purpose of the study was to find if the animal isolates were similar to the meat isolates. It would be expected that the biotypes would differ among the different animal and meat samples and each population’s isolates would be clustered within a specific biotype. Nevertheless, this was not the case because it was common to match isolates coming from various populations of animals and meat samples within a common biotype.

In summary, the removal of ceftiofur products from animal populations did not lead to an immediate reduction in the recovery of \textit{E. coli} resistant to third generation
cephalosporins. Even though the *E. coli* expressing phenotypic resistance to third generation cephalosporins and *Salmonella* spp. were retrieved from the fresh meat samples, the prevalence of these two organisms was lower in the meat samples than in the fecal samples originating from the livestock, which is indicative that the resistance gene is infrequently going into fresh meat products.
References


<table>
<thead>
<tr>
<th></th>
<th>Farm A</th>
<th>Farm B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td>E. coli*</td>
<td>Salmonella</td>
</tr>
<tr>
<td>Total</td>
<td>1763</td>
<td>0.51</td>
<td>0.07</td>
</tr>
<tr>
<td>Beef Cattle</td>
<td>700</td>
<td>0.64</td>
<td>0.09</td>
</tr>
<tr>
<td>Dairy Cattle</td>
<td>750</td>
<td>0.58</td>
<td>0.07</td>
</tr>
<tr>
<td>Swine</td>
<td>313</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Pork Ground Meat</td>
<td>42</td>
<td>0.19</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*The proportion of samples collected containing *E. coli* resistant to third generation cephalosporins.

Table 1. The proportion of fecal and ground meat samples positive for *Salmonella* spp. and *E. coli* resistant to third generation cephalosporins recovered from two farms in Ohio.
Table 2. The average number of dairy cows treated with a particular antibiotic per month per herd during the three phases of antimicrobial usage on two farms in Ohio.

*Discontinued 2006

The number represents the average number of antibiotic treatments monthly within a herd.

1 Baseline prevalence is initial sampling period when farms were operating under normal conditions.
2 Ceftiofur removal is the period associated with the removal of ceftiofur products from all the farms operating within the system.
3 Reintroduction is the period of time associated with the re-introduction of ceftiofur products to the farms.

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Baseline Prevalence 1</th>
<th>Ceftiofur Removal 2</th>
<th>Reintroduction 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farm A</td>
<td>Farm B</td>
<td>Farm A</td>
</tr>
<tr>
<td>Penicillins</td>
<td>1.37</td>
<td>3.00</td>
<td>1.57</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>0.39</td>
<td>0.22</td>
<td>0.00</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>0.00</td>
<td>0.33</td>
<td>0.00</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>1.37</td>
<td>0.89</td>
<td>0.00</td>
</tr>
<tr>
<td>Spectinomycin*</td>
<td>0.00</td>
<td>0.11</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table 3. The 8 biotypes of *E. coli* isolates recovered from coarse ground meat and livestock fecal samples collected from two farms in Ohio.

Meat represents the number of *E. coli* isolates with reduced susceptibility to ceftriaxone cultured from the ground meat products with the particular biotyping pattern.

Beef cattle represent the number of *E. coli* isolates with reduced susceptibility to ceftriaxone cultured from feedlot steers with the particular biotyping pattern.

Dairy cattle represent the number of *E. coli* isolates with reduced susceptibility to ceftriaxone cultured from dairy cows with the particular biotyping pattern.

Swine represents the number of *E. coli* isolates with reduced susceptibility to ceftriaxone cultured from finisher pigs with the particular biotyping pattern.

*Represents the most common biotype for the population.

Meat 3/11 (27%), Beef cattle 180/786 (23%), Dairy cattle 106/694 (15%), Swine 83/269 (31%)

Table 4. Number of antimicrobial treatments administered to beef, dairy, or swine populations during the each phase of the study on two farms in Ohio.
Figure 1. Dairy cattle *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio. Recovery rates for *E. coli* isolates phenotypically resistant to third generation cephalosporins from January 2006 to March 2007. January 2006 to June 2007 represents the period of initial baseline establishment. July 2006 to December 2006 marks the period when ceftiofur was removed, which is highlighted by the gray box. January 2007 to March 2007 represents the reintroduction of ceftiofur.
Figure 2. Dairy cattle *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio.
Figure 3. Beef cattle *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio. Recovery rates of *E. coli* isolates resistant to third generation cephalosporins from January 2006 to March 2007. January 2006 to June 2007 represents the period of initial baseline establishment. July 2006 to December 2006 marks the period when ceftiofur was removed, which is highlighted by the gray box. January 2007 to March 2007 represents the reintroduction of ceftiofur.
Figure 4. Beef cattle *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio.
Figure 5. Swine *E. coli* isolates expressing resistance to third generation cephalosporins recovery rates for the three phases of antibiotic use on two farms in Ohio. Recovery rates of *E. coli* isolates resistant to third generation cephalosporins from January 2006 to March 2007. January 2006 to June 2007 represents the period of initial baseline establishment. July 2006 to December 2006 marks the period when ceftiofur was removed, which is highlighted by the gray box. January 2007 to March 2007 represents the reintroduction of ceftiofur. Farm B closed its swine operation in August of 2006.
Figure 6. Swine *Salmonella* spp. isolate recovery rates for the three phases of antibiotic use on two farms in Ohio.
Bibliography


