Studies on *Salmonella enterica* and *Escherichia coli* with a focus on ceftiofur and the genetic resistance determinant *bla*$_{\text{CMY-2}}$

Dissertation

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By

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Abstract

There is increasing debate regarding the role of agricultural use of antimicrobials on the emergence and increasing recovery of multi-drug resistant organisms in food products and humans. Resistance to ceftiofur and other third generation cephalosporins in *Salmonella enterica* and *Escherichia coli* is increasing in recent years. This resistance is commonly mediated by the β-lactamase gene, \( \text{bla}_{\text{CMY-2}} \) in the United States.

Our first study was a clinical trial of the effect of vaccination with a commercially available *Salmonella* vaccine on the recovery of *Salmonella* from subclinically infected dairy cattle.

The second study was a large field based study of 50 dairy herds in Ohio. The role of the use of ceftiofur on the farms and our ability to recover *Salmonella* species and *E. coli* with reduced susceptibility to third generation cephalosporin drugs was investigated.

The third study involved administration of ceftiofur to various populations of cattle. We injected brood cows, feedlot animals and dairy cows with ceftiofur to determine the effect of the drug on the fecal *E. coli* population.

The fourth and final study for this dissertation involved genetic sequencing of \( \text{bla}_{\text{CMY}} \) genes from *E. coli* and *Salmonella* species recovered from a variety of food animals, humans and retail meat products.
Dedicated to Chelsea and Sam
Acknowledgments

I am very grateful to my advisor, Dr. Thomas Wittum, for his guidance and support throughout my graduate research training, and to my graduate committee members; Dr. Julie Funk, Dr. Armando Hoet, Dr. Wondwossen Gebreyes and Dr. Richard Meiring, for their helpful input and advice.

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One of the greatest things that happened during my graduate school career was the birth of my son Sam. Sam has provided perspective, which sometimes got lost in the depths of my studies and work.

Finally, none of this would have been possible without my wife Chelsea, who is simply THE BEST!
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Chapter 1: Introduction

My career as a practicing veterinarian started in July of 1999 after graduation from the Atlantic Veterinary College in Prince Edward Island, Canada. My first job was in a large predominantly dairy practice in Reedsville, Wisconsin. I worked for Paul Calonder, Lyle Holschbach, Terry Hruby, Keith Tuttle, and Ted Greif and worked with Bud Buchner and John Degner. I learned from them what it took to be a successful dairy practitioner. This practice allowed me to gain experience in medical and surgical conditions in dairy cattle and to see how a successful dairy practice was run. Although I only worked at Veterinary Associates for 1 year, this practice was instrumental in helping me get where I am today.

In early 2002, I had my first experience with multi-drug resistant *Salmonella* Newport. The outbreak occurred in a new dairy that was milking 3200 cows and was freshening about 20 to 30 animals per day. Cases were occurring primarily in fresh cows, but sporadic cases occurred in animals in all stages of lactation and also in breeding bulls. Treatment of the sick animals was attempted using ceftiofur and supportive care and most animals survived, but were unproductive and were culled. Necropsy results and fecal samples tested positive for *Salmonella* Newport, which was resistant to 9 different antimicrobials including ceftiofur. Later I learned that this dairy herd was a part of the nationwide outbreak of MDR-\textit{AmpC Salmonella}
Newport. This experience sparked my interest in salmonellosis and antimicrobial resistance.

While I was in private practice in Newaygo, Michigan, I was able to participate in Michigan State University’s Practice Based Ambulatory Program and was an adjunct professor in the veterinary college. In this program 4th year veterinary students at MSU were paired with participating veterinarians for 3 week blocks to experience food animal practice away from the confines of the veterinary school. Students were exposed to the day to day routine of private practice and were able to get hands-on experience in all area of veterinary medicine. These experiences with the veterinary students that rode with me helped me realize that I enjoyed teaching and wanted to be able to do more of it.

It was due to these experiences that in the autumn of 2004, I came to Ohio State and joined the laboratory of Dr. Tom Wittum. Tom and Dr. Julie Funk had recently received funding to study the relationship between ceftriaxone usage on farms and the recovery of resistant gram negative organisms, specifically *E.coli* and *Salmonella enterica*. Their grants from the USDA and the CDC along with the Department of Veterinary Preventive Medicine funded my Ph.D. training and allowed me to explore my interests in teaching, antimicrobial resistance and *Salmonella* on dairy farms.

During my graduate training I have conducted large, field based epidemiologic studies, clinical trials investigating vaccines, and molecular epidemiologic studies. Additionally, Tom gave me many opportunities to develop my teaching skills by participating in many of the courses in which he was apart of.
These were the reasons I returned to graduate school. Epidemiology is no longer the subject I hated in veterinary school. It is now something I hope to make relevant to future veterinary students and help them to make it a part of their everyday working lives.

This thesis is the result of 7 years of work. Included in it are chapters on salmonellosis and the dairy industry, a summary of the current knowledge of the \( \textit{bla}_{\text{CMY-2}} \) gene, a cross-sectional study of 50 Ohio dairy herds, a pilot study in which we investigated the genetic sequence of the \( \textit{bla}_{\text{CMY-2}} \) gene from 54 bacterial isolates, a trial where we injected cattle with ceftiofur and monitored the fecal commensal \( \textit{E. coli} \) and \( \textit{E. coli} \) with reduced susceptibility to ceftriaxone and finally a clinical trial where we examined the efficacy of a commercially available \( \textit{Salmonella} \) Newport vaccine on the shedding of \( \textit{Salmonella} \) in 2 dairy herds. I hope that others find this work meaningful and that it contributes to the science of antimicrobial resistance and the epidemiology of salmonellosis on dairy farms.
Chapter 2: *Salmonella enterica* and the dairy industry

Introduction

The prevention and control of *Salmonella enterica* infections on dairy farms in the United States is a difficult and complex issue.\(^1\) Clinical cases of salmonellosis affect the profitability of the farm due to the resulting morbidity, mortality, and labor costs which decrease saleable milk and increase the costs of production.\(^2-4\) Veterinarians dealing with *S. enterica* infections in client herds benefit from understanding its epidemiology; however much of the scientific literature has focused on individual animal infections by specific serovars of *S. enterica* that are frequently recovered, such as *S. Newport* or *S. Typhimurium*. In addition, there is considerable literature concerning the epidemiology of the host-adapted serovar *S. Dublin* which may impact dairy cattle populations differently than the non-host adapted serovars. As the dairy industry in the US has changed over the past 20 years, so has the epidemiology of *Salmonella* infections in dairy cattle populations. This manuscript attempts to describe this evolution and provide an update on the current state of knowledge.
Over the past 30 years, the dairy industry in the United States has changed from many small, self-sustaining farms distributed widely throughout the country to an industry with far fewer, but larger farms centralized into geographically concentrated regions. While the number of dairy farms in the US has dropped dramatically, the total number of dairy cows has remained relatively constant. The NARMS 2002 data indicated that larger herd size was a risk factor for the recovery of *S. enterica*. Others have reported a similar association between number of cows and risk of *Salmonella*. Thus the evolution of the dairy industry to larger farms may be conducive to the dissemination of *Salmonella* in dairy cattle.

Modern dairy farms typically operate with a continuous flow of animals in order to maintain and increase herd milk production levels. New heifers are added to the herd as late lactation cows are dried off and fresh cows enter production. Often barns are overstocked because producers want to maximize profits from saleable milk. If stalls in barns are not used to their full potential then maximum profits cannot be obtained. This continuous flow of animals together with high stocking densities is conducive to the continuous sharing of enteric flora between cows in the herd. Thus, *S. enterica* infection can be chronically maintained in a herd through a continuous cycle of exposure, subclinical infection, shedding of the organism, contamination of the environment, and clearing of the infection in the individual animal.
Pathogenesis on dairy farms

One of the most difficult things to do in an outbreak is to determine how the Salmonella was originally introduced into the herd. Fecally contaminated feedstuffs, unpasteurized milk and colostrum, or saliva are common sources of infection of dairy cows. Smith et al. have reported the infectious dose of S. enterica required to produce clinical disease to be approximately $10^9$ organisms in healthy adult cows while others give a range of values from $10^4$ to $10^{11}$. Ingestion of a large number of S. enterica by the cow increases the probability that some organisms will survive to the intestinal tract and be able to cause disease but there are likely other cow factors that are also important in determining whether or not a cow will become clinically ill. It is important to remember that S. enterica is frequently an opportunistic pathogen and that other diseases may predispose individuals to infections.

Some have proposed that there is a difference in virulence among strains of S. enterica in terms of its ability to cause disease. In humans it has been proposed that multi-drug resistant S. enterica Typhimurium has caused increased morbidity and mortality controlling for age and other co-infections. However, in a study of S. enterica Newport enteritis, there was no difference in clinical outcome in people infected by multi-drug resistant S. enterica Newport compared to people infected with pan-susceptible S. enterica Newport. It remains to be determined if there is a difference in virulence among strains of S. enterica affecting cattle.
In all mammals there are two distinct forms of the disease caused by *S. enterica*; enteric and extra-intestinal. Both forms of the disease have the same pathogenesis until they reach the intestinal tract. Intestinal forms of the disease may infect the mesenteric lymph nodes, but the immune system prevents systemic spread of the organism and the major clinical sign is diarrhea. In the extra-intestinal form of the disease, the immune system cannot control the spread of the infection and meningitis, pneumonia, or arthritis may occur.

Subclinical infection

If the herd veterinarian had the opportunity to culture the entire dairy herd they would realize that sick cattle frequently are just the tip of the iceberg in an outbreak of salmonellosis. Most infections occur in cattle which show no adverse clinical signs and often appear normal and are overlooked when outbreaks occur. Subclinical infections are common in dairy cattle in the United States\(^7,\,16\) whether or not the herd is experiencing a salmonellosis outbreak.

A high prevalence of *S. enterica* in fecal samples from herds which are not experiencing clinical outbreaks has been reported\(^\,17,\,18,\,4\) and it so common that some researchers consider *Salmonella* a part of the normal enteric flora in cattle.\(^\,16\) This theory is controversial\(^\,13\) but there is evidence in dairy herds based on fecal sampling over a number of years that this may be true. In an Ohio dairy herd that has been the subject of repeated fecal sampling over a 12 year period, a single clone of *S. enterica*
serovar Kentucky was recovered from multiple cows over that time span (Wittum, unpublished data). There had been no cases of diarrhea attributed to S. enterica Kentucky during the sampling time frame. On this farm, it appeared that S. enterica Kentucky was non-pathogenic and was occupying a niche in the GI tract of the cattle on the farm. It is interesting to note that others have recovered serovar Kentucky from the feces of cows demonstrating signs of salmonellosis.4

The same phenomenon has occurred in other situations but with a different serotype. A subclinical outbreak of S. enterica Cerro was described while monitoring a dairy because of a clinical outbreak of S. enterica Typhimurium var. Copenhagen.19 They were able to track the introduction and maintenance of the S. enterica Cerro for 2 years. This suggests that it is possible for these normal inhabitants of the GI tract to cause disease, likely depending on management or environmental factors on the farm.

Risk factors for salmonellosis on dairy farms

A common route of S. enterica transmission on dairy farms may be Salmonella contamination of feedstuffs20 and there have been reports implicating feedstuffs as a vehicle for S. enterica infecting cattle and causing both clinical and subclinical disease.8,21-23 Contamination may occur on the farm during storage, feeding, growing or harvesting. Feedstuffs may become contaminated by wildlife, or by livestock waste in run-off or irrigation water.24,20,9 For example, silage is often
stored on concrete slabs in large piles where rodents and birds may easily contaminate these feedstuffs.

In their work on *S. enterica* in swine feed, Harris et al. found that farms that used some means to keep birds away from pigs and feed were less likely to have *S. enterica* contaminated feedstuffs.\textsuperscript{25} In their study of cattle feed in the northwest United States, Krytenburg et al. were able to isolate *S. enterica* from 29 (9.8\%) of 295 feed samples taken from both dairy and beef operations.\textsuperscript{22} Herd size has been consistently reported to be an important main risk factor for the recovery of *S. enterica* from dairy cattle.\textsuperscript{3, 18, 26, 26} Increased numbers of cows on a farm may be responsible for the increased risk of recovery of *Salmonella* on dairy farms or it may be a proxy for some unmeasured factor on larger dairy farms.

Dairy herds which experience high rates of metabolic diseases such as ketosis or hypocalcemia appear to be at increased risk for clinical outbreaks of salmonellosis.\textsuperscript{9} Feeding practices that favor subacute rumen acidosis where lactate is the major molecule present increase the risk of infection in affected cows. House et al. suggest that lactate is less able to kill *S. enterica* compared to acetate, proprionate or butyrate, thus *S. enterica* are able to multiply and increase in number in the rumen.\textsuperscript{27}
Herd consequences of infection

Animals that are subclinical shedders of *S. enterica* are able to transmit *S. enterica* to other animals on the farm, contaminate the farm environment, and are a source of *S. enterica* to workers or consumers of products derived from them. Cattle which are subclinically shedding *S. enterica* are thought to maintain the organism on the farm by passing the organism into the environment which infects naïve cattle or those whose immunity has waned or been compromised.\(^{28}\) As more cows become infected, more environmental contamination will occur until the majority of the animals in the pen have been exposed. It has been reported that various serovars are able to survive in the environment of dairy farms and have been shown to survive up to 184 days in manure.\(^{29,30}\) Attempting to control *S. enterica* on dairy farms through identification of individual shedding cows will therefore likely be ineffective and result in needless cost of time and money.

Diagnosis of infection

The presumptive diagnosis of *S. enterica* infection in a dairy cow often leads to a predictable series of events. Affected cattle may present a wide range of clinical signs ranging from subclinical infection, enteritis, abortion, pneumonia or death.\(^9\) Fecal samples are collected from sick cows, submitted to a veterinary diagnostic laboratory for *Salmonella* culture and antimicrobial sensitivity. Once the veterinary
diagnostic laboratory has identified *S. enterica* from a diagnostic sample, the isolate is forwarded to the National Veterinary Diagnostic Laboratory for serotyping. During this time some veterinarians and producers may try to segregate clinically affected cows, which were *S. enterica* positive on fecal culture, away from healthy cattle. Fecal culture of *S. enterica* is currently considered the gold standard in determining the infection status in cattle. However, the sensitivity of this procedure is relatively low\(^3^1\) and other testing methods such as ELISA and PCR have been developed in attempts to improve sensitivity and speed. Specificity of *Salmonella* culture is likely high due to the serial confirmation of presumptive isolates.

*S. enterica* Dublin infections of individual animals are believed to be especially important to diagnose on dairy farms so that chronic, high volume shedders of the organism can be identified and removed from the herd. Detection of these chronic shedders using culture based detection methods is difficult\(^3^2\) because these cows only shed salmonellae 3-4% of the time. As a result it has been suggested that fecal culture tends to underestimate the true prevalence of positive cows.\(^3^3\) Various techniques including fecal culture, serial fecal culture, and various serum antibody tests have been used in an attempt to detect the subclinical shedders of *S. enterica* Dublin.

Researchers have generated a large amount of data on the hematologic differences between cattle acutely infected with *S. enterica*, including serotype Dublin, cattle that had recovered and were no longer shedding *S. enterica* and cattle that were chronically shedding the bacteria.\(^3^2,3^4-3^6\) However, this data has not resulted in reliable antigen/antibody detection methods for most serotypes of *S.*
*Salmonella enterica* being developed. Serology has been used in an attempt to detect chronic carriers of *S. enterica* Dublin in Denmark, although control has not been successful using serology alone.\(^3^7\) This serological approach has not yet been adapted to detect the more common non-Dublin serotypes.

*Salmonella enterica* serovars causing disease in cattle

Prior to the early 1970’s *S. enterica* Dublin was most commonly recovered from dairy cattle west of the Rocky Mountains.\(^3^8,^3^9\) Dairy herds in the western states at that time tended to be larger and were more intensively managed than most dairies in the east. *S. enterica* Dublin was a major pathogen of both calves and adult cows in these herds, and major research efforts were undertaken in an attempt to understand and control this pathogen.

Salmonellosis can result from infection with any of the approximately 2500 serovars of *Salmonella*. Some serovars appear to play a more important role than others in bovine salmonellosis because of their frequent recovery from clinical cases. According to The National Antimicrobial Resistance Monitoring System (NARMS) in 2005 the top 13 serovars recovered from cattle in the United States were Montevideo, Newport, Meunster, Anatum, Typhimurium var. 5, Typhimurium, Mbandaka, Agona, Dublin, Cerro, Reading, Meleagridis and Kentucky.\(^4^0\) The top serovars recovered from diagnostic samples from dairy cattle in 2005 were Newport,
Typhimurium and Typhimurium var. 5. Although these serotypes were recovered most frequently, other serovars can and do cause clinical outbreaks of bovine salmonellosis.

Much of the information regarding bovine salmonellosis on dairy farms in the scientific literature is specific to the epidemiology of *S. enterica* Dublin infection. It is the bovine host specific serovar and this has particular importance on how it may be introduced onto naïve dairies. Host specific serotypes of *Salmonella* typically cause clinical disease in young, immune-compromised or immune immature individuals. Many times this serovar is introduced onto dairy farms by infected carrier animals; however it can also be introduced in other manners as well. As such, dairy farms that are actively expanding increase their risk of introduction of *S. enterica* on their farms by buying replacement animals.

Knowing the serotype

Why do we serotype *S. enterica?* Is it important for veterinarians to know which serotype is causing disease on farms? Unless the outbreaks on these farms are due to *S. enterica* serovar Dublin, there is little evidence that serotyping is important for reasons other than epidemiologic tracking. For most serotypes of *S. enterica*, knowing the serotype provides little useful information for control on the farm. Most veterinarians are better off knowing culture and sensitivity results to aid in their treatment of affected animals.
Then why do we serotype? Serotype information is useful for scientists who study *S. enterica* and for epidemiological tracking of specific serovars within and between herds, and across larger geographical areas. Serotyping allows us to track which serotypes are spreading and also to identify common sources of infections in large outbreaks. There is the belief some *S. enterica* are more “virulent” than others and that serotyping will help in determining this. However if a strain of a specific serotype of *S. enterica* is causing disease on a farm, we will not care how “virulent” it is or is not supposed to be. Instead of looking for the similarities among *S. enterica*, we have focused on how different they are.

I feel that looking for the similarities between the different *S. enterica* serotypes in terms of their epidemiology may allow for effective broad control of *S. enterica* on dairy farms. Veterinarians and dairy producers tend to look at each serotype individually and focus on the perceived differences. When it comes down to it, I believe that the diagnosis could be either Dublin or non-Dublin along with antimicrobial sensitivity results. Then if the diagnosis is *S. Dublin*, the farm may want to institute an eradication program to eliminate carrier animals on the farm depending on the farm’s goals and economics.
Serovar shifting/serotype replacement

When reviewing the literature of *S. enterica* infections in cattle it becomes clear that at different times a single or very few serovars will be the dominant serotypes recovered from clinical and non-clinical samples from cattle. Over time however, the frequency of recovery of the previously predominant serotype will decrease, and it will be replaced by a new dominant serotype. This process is called serotype replacement, and is supported by the theory that one serovar of *S. enterica* becomes prominent due to a specific selection pressure which allows the wide geographic dissemination of that specific serovar. Selection pressure might be in the form of the use of a specific antimicrobial drug, disinfectant, feedstuff, or management practice.

An example of serotype replacement occurred in the early 1960’s in Great Britain. During that time *S. enterica* serovar Dublin was recovered infrequently from cattle and *S. Typhimurium* was recovered the most frequently. Cattle were typically housed on pastures and calves were raised locally on their home farm before being sold for slaughter. In the mid-60’s cattle started being housed in barns more extensively and calves were more frequently sold through markets for fattening before slaughter. After this shift in management of cattle, *S. Dublin* became the most common serotype of *S. enterica* reported from veterinary diagnostic laboratories. The literature suggests that this change in the frequency of recovery of the two serotypes was related to the changes in management of cattle in England. The movement of
animals and the intensive methods of raising cattle were hypothesized to be the reasons for the “explosion” of *S. enterica* Dublin in Great Britain at that time.

This phenomenon has also been observed in the United States. In the early 1980’s in California and other western states, *S. enterica* Dublin was isolated frequently from dairy cattle and dairy farms. This serotype seems to have been replaced by *S. enterica* Typhimurium definitive type 104 (DT104). This is a serovar that became a dominant serotype worldwide in human and animal populations from the 1980’s through the 1990’s and was responsible for disease that was refractive to treatment on a worldwide scale. This serotype replaced *S. enterica* Dublin in the US as the predominant serotype recovered from dairy cattle and was resistant to many of the commonly used therapeutic agents. The next serotype to come into prominence in dairy cattle populations was the multi-drug resistant (MDR) *S. enterica* Newport. In contrast to DT104, which is a chromosomally mediated, the resistance determinant in *S. enterica* Newport was a plasmid mediated ampC. MDR *S. enterica* Newport infections in humans have been associated with fresh meat and dairy products. The next serotype that will take its place is yet unknown.

*Salmonella* control on dairy farms

There has been a tremendous research effort attempting to better understand the epidemiology of *S. enterica*. Disease due to *S. enterica* has long been recognized
in cattle, birds, swine and people. Why have we not made greater progress in the control of salmonellosis on dairy farms? One reason may be that while much epidemiological research has been done regarding \( S. \text{enterica} \) on dairy farms, no effective interventions have been identified. Numerous risk factors for the introduction of \( S. \text{enterica} \) on farms and for maintenance of \( S. \text{enterica} \) have been reported\(^1, 7, 26, 50, 51 \), but no successful intervention trials are available. In addition, few of the available control strategies have been rigorously evaluated under field conditions.

Hancock et al. gave a brief discussion on the relationship of control efforts to the prevalence of \( S. \text{enterica} \) Typhimurium DT104 globally and locally at the farm level.\(^{24} \) In their discussion they talk about on-farm biosecurity measures to prevent the contamination of feedstuffs by birds and cats. It is well understood that \( S. \text{enterica} \) can be detected in a wide range of animal species and that it is shed in their feces. Dairy farms need to have rodent, wildlife, and domestic animal control measures on their farms to help control salmonellosis problems. This includes eliminating dogs and cats from the farm premises.

\( S. \text{enterica} \) organisms are shed in very high numbers from clinically ill animals. We also know that various diseases or conditions can decrease the immunity of an animal and make them more susceptible to infection by \( \text{Salmonella} \). Cobbold et al. have shown that herds are more likely to have salmonellosis if they house fresh cows and sick cows together.\(^{29} \) Therefore farms should not house sick animals near periparturient animals. Doing so places the periparturient animals at high risk for becoming infected.
The placement of the sick pen on a dairy farm is very important. It should be placed away from the immune suppressed/immune immature animals on the farm, primarily calves and periparturient animals. It should also be emphasized that human and cattle movement in and between pens on a farm can have an impact on the recovery of *S. enterica* from cattle and the environment of these pens. Cattle should be handled in a manner that limits the spread of *S. enterica* on the farm. Suggestions include having farm workers, including veterinarians, moving from calves to cows and from animals that are most susceptible to those who are least susceptible. This might mean that calves and fresh cow are handled first thing in the morning when contamination of employees and equipment may be the lowest. Additionally, handling sick animals at the end of the day or shift and then properly cleaning and disinfecting equipment, boots and clothing may help limit the spread of *S. enterica* on the farm.

All-in-all-out (AIAO) production practices may be one reason why some swine units have been successful in eliminating and preventing introduction of strains of *S. enterica* on their farm. Cattle are frequently housed in varying age groups within the same barn or on the same farm. Separation between age groups is a well accepted method to prevent younger animals from being exposed to pathogens from older animals. AIAO production systems can also take advantage of cleaning and disinfection between production groups. Age segregation and AIAO management are used effectively in the swine and poultry industries for disease prevention and control and should be adopted by the dairy industry; however this may be difficult in the face of current intensive production methods, dairy cow physiology and housing.
Vaccination, using autogenous vaccines has been practiced since the 1940’s in an effort to control the same serotypes frequently causing problems today.\textsuperscript{54} However, there are no known peer-reviewed articles describing trials that evaluate the ability of an \textit{S. enterica} vaccine to prevent clinical disease in cattle. In spite of this, some producers and practicing veterinarians continue to vaccinate dairy cattle with \textit{S. enterica} vaccines.

Vaccination is relatively easily done by procuring the number of doses needed to vaccinate the entire herd, locking up the cows and then injecting them. Producers accept the temporary drop in milk and feel better when it happens because they “know that the vaccine worked.” If the infection is spread to the herd by a point source, such as feed, then the entire herd is naturally immunized and one can expect the epidemic curve to gradually tail off after two to three weeks. Every cow has been exposed and is “naturally immunized.” If one were to vaccinate at the height of the outbreak, then one might attribute the tailing off of cases to the vaccination of the cows. Then in the face of the next herd outbreak the farmer or veterinarian vaccinates again and hopes to see the same effect because they have attributed the decreased number of cases to the vaccine and not to the natural immunization that occurred via the passing around of the organism.

Different types of vaccines including killed bacterins, modified live, autogenous, and core antigen vaccines have been used with limited success to control this \textit{S. enterica} on dairy farms.\textsuperscript{55-60} The most recent introduction of a \textit{S. enterica} vaccine is the Siderophore Receptor and Porins vaccine by Agrilabs. This is a subunit vaccine that targets the iron acquisition proteins of \textit{S. enterica}. This vaccine
purportedly induces an antibody response to siderophores and the antibodies attach to siderophores making them unable to acquire iron from the host. Preventing iron acquisition results in death of the bacteria. Work by Emery et al. suggested that this vaccine was effective in decreasing shedding of Salmonella and reducing temperatures in vaccinated bull calves experimentally infected with S. enterica Newport.61 Other studies have shown that vaccinated lactating dairy cattle have increased milk production as well.62,63 Our recent work suggests that this product does not prevent subclinical S. enterica shedding in dairy cows60 and others have found similar results.63
Chapter 3: The cephalosporin family of β-lactam antimicrobials and the impact of their use on the emergence of Gram-negative bacteria with reduced susceptibility to 3rd generation cephalosporins

Introduction

The cephalosporins and other β-lactam drugs are some of the most commonly prescribed and administered antimicrobial agents. The 3rd generation cephalosporin drugs are important therapeutic agents in human medicine for the treatment of many serious infections, including salmonellosis in pediatric patients. In veterinary medicine, ceftiofur is the only 3rd generation cephalosporin licensed for use in the United States in food animals, although its widespread use has come under increased scrutiny in recent years. Some believe that agricultural use of ceftiofur is responsible for the increase in 3rd generation cephalosporin resistant Salmonella enterica isolated from humans in the United States due to transmission via the food chain. Most 3rd generation cephalosporin resistance in Salmonella enterica is mediated by blacMY-2. The epidemiology of this gene has been elucidated but is not fully understood. In this chapter, the existing literature pertaining to blacMY-2 and
attempts to clarify the epidemiology in both human and veterinary medicine will be summarized. The literature regarding the relationship of \textit{bla}_{\text{CMY-2}} to ceftiofur and other cephalosporin usage in veterinary medicine will be limited to their use in the U.S. dairy industry.

### History of the cephalosporin family of antimicrobials

The cephalosporin drugs are derived from the parent compound, cephalosporin C, which was discovered in 1945.\textsuperscript{77} It was recovered from the fungus \textit{Cephalosporium acremonium}, which was isolated from a sample of human sewage.\textsuperscript{77} Since that time many other antimicrobial agents have been developed from cephalosporin C. The β-lactam ring structure, present in all β-lactam drugs is responsible for the antimicrobial activity of these compounds. Changes to the various side-chains give each cephalosporin drug its unique properties.\textsuperscript{78}

### The generations of the cephalosporin family of antimicrobials

Cephalosporin drugs can be broken into four different generations. These drugs are placed into these different families based on the time in which the drug was discovered as well as their pharmacologic activity.\textsuperscript{77} In general, the higher the generation, the greater the efficacy against Gram-negative pathogens. In addition,
drugs within each generation share approximately the same spectrum of activity and are used in human and veterinary medicine in similar ways.

Use of cephalosporin family of antimicrobials in dairy cattle

The 1st generation cephalosporin antimicrobial drugs are primarily used in dairy cattle for the treatment and prevention of contagious staphylococcal and streptococcal mastitis. Examples of these drugs are cephlothin and cepaphirin. Both drugs are commonly administered in sodium or benzathine formulations and they are given by intramammary infusion. Treatment failure is common due to the intrinsic properties of the pathogen but additionally the production of β-lactamases by Staphylococcus aureus from clinical cases of bovine mastitis has been shown to affect the cure rate in clinically infected animals.

Currently there are no 2nd generation cephalosporin drugs licensed for use in dairy cattle in the United States.

Ceftiofur, the only third generation cephalosporin drug licensed for use in food animals in the United States, is very commonly used on commercial dairy farms in the US. In a recent study conducted by our laboratory, 92% of Ohio dairy farms reported the use of ceftiofur in their lactating cows. Besides its efficacy, the main reason ceftiofur is so commonly used on dairy farms is its short slaughter and milk withholding times (Table 3.1). Dairy producers often prefer to use ceftiofur rather than other available drugs because the risk of a milk residue violation is lower.
Ceftiofur, is available in many formulations; ceftiofur sodium (Naxcel, Ceftiflex), ceftiofur hydrochloride (Excenel) and as a crystalline-free acid (Excede). It is also formulated in 2 intramammary forms (Spectramast LC and DC) for the treatment and prevention of bovine mastitis.

Ceftiofur is indicated for use in dairy cattle for the treatment of bovine respiratory disease, bovine interdigital necrobacillosis, acute postpartum metritis, and mastitis caused by non-contagious mastitis pathogens. It also has label claims for the prevention of mastitis caused by *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae*. Complete ceftiofur label indications for dairy cattle are summarized in Table 1.

Additionally ceftiofur is frequently used in an extra-label manner for a variety of conditions on dairy farms. It has been reported to be effective in the treatment of experimental salmonellosis in dairy calves. 

Currently there are no 4th generation cephalosporin drugs licensed for use in dairy cattle; however cefquinome has been evaluated by the FDA for the treatment of bovine respiratory disease in cattle but is not yet approved. This drug (cefquinome sulphate) has been approved for use in cattle in Europe since 1994 for this use and has also been formulated into an intramammary product called Cobactan (Cephaguard in UK). The injectable formulation has a pre-slaughter withdrawal period of 13 days but this formulation may not be used in dairy cattle and so does not have a milk withdrawal time. Cobactan is indicated for the treatment of clinical mastitis in the lactating cow caused by *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Escherichia coli* and also for the treatment and prevention
of subclinical mastitis, at dry off, caused by the following organisms: \textit{Streptococcus uberis}, \textit{Streptococcus dysgalactiae}, \textit{Streptococcus agalactiae}, \textit{Staphylococcus aureus}, coagulase negative \textit{staphylococci}. The slaughter withholding time for the lactating cow formulation is 4 days and the milk withholding time is 5 days. For the dry cow formulation the slaughter withholding time is 2 days and there a milk withdrawal time of 49 days after treatment.

Use of the cephalosporin family of antimicrobials in human medicine

The first generation cephalosporin drugs are commonly used in the empirical treatment of skin and soft tissue infections. They are also administered intraoperatively and for the treatment of biliary tract infections.

The second generation cephalosporins are mainly used to treat respiratory tract infections but their use is limited due to the effectiveness of the third generation cephalosporin drugs. They may also be used in the treatment of conditions where the likely agents are anaerobic organisms or facultative Gram-negative organisms.

The third generation cephalosporin drugs, such as ceftriaxone and cefotaxime, are considered to be the drugs of choice for many infections caused by members of the Enterobacteriaceae. Ceftriaxone is commonly used because it may be administered as a once daily injection and because it has broad spectrum bactericidal effects. Ceftriaxone is the treatment of choice for all forms of gonorrhea, severe
forms of Lyme Disease\textsuperscript{92} and in pediatric cases of salmonellosis.\textsuperscript{93} It is also used empirically to treat meningitis due to its distribution to the cerebrospinal fluid.\textsuperscript{89}

Cefepime, a 4\textsuperscript{th} generation cephalosporin, is frequently used in community acquired pneumonia where resistance too many of the earlier generation cephalosporin drugs is a concern.\textsuperscript{94} Recently the United States Food and Drug Administration issued a warning regarding the safety of cefepime\textsuperscript{95} in response to a report in the The Lancet Infectious Diseases journal which reported higher mortality in patients treated with cefepime compared to other β-lactam antimicrobials.\textsuperscript{96}

Resistance to the cephalosporin family of antimicrobials

The continued development of the successive generations of the cephalosporin antimicrobials was largely in response to the development of resistance in pathogens which resulted in treatment failures in patients. Selection pressure is a term frequently used in conjunction with antimicrobial resistance to explain the emergence of resistance in species or groups of bacteria in response to antibiotic use. The microbiological flora of an animals or human is made up of both susceptible and resistant flora. The susceptible, commensal flora are often found in higher numbers and their presence can inhibit the growth of pathogenic organisms in the patient.\textsuperscript{97} If these susceptible organisms are reduced or eliminated by an antimicrobial, then the resistant, sometimes pathogenic organisms can flourish and fill the void left by the susceptible organisms.
Mechanisms of resistance in Gram negative bacteria to the cephalosporin family of antimicrobials

There are 4 mechanisms by which Gram-negative bacteria are known to resist the effects of β-lactam drugs: 1) the production of β-lactamases, 2) mutations in penicillin binding proteins in the bacterial cell wall, 3) the loss of porins in the cell which the β-lactam drugs diffuse and 4) the acquisition of efflux pumps, which actively pump antimicrobial drugs out of the bacterial cell. 98

The most common means of resistance to β-lactam antimicrobial drugs in members of the Enterobacteriaceae family is the acquisition of genes that encode for the production of β-lactamases.71,99,100 There are greater than 340 different β-lactamases reported,101 all of which essentially work in the same manner, although they may have differing substrates on which they work. Because of the large number of known β-lactamase enzymes, attempts have been made to classify them. The Ambler method groups enzymes according to their genetic similarity.102 The Ambler class A and the Ambler class C β-lactamases are produced by important Gram-negative organisms in human and veterinary medicine and will be discussed in the following paragraphs.

Members of the Ambler class A β-lactamases are common β-lactamases reported in the literature with genes located on chromosomes, plasmids and transposons.98,103,104 TEM-1 is the most common plasmid mediated β-lactamase105 and members of the TEM family are the most common β-lactamases found in Salmonella enterica.106 TEM-1 was first reported in E.coli in the early
The Ambler class A group also includes the SHV-1 family of lactamases, which are most commonly found in the genus *Klebsiella*.\textsuperscript{105,108}

Both the TEM-1 and SHV-1 families are active against the potentiated penicillins, ampicillin and amoxicillin; however single point mutations in the genetic sequence of these enzymes can broaden their spectrum of activity to include the extended spectrum cephalosporins.\textsuperscript{109} The TEM and SHV enzymes with activity against the expanded spectrum cephalosporins are called expanded spectrum β-lactamases or ESBLs. These enzymes were discovered shortly after ampicillin and amoxicillin were first used therapeutically in humans.\textsuperscript{110} ESBLs are able to hydrolyze the β-lactam ring of members of the 3\textsuperscript{rd} generation cephalosporins including ceftriaxone and ceftazidime in human medicine and ceftiofur in veterinary medicine. ESBLs are typically believed to be susceptible to the cephemycins of the 2\textsuperscript{nd} generation cephalosporins\textsuperscript{98} and also 3\textsuperscript{rd} generation cephalosporins combined with a β-lactamase inhibitor such as clavulanic acid. However in rare instances some ESBLs will confer resistance to the β-lactamase inhibitors\textsuperscript{111} making differentiation of these enzymes and the *ampC* β-lactamases difficult.

ESBLs among members of the *Enterobacteriaceae* are the most important source of cephalosporin resistance among isolates from human patients,\textsuperscript{112} however at this time they are not an important mechanism of 3rd generation cephalosporin resistance in veterinary medicine.\textsuperscript{113}

Carattoli has summarized the reports of ESBL producing *E.coli* and *Salmonella enterica* isolated from veterinary medicine and from food.\textsuperscript{113} Of the 26 reports described there has only been a single case of an isolate containing an ESBL
in the United States, which was recovered from a horse.\textsuperscript{114, 115} The remaining 25 cases were all reported from Europe and the Far East. Whether these enzymes will be important in the future is yet to be determined. The low numbers of reports may be due to lack of focus on these enzymes by those of the veterinary research community or they may not be being selected for by the antimicrobial use practices in the veterinary community.

The CTX-M family of $\beta$-lactamase enzymes

Another important family of Ambler class A $\beta$-lactamases which is responsible for 3\textsuperscript{rd} generation cephalosporin resistance in human medicine is the CTX-M family of enzymes. These enzymes are unrelated to the TEM or SHV families of ESBLs and are able to hydrolyze cefotaxime and ceftriaxone but are ineffective against ceftazidime.\textsuperscript{116} There are approximately 25 CTX-M enzymes and they are thought to be descendents of the chromosomal $\beta$-lactamase of \textit{Kluyvera ascorbata}. This group of enzymes is mainly found in \textit{E.coli}, \textit{Salmonella enterica}, and \textit{Klebsiella pneumoniae}.\textsuperscript{117} This family is an important cause of 3\textsuperscript{rd} generation cephalosporin resistance worldwide, but had not been associated with any cases in humans in the United States prior to 2008.\textsuperscript{118} However in 2008 a CTX-M producing \textit{Salmonella} was recovered from a patient in Georgia.\textsuperscript{119} These enzymes have been rarely found in veterinary medicine \textsuperscript{117, 120, 121} and had not been reported in isolates of
veterinary origin in the United States prior to 2010 when they were found in isolates of *E.coli* from dairy cattle in Ohio.\textsuperscript{122}

The ampC family of \(\beta\)-lactamase enzymes

A fourth class of enzymes that confers resistance to the expanded spectrum cephalosporins are the ampC family of enzymes. These enzymes are in the Ambler Class C group of enzymes and as such they differ from the TEM and SHV families. The ampC group is descended from the chromosomal ampC enzymes of *Citrobacter*, *Enterobacter*, *Morganella*, *Hafnia* and others.\textsuperscript{100} These enzymes confer resistance to the penicillins, the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generations of cephalosporins and also against the \(\beta\)-lactamase inhibitors.\textsuperscript{123-125} The ability to confer resistance to the cephemycins (2\textsuperscript{nd} generation) and \(\beta\)-lactamase inhibitors is an important phenotypic means of differentiating the ampC enzymes from the most of the ESBLs.

AmpC enzymes are found on the chromosome in many of the enterobacteriaceae,\textsuperscript{100} and their expression is induced by the presence of \(\beta\)-lactam antimicrobials.\textsuperscript{126-128} *E.coli* has a chromosomal ampC enzyme, which is not inducible, and normally does not produce enough enzyme to be clinically important.\textsuperscript{129,130} If the organism acquires a plasmid containing a gene encoding for an ampC enzyme such as CMY-2, it is able to produce clinically important levels of the \(\beta\)-lactamase enzyme and confer resistance to 3\textsuperscript{rd} generation cephalosporin drugs.
At some point in their history, the chromosomal ampC genes are thought to have translocated to a mobile plasmid and as a result are now found in organisms that do not have a chromosomal version of the gene.\textsuperscript{131, 132} These plasmid-borne genes are also no longer inducible\textsuperscript{126, 128, 133, 134} and are now constitutively expressed at high levels.\textsuperscript{98}

The $\text{BLA}_{\text{CMY-2}}$ family of $\beta$-lactamase enzymes

The most common enzyme in the ampC family is $\text{bla}_{\text{CMY-2}}$.\textsuperscript{100, 135, 136} It is now found world-wide in multiple species of pathogenic and non-pathogenic members of the $\text{Enterobacteriaceae}$. For those interested in food safety and food animals, $\text{Salmonella}$ and $\text{E. coli}$ are some of the most important. $\text{E. coli}$, of which there is considerable diversity in dairy cattle\textsuperscript{137} can be a pathogen but is largely studied because most serotypes are commensal organisms. Commensals are thought to be reservoirs for antimicrobial resistance genes that can be shared with pathogens such as $\text{Salmonella}$.\textsuperscript{138} $\text{Salmonella}$ on the other hand is a frequent cause of foodborne illness in humans and is a common pathogen in food animal species.\textsuperscript{84} $\text{Klebsiella}$ has rarely if ever been implicated in a food animal outbreak and $\text{Shigella}$ is a human pathogen without a known food animal reservoir.

Most of the early reports of CMY-2 were simple case reports in which resistant bacteria from sick individuals were isolated. The first report of CMY-2 came from $\text{Klebsiella pneumoniae}$ isolated from a patient with pyelonephritis in
Athens, Greece in 1990. The antibiotic resistance profile of the isolate suggested that the enzyme was a plasmidic cephamycinase. Plasmid DNA was isolated from this organism and the gene was found to be 381 amino acids in length (1146 bp).

The first identification of CMY-2 in the United States from a domestically acquired infection occurred in Nebraska in 1998. *Salmonella enterica* Typhimurium were recovered from a child with appendicitis and also from cattle the child’s father was treating for diarrhea. The isolates were subsequently found to be resistant to the majority of the β-lactam drugs. Interestingly, the impact of this resistant pathogen on the patient seems to have been minimal as no other treatment was noted and the boy’s recovery was reported as uneventful.

Prior to and during the boy’s illness, the boy’s father, a veterinarian, was treating some diarrheic calves with ceftiofur and fecal samples from the animals contained *Salmonella enterica* Typhimurium with a similar resistance pattern as the boys. The Centers for Disease Control (CDC) characterized the boy’s and the cattle’s *Salmonella enterica* isolates and found that the boy’s and one of the cattle *Salmonella enterica* were virtually identical. Both were positive for *bla*$_{CMY-2}$ and were found to be 100% identical to the known plasmidic CMY-2 gene. The authors speculated that direct zoonotic transmission of the pathogen occurred and caused the child’s illness, although a true cause and effect relationship was never established due to limits of the investigation.

Since the first discovery of the *bla*$_{CMY-2}$ enzyme in the US, it has rapidly expanded its range and is now found in many different species and in multiple serotypes of *Salmonella enterica*. *Salmonella enterica* Newport has frequently
been found to be a carrier of $\text{bla}_{\text{CMY}-2}$ and has become associated with the United States dairy industry.\textsuperscript{49,141} This bacterium and the associated $\beta$-lactamase gene are responsible for an outbreak of salmonellosis in the United States in both dairy cattle and humans that occurred throughout the early 2000’s.\textsuperscript{47,49,141,142} Humans have been affected by $\text{Salmonella enterica}$ Newport carrying $\text{bla}_{\text{CMY}-2}$ when consuming undercooked ground beef dairy cattle and also following exposure to dairy farms.\textsuperscript{143} Currently, $\text{Salmonella enterica}$ Newport carrying $\text{bla}_{\text{CMY}-2}$ is the most common $\text{Salmonella enterica}$ serotype isolated from diagnostic samples from dairy cattle in the United States.\textsuperscript{41}

Many of the isolates recovered from cases involved in the early 2000 outbreak, when examined by pulse field electrophoresis were determined to be indistinguishable, indicating the clonal spread of the $\text{Salmonella enterica}$ Newport and the associated $\text{bla}_{\text{CMY}-2}$ genetic element. Clonal dissemination of organisms harboring $\text{bla}_{\text{CMY}-2}$ is a common means for the dissemination of the gene.\textsuperscript{144} In this case the gene is transferred from mother to daughter cell and there is no dissemination of the gene itself without the bacterium.

The $\text{bla}_{\text{CMY}-2}$ containing plasmids

In contrast to clonal dissemination of $\text{bla}_{\text{CMY}-2}$, another common means of dissemination is via conjugation. An allele of $\text{bla}_{\text{CMY}-2}$ was first detected in $\text{Salmonella enterica}$ Senftenberg isolated from an Algerian child infected in 1994.\textsuperscript{145}
The authors were able to pass the class C resistance pattern to *E. coli* via conjugation experiments, which is the first evidence that the gene was located on a conjugative plasmid. The gene was then cloned and sequenced and found to be 99.5% homologous to the parent CMY-2 gene from *Klebsiella pneumoniae*. This is the report of *bla<sub>CMY-2</sub>* being carried on a large 170 kb plasmid.

Carattoli et al. reported that the *bla<sub>CMY-2</sub>* genetic element has been found on 3 different types of plasmids in *Salmonella* and *E. coli*. These plasmids designated A, B and C, are large plasmids of about 80-90 kb in size. Additionally, Winoker, et al. found *bla<sub>CMY-2</sub>* on a smaller plasmid (approx. 10kb) and designated it D. Plasmids A and C are known to harbor *bla<sub>CMY-2</sub>* and other antimicrobial resistance genes, thus conferring both β-lactamase resistance and other types of resistance. Plasmid type B on the other hand has been shown to only confer β-lactamase resistance. These plasmids have been determined to be low copy number plasmids. Interestingly, all three plasmid types share a common plasmid backbone as shown by RFLP analysis.

The *bla<sub>CMY-2</sub>* gene has been found on both conjugative and non-conjugative plasmids. Conjugative plasmids are the most common method bacterial organisms share genetic material. Non-conjugative plasmids suggest alternate modes of transfer of the *bla<sub>CMY-2</sub>* genes, such as transposons, transduction or integrons.
The clinical impact of the ampC family of β-lactamase enzymes

Pathogens which produce the CMY-2 enzyme can be challenging for clinicians to effectively treat, and have been implicated in treatment failures in both human and veterinary medicine. The blaCMY-2 β-lactamase gene will confer resistance to most of the cephalosporins and penicillins. In human medicine there are still treatment options for pathogens harboring this gene. For instance, most ampC organisms are still susceptible to the 4th generation cephalosporin drugs, such as cefepime, and the carbapenems drugs, imipenem and meropenem.

In veterinary medicine, especially in food animal practice in the US, treatment options are severely limited as there are no approved 4th generation cephalosporin drugs. ampC producers inactivate ceftiofur and although 4th generation drugs could be used in an extra-label manner under the Animal Medicinal Drug Use Clarification Act, their use in outbreak situations would not be cost effective. In Europe, use of cefquinome (Cobactan) should be effective in the treatment of bacterial organism producing ampC enzymes.

Laboratory detection and confirmation of CMY-2 organisms

In our laboratory, where we study the epidemiology of the blaCMY-2 gene in Salmonella enterica and Escherichia coli in cattle and swine populations, we use a 2 stage selection process to isolate presumptive blaCMY-2 containing organisms and
then confirm the genotype using a polymerase chain reaction (PCR). Our selection process is as follows; the initial samples or isolates are inoculated into nutrient broth containing the cephapycin drug cefoxitin at the concentration of 4μg/ml and incubated at 37˚C for 24hrs. Next a sterile swab is used to inoculate MacConkey Agar containing 8μg/ml of the 3rd generation cephalosporin drug, ceftriaxone, and incubated at 37˚C for 24hrs. Those organisms that are recovered through this process are resistant to ceftriaxone.

We have found that approximately 85-90% of the organisms recovered from fecal samples using the 2-step selection process will be positive on the blaCMY-2 PCR. It is possible that the other 10-15% of isolates are ESBL producers with resistance to the cephapycins and the β-lactamase inhibitors. Additionally, they could be other members of the blaCMY gene family or another ampC family which the primers do not react with.

The CMY-2 enzyme is known as the parent enzyme of the CMY-2 family. Genetic sequencing of the enzymes in the family shows that all members are from 95 – 99% homologous to CMY-2. There are approximately 20 members of this family, all of which have been given unique designations of CMY-XX, where XX is a number.

There are many different primer pairs reported for the detection of the blaCMY-2 genetic sequence. Some are for detection of the entire 1,146 length of the blaCMY-2 gene, while others are only for the detection of a partial sequence of the blaCMY-2 gene. In our studies of the blaCMY-2 gene we used the consensus primers described by Koeck and Winokur and followed the PCR protocol described by
Winokur. The PCR reaction conditions for the others primers pairs can be found in their respective references.

The dissemination of the CMY-2

Ceftiofur resistant organisms have been recovered from humans, food animals and food. *Salmonella enterica* serovar Newport demonstrates this. It as been frequently recovered from dairy cattle and compared to other serovars is frequently resistant to ceftiofur. Some have stated that this is likely a result of the use of ceftiofur on dairy farms but some studies have shown no association between ceftiofur use and the recovery of resistant/reduced susceptibility to ceftiofur. *Salmonella*. There is conflicting experimental evidence as to how the CMY-2 genetic element has been selected for on dairy farms.

We know that ceftiofur is frequently used on dairies due to it positive pharmacologic and food safety properties. Tragesser et al., found that individual treatment of animals with ceftiofur was not associated with the recovery of *E.coli* with reduced susceptibility to ceftriaxone, however Lowrance et al. and Jiang, et al. found that upon administration of ceftiofur there is a rapid and profound drop in the susceptible commensal flora and an increase in the number of 3rd generation cephalosporin resistant *E.coli*. This relationship between susceptible and resistant is maintained out to approximately 17 days at which time the susceptible flora overtakes the resistant flora and hide the resistant flora. The difference in these finding was that
Tragesser looked at cows treated up to 6 months in the past while Lowrance and the other paper monitored cattle immediately after administration of ceftiofur. It seems that the effect of ceftiofur on the selection of resistant flora is immediate yet temporary phenomenon in the GI tract of the cow. This may also explain why Tragesser did find a herd effect of ceftiofur use on the recovery of resistant organisms.

We know that the plasmids containing $bla_{CMY-2}$ in human and animal derived organisms are virtually identical and that some of these plasmids are capable of being shared with other organisms. We also know that ceftiofur resistant pathogens of food animal origin enter the food chain and also people have become ill from eating foods of animal origin but we still do not have the answer as to whether or not agricultural use of ceftiofur is the reason for increased resistance to 3$^{rd}$ generation cephalosporins in humans $^{162}$. We may never find the answer to this question but it is likely that both human and veterinary usage of 3$^{rd}$ generation cephalosporin drugs contributes in some fashion to the increased recovery of organisms resistant to 3rd generation cephalosporin drugs.

**Conclusions**

There is little doubt that with every cow, pig, chicken or human treated with 3$^{rd}$ generation cephalosporin antimicrobials, resistant organisms are selected for in their GI tract. These organisms rapidly expand their population in the absence of the
susceptible flora and these resistant organisms are then shed in the feces of treated animals. What is lacking in our knowledge of the epidemiology of $bla_{CMY-2}$ is the frequency with which organisms derived from foods of animal origin infect humans and how often it causes disease in people. Once this is elucidated then the true effect of agricultural ceftiofur use on the public health will be known.
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<td>Ceftiofur hydrochloride</td>
<td>Spectramast DC</td>
<td>Treatment of treatment of Staphylococcus aureus, <em>Streptococcus dysgalactiae</em> and <em>Streptococcus uberis</em> subclinical mastitis in dairy cattle at the time of dry off</td>
<td>16</td>
<td>0+</td>
</tr>
</tbody>
</table>

Table 3.1. Indications, pre-slaughter withdrawal period (meat) and milk withholding time (milk) for the various formulations of ceftiofur.

*  Bovine Respiratory Disease Complex

**  Acute Bovine Interdigital Necrobacillosis

***  Acute Postpartum Metritis

+  0 if a cow has a minimum 30 day dry period otherwise, if greater then test
Chapter 4: The Effect of Vaccination with a Commercial Subunit Vaccine on the Shedding of *Salmonella enterica* in Non-Clinically Infected Dairy Cows

Abstract

The objective of our study was to estimate the efficacy of a commercially available *Salmonella enterica* subunit vaccine on the subclinical shedding of *Salmonella enterica* in dairy herds. We conducted our trial using 175 mature cows on 2 dairy farms in Ohio with a history of *Salmonella* infection. We systematically randomized twenty-five percent of the mature cows from each herd to receive the *Salmonella enterica* subunit vaccine following label guidelines. The remaining 75% of cows in each herd served as non-vaccinated controls. Fecal samples were collected from all cows at the time of initial vaccination (day 0), booster vaccination (day 14), 2 weeks following the booster vaccination (day 28), and 10 weeks following the start of the trial (day 70). All samples were processed on the day of collection and cultured for the presence of *Salmonella enterica*. We obtained a total of 651 fecal samples over the entire study period. *Salmonella enterica* were recovered from 46 (7.1%) of the samples. The shedding of *Salmonella enterica* was similar for vaccinated and non-vaccinated control cows on each of the collection dates. Our results indicated that there was no evidence that extralabel vaccination with this
commercial subunit *Salmonella enterica* vaccine reduced shedding of *Salmonella enterica* in non-clinically infected dairy cows in these 2 dairy herds.

**Introduction**

An outbreak of salmonellosis on a dairy farm can have a large impact on the dairy enterprise's profitability as well as on the welfare of animals in the herd. Morbidity, mortality, and labor costs can all increase which reduces saleable milk and increases the cost of production. When a *Salmonella enterica* (*Salmonella*) outbreak occurs, producers and their veterinarians are faced with difficult treatment and control options. This difficulty is compounded when an outbreak is caused by a strain of *Salmonella* that is resistant to antimicrobial drugs commonly used in dairy cattle. Antimicrobial therapy options are limited in these situations and producers and their veterinarians may attempt vaccination of the herd to help control the outbreak and to prevent additional cases. There are a limited number of commercial *Salmonella* vaccines approved for use in dairy cattle for which the field efficacy has been established. Various experimental and field trials using different types of vaccines have been reported with highly variable results. In addition, autogenous vaccines designed to stimulate an immune response against the outbreak strain can be produced, but the efficacy of these products in dairy cattle is questionable or has not been reported.

A commercially available vaccine, Siderophore Receptor and Porins (SRP), was recently introduced for the control of clinical salmonellosis caused by *Salmonella* serovar
Newport (Salmonella Newport; Agrilabs, St. Joseph, MO). This vaccine is labeled for use in healthy cattle six months of age or older as an aid in the control of disease and fecal shedding caused by infection with Salmonella Newport. The vaccine induces an antibody response against siderophores and their receptors, which are proteins that are produced by Salmonella and other organisms to scavenge iron when they are under iron-deficient conditions. It is suggested by the manufacturer that this vaccine acts in a bacteriocidal way by inhibiting the iron transport system of the bacteria which results in death of the organism. Siderophores are known to be highly conserved in most Gram-negative organisms.64 Emery, et al. have suggested that a vaccine targeting siderophores may have decreased the fecal shedding and rectal temperature of Holstein bull calves challenged with Salmonella Newport61 while other vaccines targeting siderophores may have been associated with increased milk production in fresh cows on an expansion dairy.62 The efficacy of this vaccine has been poorly understood under field conditions.

Although the SRP vaccine is labeled for the control of clinical disease and shedding of Salmonella Newport, many veterinarians have used the vaccine, in an extra label manner, in an attempt to control salmonellosis problems attributed to other serovars of Salmonella. However the efficacy of the extra label use of this product for this purpose in dairy cattle has not been reported. Anecdotal evidence reported in the lay press and from field reports has suggested that the SRP vaccine is effective in preventing clinical disease in cattle and as a result, use of the product has increased.

The non-clinical shedding of Salmonella is a common occurrence on U.S. dairy farms.7,65 Cull dairy cows also commonly enter the food supply in the US as fresh meat products. When apparently healthy cull cattle are shedding Salmonella at the time of
slaughter, they represent a potential food safety risk to the consumer. Currently there are no vaccines labeled for use to eliminate subclinical shedding of \textit{Salmonella} in cattle and there are no reports on the efficacy of the SRP vaccine for this purpose. We have hypothesized that the SRP vaccine may be effective in reducing non-clinical shedding of \textit{Salmonella} by commercial dairy cows, and thus may be a useful preharvest food safety intervention. Therefore, the objective of this study was to determine if vaccination with a commercial subunit vaccine that targets siderophores reduces fecal shedding of \textit{Salmonella} in non-clinically infected dairy cows.

Materials and Methods

\textbf{Study Population}

All mature cows (n = 175) in two commercial dairy herds located in Ohio were included in this study. These 2 farms were chosen for inclusion into the study because both farms were known to have a history of subclinical fecal shedding of \textit{Salmonella} and also both farms had a history of collaboration with the investigators. Farm 1, a seasonal dairy, milked 91 cows per day while Farm 2 milked 84 cows per day. Farm 1 had a rolling herd average of approximately 23,000 lbs and Farm 2 had a rolling herd average of approximately 18,000 lbs. No animals had been introduced into either herd in recent years. Artificial insemination was used as the sole means of reproduction and herd bulls were not used. Lactating cows on each farm were housed in free-stalls that were bedded with sand. Both farms housed dry cows in loose
housing. The study herds had a history of chronic, non-clinical, *Salmonella* shedding. Farm 1 had a history of subclinical fecal shedding of unknown serovars of *Salmonella* and Farm 2 also had a history of subclinical shedding of unknown serovars of *Salmonella*, although one isolate from this farm had been identified as *Salmonella enterica* serovar Bovis-morbificans.

**Treatment Randomization**

In each herd, 25% of the lactating cows were randomly assigned to receive the SRP vaccine treatment. The remaining 75% of the lactating cows served as non-vaccinated controls. This distribution of the treatment groups was chosen to reduce the potential effect of herd immunity on the shedding of *Salmonella* in these herds. By allowing 75% of the cows to remain unvaccinated, we believed that vaccine efficacy could be fairly assessed in the presence of a normal challenge exposure.

Based on our previous work with the herds in this study, we expected to recover *Salmonella* from 30% of the samples that we collected. We hypothesized that if the vaccine were efficacious it would reduce subclinical shedding from 30% to 0%. In order to be 95% confident and have a statistical power of 80% to detect a 30% drop in shedding using the 3:1 ratio of controls to vaccinates, we needed herds of approximately 76 cows each. From this herd size, we would need 57 control cows and 19 treatment cows to appropriately evaluate the efficacy of the vaccine. To detect a reduction in *Salmonella* shedding from 30% to 15%, we would need a larger sample size of 368 cows. From this number of cows, we would need 276 control cows and 92 treatment cows to detect this difference. Based on our actual sample size, we
would be able to detect a drop in detection of *Salmonella enterica* from 30% to 8.75%. Some may feel that it is not possible that a vaccine alone could cause of drop in shedding to 0%. However, we feel for the vaccine to be an effective preharvest food safety intervention that this is what it must be able to do.

Cows were selected for treatment group assignment using systematic randomization based on the order in which they came through the animal-handling system at the farm. All mature cows on each farm were processed on each collection date and every fourth animal received the *Salmonella* vaccine. The remaining animals in the herd served as non-vaccinated controls. No other selection criteria were used to assign cows into treatment groups.

**Vaccination Protocol**

The vaccine tested in this study was the *Salmonella* Newport Bacterial Extract, Conditional License, Siderophore Receptors and Porins vaccine (AgriLabs, St. Joseph, MO). The vaccine was administered according to the label directions with single use needles by trained laboratory technicians. Two ml of vaccine was injected subcutaneously in the neck on day 0 and a booster dose of vaccine was given subcutaneously in the neck on day 14. Control cows were given a single 2 ml subcutaneous injection of sterile water in place of the vaccine, and otherwise were handled the same as treatment cows. All vaccinations on each day were administered by the same person.
Sample processing and *Salmonella enterica* culture

Fresh fecal samples were collected from all cows at the time of initial vaccination (day 0) and at the time of booster vaccination (day 14). A third fecal sample was obtained approximately 2 weeks later (day 28) and a final sample was collected approximately 10 weeks (day 70) after the trial was initiated. The study was conducted in 2 different seasons. Farm 1 was sampled in the fall of 2006 and Farm 2 was sampled in the summer of 2006. While the fall and spring seasons have been associated with increased shedding of *Salmonella*, because the vaccinates and controls that were compared to each other were sampled at the same time, this should have little if any effect on our results.

Approximately 25g of feces was obtained from the rectum from each cow and placed in an individual container. New palpation sleeves were used to collect each sample from all cows to prevent cross-contamination. All samples were transported to the lab and processed within 4 h of collection. A 4g aliquot of feces was removed from each sample and incubated in 36 ml of tetrathionate broth (TTB; Becton, Dickinson and Co, Sparks MD) overnight at 37° C. The next day, 100 µl of the TTB was transferred into 10 ml of Rappaport-Vassiliadas (RV) Broth (Becton, Dickinson and Co, Sparks MD) and incubated overnight at 42° C. Sterile, cotton tipped swabs were used to inoculate xylose lysine desoxycholate 4 (XLT-4; Becton, Dickinson and Co, Sparks MD) agar plates with the RV. These plates were incubated overnight at 37° C. Black colonies on XLT-4 were considered presumptive positive for *Salmonella* and were confirmed using standard biochemical tests including Triple Sugar Iron and Urea.
Statistical Analysis

All statistical analysis was conducted using a commercial statistical package (STATA v. 9.2, College Station, TX). Comparison of fecal *Salmonella* prevalence (Proportion positive for each group) was initially compared between vaccinates and controls within farm and collection date using the Pearson $\chi^2$ test. In order to account for the clustering of cows within farms, and the lack of independence of samples from within the same herd, mixed effects logistic regression models, using the *xtlogit* command were developed. Separate models were developed for each sampling day with the herd included in each model as a random effect. The outcome variable of interest was *Salmonella* culture result (i.e. *Salmonella* presence yes/no) at the individual cow level. Odds ratios and their 95% confidence intervals to estimate the association between vaccination and recovery of *Salmonella* were calculated from the logistic regression model for each sampling day.

Results

A total of 651 fecal samples were collected over the entire study period and *Salmonella* were recovered from 46 (7.1%) of them. Fourteen (30%) of the samples from which *Salmonella* were recovered came from vaccinates and 32 (70%) came from unvaccinated controls. The proportion of samples from which *Salmonella* were recovered, for each collection time, is presented in Table 4.1.
A total of 335 fecal samples were collected from 94 cows on Farm 1 (Fig.4.1a). Eighteen cows in this herd were lost to follow-up (9 control cows and 3 vaccinated cows) prior to collection of the day 70 sample. For the entire study period, the percentage of cows from farm 1 from which Salmonella were recovered was 26.8%. No cows in the herd had more than 1 sample from which Salmonella were recovered.

The percentage of cows with a sample in which Salmonella were recovered from Farm 1 on day 0 was 5.32% (5/94). The proportion of samples from vaccinates and controls from which Salmonella were recovered was 3/23 (13%) and 2/71 (2.8%) respectively (p = 0.058). The proportions of samples from which Salmonella were recovered from cows in each group for Farm 1 were not different at any of the collection dates, except for the initial collection time (day 0). The results are summarized in Table 4.2a.

A total of 316 fecal samples were collected from 81 cows on Farm 2 (Fig.4.1b). Three control cows were either sold or died during the period of study and therefore did not contribute all four fecal samples. The percentage of samples from which Salmonella were recovered on farm 2 for the entire study period was 25%. Two control cows had multiple samples that were positive. One of these control cows was positive on day 0 and day 14, while the other was positive on day 28 and day 70. We were unable to recover Salmonella from these 2 cows on the other collection days.

The proportion of samples on day 0 from which Salmonella were recovered from Farm 2 was 9/81 (11.11%). Three of 20 (15%) treatment cows and 6/61 (9.8%)
of the control cows had samples from which *Salmonella* were recovered (p = 0.524). The fecal shedding of *Salmonella* for treatment and control cows was not found to be different on any of the subsequent collection dates. Results for Farm 2 are summarized in Table 4.2b.

Odds ratios and their 95% confidence intervals to estimate the effect of vaccination on *Salmonella* shedding while adjusting for the effect of the herd using the mixed effects logistic regression model are summarized in Table 4.3. On day 0, the adjusted odds of recovering *Salmonella enterica* from a vaccinated cow were 2.5 (95% C.I., 0.82-7.69) times the adjusted odds of recovering *Salmonella enterica* from a unvaccinated cow. As we move further into the study to collections days 14, 28, and 70, the adjusted odds of recovery of *Salmonella enterica* become relatively equal for the 2 groups and hovers around the null value of 1.

**Discussion**

The use of vaccines to control *Salmonella* on dairy farms is a common practice in the United States. Field trials testing the SRP vaccine from peer-reviewed journals are lacking but anecdotal reports suggest that these vaccines may be effective in limiting the number of affected cattle during a clinical outbreak or that they may be effective in preventing future clinical outbreaks from occurring. The effect of vaccination on the reduction of clinical cases was not assessed in this study. We were interested in the vaccine as pre-harvest food safety intervention and wanted
to evaluate its effects on subclinical *Salmonella* shedding. The results of this study suggest that the extra label usage of the commercial subunit vaccine targeting bacterial iron-scavenging siderophore proteins was not effective in preventing non-clinical shedding of *Salmonella* by dairy cattle on these 2 dairy farms.

We originally hypothesized that the vaccine would be effective in controlling subclinical shedding of *Salmonella* in dairy cows. This hypothesis was based on anecdotal evidence gathered on farms and the fact that subunit vaccines targeting siderophores have been found to be efficacious in reducing shedding in cattle infected with other bacterial species. Siderophores enable bacteria to remove iron from its environment for its own metabolic needs. To inhibit the growth of *Salmonella* the cow limits the amount of iron available to invading bacteria by secreting its own iron-binding molecules, such as lactoferrin. Lactoferrin has been found to have bacteriostatic properties in the gut by binding to free iron and also has been shown to have bacteriocidal properties as well.

When making the decision to use a vaccine on a dairy farm, it is important to know what antigen the vaccine is targeting. Siderophore proteins are very well conserved in gram-negative organisms and over 99% of *Salmonella* produce the siderophores enterobactin and aerobactin, thus making them good targets for a vaccine. This, along with reports of the successful use of the vaccine on dairy operations suggested that the vaccine was effective against infections due to *Salmonella* Newport and a wide range of other serovars of *Salmonella*. Therefore, we expected that this vaccine would decrease the prevalence of *Salmonella* in subclinical cows.
It is possible that the vaccine was ineffective in stopping non-clinical shedding in the study herds because it does not protect against the specific *Salmonella* serovars present on these farms. Kingsley, et al. have reported that most host adapted *Salmonella enterica* produce no aerobactin and minimal levels of enterobactin.\(^7\) If the SRP vaccine is targeting either or both of these siderophores and the *Salmonella* recovered from the subclinical cows in the two herds in our study are similar to these host adapted serovars, then the SRP vaccine will likely be ineffective.

On commercial dairy farms it is also common to recover multiple serovars of *Salmonella* from animals or the environment, especially in subclinically infected herds.\(^7,\,59,\,65\) Although we did not determine the serovars of the *Salmonella* recovered in this study, it is possible that there was a serovar shift in animals that received the SRP vaccine. Vaccination may have reduced or eliminated one serovar and allowed another serovar to expand and fill the niche left by the eliminated serovar. If this were the case, our detection techniques would not have allowed us to detect this shift in serovar in the herds studied, and prevalence of *Salmonella* shedding would appear to be unchanged.

The cow-level prevalence of *Salmonella* over the entire study period was 26.8 and 25% respectively for Farm 1 and Farm 2. These levels were never reached at any of the individual collection dates at either farm. This demonstrates the problem with culture methods for the detection of *Salmonella* in dairy cattle and other species of animals. *Salmonella* organisms are shed in feces in low numbers and are also shed intermittently.\(^12\) Therefore, other detection methods that are more sensitive than microbiological culture methods should be developed for more accurate prevalence
levels to be determined. In addition, we did not attempt to quantify the number of *Salmonella* present in fecal samples. It is possible that the vaccine reduced the number of bacteria shed by vaccinated cows, without completely eliminating shedding.

The serial sampling methods used in this study show how difficult it is to monitor or determine *Salmonella* prevalence using traditional means. There was no way to determine if these herds had carrier animals that persistently shed *Salmonella enterica* or if their history of subclinical fecal shedding was due to environmental contamination which resulted in passive shedding by the cows. In subclinically infected herds the environment is usually heavily contaminated thus cows are constantly exposed to *Salmonella enterica* via the environment. From a food safety, animal welfare, and public perception standpoint, it does not matter that these herds may have high exposure due to the environment. Given the appropriate conditions, people and cows can become ill from *Salmonella* in these types of situations but it may be impractical to expect that subclinical shedding can be significantly reduced solely by the use of a vaccine.
### Table 4.1

<table>
<thead>
<tr>
<th></th>
<th>Number of samples (nv* + nc**)</th>
<th>Total Positive N (%)</th>
<th>Vaccinates Positive N (%)</th>
<th>Controls Positive N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td>175 (43, 132)</td>
<td>14 (8%)</td>
<td>6 (14%)</td>
<td>8 (6.1)</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td>162 (40, 122)</td>
<td>4 (2.5%)</td>
<td>1 (2.5%)</td>
<td>3 (2.5%)</td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td>160 (40, 120)</td>
<td>17 (10.6%)</td>
<td>4 (10.0%)</td>
<td>13 (10.8%)</td>
</tr>
<tr>
<td><strong>Day 70</strong></td>
<td>154 (39, 115)</td>
<td>11 (7.1%)</td>
<td>3 (7.7%)</td>
<td>8 (7.0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>651 (162, 489)</td>
<td>46 (7.4%)</td>
<td>14 (8.6%)</td>
<td>32 (6.5%)</td>
</tr>
</tbody>
</table>

Table 4.1. The number and percentage of fecal samples positive for *Salmonella enterica* from all lactating cows on two dairy farms in Ohio by vaccination status at each collection day.

* nv = total number of vaccinates  
** nc = total number of controls
Figure 4.1. The percentage of samples positive for *Salmonella enterica* from all lactating cows on Farm1 (a.) and Farm 2 (b.) by vaccination status for each collection day.
Table 4.2. The proportion, percentage and p-value generated from $\chi^2$ analysis of *Salmonella enterica* positive samples from all lactating cows on Farm1 and Farm 2 by vaccination status for each collection day.

<table>
<thead>
<tr>
<th>Farm 1</th>
<th>Vaccinates (number positive/total)</th>
<th>Controls (number positive/total)</th>
<th>p - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 335</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
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<tr>
<td>Day 0</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
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<tr>
<td>Day 14</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
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<tr>
<td>Day 28</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
</tr>
<tr>
<td>Day 70</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Farm 2</th>
<th>Vaccinates (number positive/total)</th>
<th>Controls (number positive/total)</th>
<th>p - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 316</td>
<td><img src="#" alt="Table data" /></td>
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</tr>
<tr>
<td>Day 0</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
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<tr>
<td>Day 14</td>
<td><img src="#" alt="Table data" /></td>
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<tr>
<td>Day 28</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
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</tr>
<tr>
<td>Day 70</td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
<td><img src="#" alt="Table data" /></td>
</tr>
<tr>
<td>Collection Day</td>
<td>Odds Ratio</td>
<td>Odds Ratio, 95% CI</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>2.51</td>
<td>0.82, 7.69</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>1.00</td>
<td>0.10, 9.90</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.88</td>
<td>0.27, 2.89</td>
<td></td>
</tr>
<tr>
<td>Day 70</td>
<td>1.11</td>
<td>0.28, 4.44</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. The adjusted odds of recovering *Salmonella* from a SRP vaccinated cow compared to an unvaccinated control cow being positive on two dairy farms in Ohio for each collection day.

Separate mixed effect logistic regression models were developed for each collection day with treatment as a fixed effect and herd as a random effect to control for the effect of clustering in the herd.
Chapter 5: *Escherichia coli* and *Salmonella enterica* with reduced susceptibility to ceftriaxone among Ohio dairy farms

Abstract

Our objective was to estimate the relationship between the therapeutic use of ceftiofur and the recovery of fecal *Escherichia coli* (*E. coli*) and *S. enterica enterica* (*S. enterica*) with reduced susceptibility to ceftriaxone in dairy cattle. We collected fecal samples from 3840 mature dairy cows on 50 dairy farms in Ohio. On each farm fecal samples were obtained from up to 100 mature dairy cows. Samples were screened for the presence of *E. coli* and *S. enterica* with reduced susceptibility to ceftriaxone using selective media. A mixed effect logistic regression model was constructed to estimate the association between ceftiofur use and the recovery of *E. coli* with reduced susceptibility to ceftriaxone. We recovered *E. coli* with reduced susceptibility to ceftriaxone from 92% (46/50) of herds in the study and from 60.9% (2338/3840) of the cows sampled. *S. enterica* were recovered from 44% (22/50) of the herds in the study and from 9.9% (382/3840) of the cows that were sampled. All of the 382 isolates of *S. enterica* recovered were susceptible to the third generation
drug, ceftiraxone. There was no association between ceftiofur use and recovery of *E. coli* with reduced susceptibility to ceftiraxone at the herd level. However, we were more likely to recover *E. coli* with reduced susceptibility to ceftiraxone from cows in herds from which we also recovered *S. enterica* on the day of collection (odds ratio, 29.0, CI (OR), 3.7 - 224.1) than from herds which we did not recover *S. enterica*. We found no evidence that the use of ceftiofur on commercial dairy farms increases the prevalence or dissemination of *S. enterica* or *E. coli* with reduced susceptibility to ceftiraxone.
Introduction

Agricultural use of antimicrobial drugs has become a controversial issue. Some feel that the use of antimicrobials in agriculture should be severely restricted or even eliminated. Others believe that only non-therapeutic use of antimicrobials should be eliminated, while others feel that there should be minimal regulations regarding antimicrobial usage in agriculture. Many agree that when antimicrobial drugs are administered to an individual that these drugs can act as selective agents in the gastrointestinal tract by killing susceptible commensal and pathogenic organisms thereby allowing resistant organisms to fill the niche left by the eliminated susceptible bacteria. This is thought to promote the emergence of resistant microorganisms. If there are bacteria present in the gastrointestinal tract that are resistant to an antimicrobial administered to the animal, then these resistant bacteria may amplify due to the lack of inhibition by the faster growing, sensitive, commensal bacteria of the gut. These resistant bacteria can then be shed in large numbers in the fecal material of the treated animals and can infect other members of the herd.

So while we know that resistant bacteria occur in food animals when antimicrobials are used, we do not know if agricultural antimicrobial use constitutes a public health risk. Opponents of agricultural antimicrobial usage strongly believe that it causes antimicrobial resistance to develop in food animals, which results in antimicrobial resistant organisms that contaminate the food supply and infect consumers. Unfortunately, there is very limited data on the frequency at which this occurs and to what extent. Additionally, opponents of agricultural antimicrobial
usage believe that food animals are a reservoir for resistance genes and resistance organisms that infect people.\textsuperscript{72}

Ceftiofur is the only third generation cephalosporin drug licensed for use in food animals and is commonly used on dairy farms for a variety of different conditions.\textsuperscript{82,83} One reason that ceftiofur is popular among milk and beef producers is that time from which an animal is treated with ceftiofur to the time in which milk or meat derived from that animal can be marketed is relatively short. This means that the likelihood for shipping milk or presenting an animal for slaughter that is adulterated with ceftiofur is low, if it is used according to label directions.

Ceftriaxone is a third generation cephalosporin drug used in human medicine which is similar to ceftiofur and it is the treatment of choice for pediatric cases of salmonellosis and in adult cases of fluoroquinolone-resistant salmonellosis.\textsuperscript{72} The United States Food and Drug Administration has designated that the third generation cephalosporins are a critically important class of drugs for human medicine, the highest classification possible.\textsuperscript{166,167} As a result the use of all third generation cephalosporin drugs and especially the use of ceftiofur in agriculture, has come under increased scrutiny.

Resistance to ceftriaxone and other third generation cephalosporins in \textit{S. enterica} seems to be increasing. The prevalence of antimicrobial resistant \textit{S. enterica} has been monitored in the United States since 1996 by the National Antimicrobial Resistance Monitoring System and their data suggests that since 1997 the prevalence of ceftiofur resistant \textit{S. enterica} has risen from 0.0\% to over 21.6\% in cattle\textsuperscript{168} and that the prevalence in humans has risen from 0.2\% in 1996 to 3.4\% in 2004.\textsuperscript{169}
Resistance to third generation cephalosporin drugs in *S. enterica* is predominantly mediated by the β-lactamase, *bla*<sub>CMY-2</sub> in the United States. 72, 146 *Bla*<sub>CMY-2</sub> confers resistance to the first, second and third generation cephalosporins, and the potentiated penicillins and it is also resistant to the effects of the β-lactamase inhibitors such as clavulanic acid. Due to the fact that *E. coli* are a commonly occurring member of the fecal flora of animals and that they are closely related to the *S. enterica* genus, some have proposed that they maybe a reservoir for antimicrobial resistance genes, including *bla*<sub>CMY-2</sub>.48, 160

We hypothesized that the recovery of *E. coli* and *S. enterica* with reduced susceptibility to ceftriaxone is more common in dairy herds where ceftiofur is used more frequently compared to farms where ceftiofur is used less frequently used. Therefore, the objectives of the study were to estimate the prevalence of *E. coli* and *S. enterica* with reduced susceptibility to ceftriaxone on Ohio dairy farms and to investigate the association between *E. coli* with reduced susceptibility to ceftriaxone and ceftiofur usage on Ohio dairy farms.

Materials and Method

Study Population

To study our hypothesis and complete our objectives, a cross-sectional study of a convenience sample of 50 Ohio dairy herds was conducted. We contacted nine veterinarians in private practice from across the State of Ohio for help in the
recruitment of prospective dairy herd study participants. Herds were voluntarily enrolled in the study if they permitted the investigators to collect fecal samples from their cows and if they would agree to answer survey questions regarding demographic and antimicrobial usage on their farm. Herds were sampled a single time between the summer of 2004 and the spring of 2006.

Sample Collection and Processing

Fresh fecal samples were collected from all lactating cows in study herds that were milking less than 100 cows. In herds that were milking more than 100 cows, a maximum of 100 fecal samples were collected from lactating cows. Approximately 25 g of feces were obtained from the rectum of each sampled cow using individual palpation sleeves. The samples were placed in individual containers to prevent cross-contamination, transported to the laboratory, and were processed on the day of collection.

In the laboratory, selective media were used in an attempt to recover \textit{E. coli} with reduced susceptibility to ceftriaxone and \textit{S. enterica} from each fecal sample. The selection procedure for the detection of \textit{E. coli} with reduced susceptibility to ceftriaxone was a 2 stage process. A 10g aliquot of feces was placed in 90ml of nutrient broth containing 4μg/ml of cefoxitin and incubated at 37°C for 24h. The next day, MacConkey Agar that contained 8μg/ml of ceftriaxone was inoculated using sterile cotton tipped swabs and incubated for 24h. Typical lactose positive colonies were selected and confirmed as \textit{E. coli} by the indole test and sample aliquots
of typtic soy broth were inoculated with individual colonies and frozen with DMSO for preservation.

The detection of *S. enterica* was a three stage process. A 4g aliquot of feces was obtained from each sample and placed in 36 ml of tetrathionate broth which was supplemented with brilliant green and Tergitol. This was incubated for 24h at 37º C. The next day, 10ml of Rappaport-Vassiliadis Broth was inoculated with 100μl of tetrathionate broth. This was incubated for 24h at 42º C. On the third day, sterile, cotton-tipped swabs were used to inoculate xylose lysine desoxycholate 4 agar. Red colonies with black centers were considered presumptive positive for *S. enterica*. A single isolate was selected and used to inoculate MacConkey agar that was incubated for 24h at 37º C. A single colorless, lactose-negative colony was selected and used to inoculate tryptic soy broth for storage and for Triple Sugar Iron and Urea biochemical confirmation. Isolates were further confirmed as *S. enterica* by agglutination using *S. enterica* polyvalent anti-sera.

To evaluate whether reduced susceptibility to third generation cephalosporins was a characteristic of the recovered *Salmonella*, all *S. enterica* isolates were struck onto both MacConkey agar and MacConkey agar supplemented with 8μg/ml of ceftriaxone to determine if they had reduced susceptibility to ceftriaxone.

Survey data

At the time of sample collection, the dairy herd owners or managers were administered a short survey assessing basic herd demographic information and antimicrobial use on the farm, including ceftiofur. Information was collected on
cattle in all stages of production and also on antimicrobials used to treat various types of diseases on each farm. These data were analyzed and used to construct variables to be used in the data analysis.

Based on previous data collected in our laboratory, we estimated that ceftiofur was used by approximately 61% of dairy farms in Ohio. However in the present study 88% (44/50) of all study herds reported at least some ceftiofur use. Therefore, we attempted to quantify ceftiofur usage on each farm by estimating the proportion of animals on each farm that were treated with ceftiofur during a defined time period. To do this, owners of the study herds were contacted by telephone and asked to report the proportion of cows that were treated with ceftiofur in a 3 month period (January 1 – March 31, 2006) using their farm records. This proportion was then multiplied by 100 to create a continuous variable, which we used as our risk factor of interest. Of the 50 herds initially enrolled in the study, only 43 herds provided this additional information and the remaining 7 herds were not included in the final analysis.

Statistical Analysis

All data analysis was accomplished using a commercial statistical package. The outcome of interest was recovery of *E. coli* with reduced susceptibility to ceftriaxone at the individual cow level (1=recovered, 0=not recovered). No *S. enterica* with reduced susceptibility to ceftriaxone were recovered from the study herds, so no association could be investigated. The association between ceftiofur use and recovery of *E. coli* with reduced susceptibility to ceftriaxone was accomplished using the *xtmelogit* command. A mixed effects logistic regression model was constructed with the herd included in the model as a
random effect. Modeling herd as a random effect was used to account for the clustering of cows within farms and the lack of independence of samples within the same herd.

In order to construct the logistic regression model, we first entered our risk factor of primary interest, the proportion of cattle treated with ceftiofur, into the model. A forward selection process was then used to screen for potential confounders, with variables entered into the model based on the Likelihood Ratio Chi-square statistic. We calculated adjusted odds ratios and their 95% confidence intervals for the variables that remained in our final model.

Results

The mean herd size was 180 (sd=27) lactating cows and ranged from 13 to 810. The mean rolling herd average for milk production for the 50 farms was 21,452 lbs (sd=480) with an average somatic cell count of 254,125 cells/ml. Seventy–four percent of farms utilized free-stall barns for housing lactating cows and 70% milked Holstein cows exclusively. Of the 50 farms in the study, 54% reported being a closed herd for the previous 12 months. Eighty-eight percent of farms reported that they used injectable ceftiofur on their farm within the previous 12 months. Study herds had been in existence for an average of 41.13 years (.66 to 114.) A thorough account of the summary statistics of the 50 dairy herds in the study can be found in Table 5.1. A total of 3840 individual fecal samples were collected from the 50 dairy herds. *E. coli* with reduced susceptibility to ceftriaxone were recovered from at least
one of the samples from 92% (46/50) of herds sampled. *E. coli* with reduced susceptibility to ceftriaxone were recovered from 60.9% (2338/3840) individual fecal samples. The mean herd proportion of cows from which we recovered *E. coli* with reduced susceptibility to ceftriaxone was 55.6% (range, 0% to 100%). A summary of the results for the *E. coli* culture is presented in Table 5.2.

*S. enterica* were recovered from at least one of the samples from 44% (22/50) of farms and from 9.9% (382/3840) of individual fecal samples. The mean herd proportion of cows shedding *S. enterica* in positive herds was 10.2% (range, 0% to 98.3%).

All of the *S. enterica* recovered had a minimum inhibitory concentration to ceftriaxone of \( \leq 8\mu g/ml \), indicating that it was unlikely that they possessed the *bla*\(_{\text{CMY-2}}\) genetic element. A summary of the *S. enterica* culture results can be found in Table 5.2.

We did not observe an association between the proportion of cattle treated with ceftiofur and the recovery of *E. coli* with reduced susceptibility to ceftriaxone (OR, 1.16; \( P = 0.38 \); CI, 0.83 to 1.63). We were more likely to recover *E. coli* with reduced susceptibility to ceftriaxone from cows on farms where we also recovered *S. enterica* (OR = 29.0; \( P = .001 \); CI, 3.7 - 224.1). The final mixed effect logistic regression model developed to investigate the relationship between *E. coli* with reduced susceptibility to ceftriaxone and the proportion of cattle treated with ceftiofur on the farm is summarized in Table 5.3.
Discussion

We found that 88% of farms in this study reported using ceftiofur to treat cattle on their farms. We previously reported that 61% of Ohio dairy herds used ceftiofur\(^\text{155}\), suggesting that its use on dairy farms has become more common in Ohio in recent years. The veterinary use of ceftiofur and other cephalosporin drugs in dairy cattle is a common practice due to its pharmacokinetic properties that make the possibility of adulterated milk or meat entering the food chain due to human error low. In addition, there is a perception by veterinarians and dairy producers that ceftiofur is very effective in the treatment of a wide variety of disease conditions other than and including those that are indicated on its label. For example, veterinarians and producers may use ceftiofur in the treatment of undifferentiated fever or enteric disease thought to be due to \textit{S. enterica} based on previous experience or based on reports found in the literature. The extra-label use of ceftiofur in the treatment of Holstein bull calves with experimental salmonellosis has been evaluated.\(^\text{85}\) These authors found that ceftiofur treated calves had fewer days with abnormal rectal temperatures and had fewer days of diarrhea compared to untreated control calves, however no differences in mortality between the two groups were detected.

We were frequently able to recover \textit{E. coli} with reduced susceptibility to ceftriaxone from the fecal flora of dairy cows from most (92%) of the farms in the study. The average number of cows on each farm from which \textit{E. coli} with reduced susceptibility to ceftriaxone was recovered was 55.6%. These data suggest that the
bla<sub>CMY-2</sub> genetic element, which is frequently responsible for third generation cephalosporin resistance in <i>E. coli</i> and <i>S. enterica</i> from cattle<sup>48,93,138,155,170</sup>, is present on most commercial dairy farms; however the frequency with which it occurs cannot be estimated from our data. Use of ceftriaxone in our selection media only allows for detection and not quantification of organisms with reduced susceptibility to ceftriaxone that are suspected to carry the <i>bla<sub>CMY-2</sub></i> genetic element. However, we believe that in the absence of selective pressure, either in the media or in the cow, that the number of organisms with the <i>bla<sub>CMY-2</sub></i> genetic element is quite low. Because we did not attempt to quantify the number of <i>E. coli</i> with reduced susceptibility to ceftriaxone in this study, we cannot draw any conclusions as to their true frequency in the fecal flora of dairy cows.

Detection of <i>S. enterica</i> from the dairy farms in the study was similar to other reports of <i>S. enterica</i> prevalence in dairy herds.<sup>7</sup> We were unable to recover <i>S. enterica</i> with reduced susceptibility to ceftriaxone from any cows in the study. Thus, our data provide no evidence that the use of ceftiofur in dairy cattle impacts the prevalence of <i>S. enterica</i> resistant to expanded-spectrum cephalosporin drugs.

In this study, the proportion of dairy cattle on a farm that were treated with ceftiofur was not associated with the recovery of <i>E. coli</i> with reduced susceptibility to ceftriaxone after controlling for the effect of <i>S. enterica</i> status of the farms and the rolling herd average for milk production and the random effects of the herd. A previous study that examined the association between ceftiofur usage on Ohio dairy farms and the recovery of <i>E. coli</i> with reduced susceptibility to ceftriaxone reported similar findings.<sup>155</sup> They reported a cross-sectional study of 18 Ohio dairy farms and
used farm records to determine ceftiofur exposure. They did not find an association between an individual cow’s ceftiofur exposure status and the recovery of *E. coli* with reduced susceptibility to ceftriaxone. However, they were able to detect a herd level association between ceftiofur use and the recovery of *E. coli* with reduced susceptibility to ceftriaxone. As in our study, selective media was used to determine the presence of absence of *E. coli* with reduced susceptibility to ceftriaxone, and the numbers of susceptible and reduced susceptibility *E. coli* were not quantified. Our results support their observation that treating a greater number of cows with ceftiofur does not increase the dissemination of *E. coli* with reduced susceptibility to ceftriaxone in the fecal flora of cows in the herd.

Although we were unable to detect an association between level of ceftiofur use on the farm and the recovery of *E. coli* with reduced susceptibility to ceftriaxone, we did observe a strong association between the recovery of *E. coli* with reduced susceptibility to ceftriaxone and the *S. enterica* status of the farm. We found that the odds of recovering *E. coli* with reduced susceptibility to ceftriaxone were much greater on farms in which we detected *S. enterica* compared to farms in which we did not detect salmonellae. We hypothesize that farms which are *S. enterica* “positive” may have different herd characteristics and management practices, other than the use of ceftiofur, which may select for *E. coli* with reduced susceptibility to ceftriaxone. It is possible that herd-level factors such as population density that are associated with *S. enterica* infections may also be associated with the dissemination of *E. coli* with reduced susceptibility to ceftriaxone in the flora of cows.
There are few reports on studies investigating the prevalence of *E. coli* with reduced susceptibility to third generation cephalosporins on dairy farms. In our study the percentage of farms in which *E. coli* with reduced susceptibility to third generation cephalosporins was recovered was greatly increased when compared to previous studies done on Ohio dairy farms. Both studies used selective media in the laboratory for the detection of the third generation cephalosporins. While the third generation cephalosporins resistance does seem to be increasing especially in *E. coli*, caution should be exercised when evaluating the clinical implications of this finding. Bacteria with this resistance phenotype are commonly hidden by the susceptible commensal flora in the absence of selective pressure whether in the media or in the animal. Had we not used selective media, it is highly likely that we would not have been able to detect these third generation cephalosporins organisms among all the susceptible *E. coli*. The increase in the percentage of farms from which *E. coli* with reduced susceptibility to third generation cephalosporins were recovered however is concerning because it may mean that the *bla*<sub>CMY-2</sub> gene conferring this resistance phenotype is becoming more widespread and therefore it maybe more available for transfer to pathogenic organisms frequently recovered from dairy farms.
Table 5.1. Summary statistics of 50 Ohio dairy herds involved in a study investigating the relationship between on-farm ceftiofur usage and recovery of resistant *E. coli*
Table 5.2. The prevalence of *E. coli* and *S. enterica* with reduced susceptibility to third generation cephalosporins isolated from 3840 lactating cows and 50 Ohio dairy farms and the mean prevalence of positive cows per farm for the 50 Ohio dairy herds.
<table>
<thead>
<tr>
<th>Covariate</th>
<th>OR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of cows treated with ceftiofur*</td>
<td>1.16</td>
<td>0.829 – 1.63</td>
</tr>
<tr>
<td>Farm <em>S. enterica</em>Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>28.96</td>
<td>3.75 – 224.06</td>
</tr>
<tr>
<td>Negative</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Milk Rolling Herd Average**</td>
<td>1.00</td>
<td>1.0001 – 1.0008</td>
</tr>
</tbody>
</table>

* OR applies to a 1 unit increase in the percentage of cows treated with ceftiofur
** OR applies to a 10 lb (4.54 kg) increase in milk RHA

Table 5.3. Summary of the mixed effect logistic regression model building investigating the relationship between the recovery of *E. coli* with reduced susceptibility to third generation cephalosporin antimicrobial drugs and ceftiofur usage on 50 Ohio dairy farms.
Chapter 6: Impact of ceftiofur administration on the dynamics of bovine fecal coliform bacteria populations

Abstract

Ceftiofur is a third generation cephalosporin drug licensed for use in food animals in the USA. Its use by the food animal industry is controversial because of concerns regarding the potential for zoonotic food-borne transmission of enteric bacteria resistant to extended-spectrum cephalosporins.

Our objective was to describe the dynamics of ceftriaxone-resistant fecal coliform bacteria following administration of ceftiofur to cattle. Mature, commercial beef and dairy cattle and commercial weanling beef cattle on farms in Ohio were included in 3 different trials. Treatment groups received label dose injections of ceftiofur sodium IM, while controls were injected IM with sterile water. Fecal samples were collected from individual animals for bacterial quantitative and qualitative culture. For all trials, the colony forming units of (CFU) fecal coliform bacteria and fecal coliform bacteria resistant to ceftriaxone were calculated.

In Trial 1, the CFU fecal coliform bacteria resistant to ceftriaxone in the fecal flora of ceftiofur treated cows peaked at 8.5x10³cfu/g 72 hr post injection then returned to undetectable levels by 168hr. In trials 2 and 3, no amplification of fecal
coliform bacteria resistant to ceftriaxone was detected. The results of our studies demonstrate that treatment of cattle with ceftiofur at label dosages may, but does not consistently result in amplification of fecal coliform bacteria resistant to ceftriaxone and that factors in addition to the presence of the resistance gene and antimicrobial selection pressure are likely required for amplification to occur.
Introduction

Ceftiofur is a third generation cephalosporin drug approved for veterinary use in livestock. It has a broad-spectrum of activity together with a short pre-slaughter withdrawal period and no milk withholding period (depending on the formulation), making it attractive for veterinary use in food animals. However, since the first report of a domestically acquired 3rd generation cephalosporin resistant S. enterica isolate from humans which was attributed to contact with ceftiofur-treated livestock,\textsuperscript{73} 3rd generation cephalosporin resistance in both human and veterinary salmonellosis cases rapidly increased.\textsuperscript{17,174} Much of the increase was attributable to the epidemic spread of the multi-drug resistant S. Newport\textsuperscript{74} which typically carried an ampC β-lactamase gene on a large plasmid.\textsuperscript{93} Veterinary ceftiofur use has been associated with the increased recovery of the \textit{bla}_{CMY-2} ampC β-lactamase gene in the fecal flora of livestock.\textsuperscript{155,160,161,172} Because many human cephalosporin-resistant salmonellosis cases have been linked epidemiologically to direct contact with cattle,\textsuperscript{49} the consumption of unpasteurized dairy products,\textsuperscript{173,174} or undercooked ground beef,\textsuperscript{142} the agricultural use of ceftiofur in food animals has been implicated as a potential public health risk.\textsuperscript{75,175}

While the potential causal link between ceftiofur therapy in food animals and emergence or dissemination of 3rd generation cephalosporin resistant bacteria has been investigated in dairy and beef cattle, the exact relationship is not fully
understood. A study of antimicrobial resistance in fecal flora of ceftiofur-treated calves evaluated fecal shedding of ceftriaxone-resistant bacteria by 3 Holstein calves treated with ceftiofur for 5 days. The prevalence of ceftriaxone resistant coliforms from these treated calves increased by 14% within 72 h of initial treatment, remaining at this level for nearly 2 weeks. Among a cohort of 61 feedlot steers, 3 different ceftiofur dosing regimens revealed a transient increase in 3rd generation cephalosporin-resistant fecal *E. coli* shedding which persisted for approximately 2 weeks before returning to baseline levels. A comparison of ceftiofur-resistant enteric *E. coli* from 5 ceftiofur-treated dairy cows and lactation-matched commingled controls on a 150 head dairy found that the quantity of resistant *E. coli* remained low with no amplification and was only detected by a significant reduction in susceptible *E. coli* counts. Antimicrobial susceptibility of fecal *E. coli* was evaluated in group a mature dairy cows receiving either ceftiofur or penicillin for common health ailments. While treatment did not affect the prevalence of fecal *E. coli* with reduced susceptibility to ceftiofur, a marked reduction in total fecal *E. coli* was noted on days 2 and 7 following therapy in ceftiofur treated cows.

To better understand the effect of ceftiofur administration on the intestinal flora of cattle and their untreated herdmates, we designed a study to test the hypothesis that administration of ceftiofur results in the amplification of fecal coliform bacteria resistant to ceftriaxone. It has been proposed that these commensal organisms in the intestinal tract may serve as a reservoir for antimicrobial resistance genes. Therefore the objectives of this study were to describe the dynamics of fecal coliform bacteria and fecal coliform bacteria resistant to ceftriaxone in bovine
feces following administration of ceftiofur to cattle housed in an agricultural environment.

Materials and Methods

We conducted 3 field trials as part of this study using commercial cattle in different production systems. We chose to evaluate these different production systems in order to help determine the impact of ceftiofur administration on fecal flora of cattle in each of these unique situations. The cattle were housed on farms in Ohio and were maintained on typical rations. No antimicrobial feed additives were included in the any of the rations.

Within trials, the study animals were systematically randomized to either treatment or control groups. Treatment groups received 2.2mg/kg bodyweight of ceftiofur sodium IM while controls were injected IM with 10cc of sterile water. Fecal samples were repeatedly collected using individual palpation sleeves from the rectum of individual animals for 7 to 9 d post-injection. Samples were collected in sterile collection tubes and transported back to the laboratory for immediate processing.

Quantitative estimates of MPN E. coli resistant to ceftriaxone were obtained by creating a series of 10-fold dilutions of 1g of feces. These fecal dilutions were spread-plated in duplicate on MacConkey Agar and MacConkey Agar containing 8μg/ml of ceftriaxone (Mac-Cef). Ceftriaxone was used at this concentration.
because it has been used successfully in our laboratory in other studies to detect both \textit{E. coli} and \textit{Salmonella} isolates with \textit{bla}_{\text{CMY}} resistant to ceftiofur and ceftriaxone \cite{84, 155, 178}. In addition, this concentration of ceftriaxone corresponded to the CLSI susceptible breakpoint for \textit{Enterobacteriaceae} at the time of the study. The resistant breakpoint concentration for ceftriaxone established by the CLSI for \textit{Enterobacteriaceae} is currently 4μg/ml. The plates were incubated for 24h at 37°C and lactose utilizing coliform bacteria were enumerated to estimate MPN CFU/g of feces.

Qualitative determination of the presence of \textit{E. coli} resistant to ceftriaxone was accomplished using selective media as we have previously reported \cite{84, 155}. A 10 g aliquot of feces was placed in 90 mL of nutrient broth containing cefoxitin 4 μg/mL and incubated at 37°C for 24 hours. The next day, MacConkey agar that contained ceftriaxone 8 μg/mL was inoculated using a sterile cotton-tipped swab and incubated for 24 hours.

Trial 1 was conducted using 15 mature Angus beef cows. These animals were taken from pasture and placed in a single dry-lot pen and received \textit{ad libitum} mixed grass hay. Five cows served as treatment animals and received a single dose of ceftiofur (2.2 mg/kg IM), and 10 cows were commingled controls. Samples were collected from each animal at 0, 4, 8, 12, 16, 24, 36, 48, 60, 72, 96, and 168 h following injection.

Trial 2 was conducted using 15 Angus and Angus-Simmental cross beef calves. Ten calves served as treatment animals and received ceftiofur once daily for 3 days (2.2 mg/kg IM) and 5 calves served as comingled control animals. Calves were
housed on a drylot pen after weaning and remained in the same lot for the duration of the trial. Only calves included in this trial were housed in this pen, and neighboring pens were empty so that no other animals could come into direct contact with the animals in Trial 2. Calves received *ad libitum* mixed grass hay and were fed a concentrate consisting of soy hulls, cracked corn and soy bean meal fed at 1.5% of bodyweight. Samples were collected from these calves at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 120 h from the initial injection.

Trial 3 was conducted using 15 lactating Jersey cows in a 130 head commercial dairy herd which consisted of both Holstein and Jersey cows. Throughout the trial, these cows were housed normally with their herdmates in a freestall barn. The cows were fed a total mixed ration twice daily which consisted of corn silage, soy bean meal, chopped grass hay and a mineral mix. In Trial 3, 10 cows received ceftiofur once daily for 3 days (2.2 mg/kg IM) and 5 cows served as controls. Fecal samples were collected from the cows at 0, 12, 24, 36, 48, 60, 72, 84, 96, 120, 144, 168, 180, 192 and 216h relative to the first injection of ceftiofur.

Use of the animals and all procedures in each trial were approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Results

Trial 1. Fecal coliform bacteria resistant to ceftriaxone were first detected in the fecal flora of cows treated with ceftiofur at 24 hr post injection (Figure 6.1B).
The CFU fecal coliform bacteria resistant to ceftriaxone in the fecal flora of treated cows peaked at $8.5 \times 10^3$ cfu/g 72 hr post injection then returned to undetectable levels by 168hr. Total CFU fecal coliform bacteria dropped markedly in treated cows beginning 8 hr post injection while concurrently increasing in control cows (Figure 6.1A).

**Trial 2.** We recovered fecal coliform bacteria resistant to ceftriaxone from a single control animal one week prior to the start of the trial, indicating its presence in the fecal flora of the study animals. Approximately 12 hr following ceftiofur administration, CFU total fecal coliform bacteria dropped to near or below detectable levels in the treated calves while remaining near baseline levels in untreated calves. We did not recover any fecal coliform bacteria resistant to ceftriaxone from any of the study animals using our quantitative method. We did recover fecal coliform bacteria resistant to ceftriaxone from 1 control calf and 2 treated calves at 84 hr using our qualitative method. We also recovered fecal coliform bacteria resistant to ceftriaxone at 120 hrs post injection from 3 treated calves and at 144 hrs from 7 treated calves and 1 control calf. See Figure 6.2 for a summary of the CFU results of non-selective media isolates.

**Trial 3.** Prior to the beginning of Trial 3, we detected fecal coliform bacteria resistant to ceftriaxone from the fecal flora of one of the dairy cows in the study population. After treatment with ceftiofur, CFU total fecal coliform bacteria counts decreased below detectable limits by 24 hr in the treated animals and then rose back to the level of the control cows by 96 hr. No amplification of fecal coliform bacteria resistant to ceftriaxone was detected in any of the animals using our quantitative
method, although isolates resistant to ceftriaxone were recovered from feces of both treated and control animals using our qualitative method throughout the study. See Figure 3 for a summary of the CFU results.

Discussion

Our results suggest that administration of ceftiofur to either beef or dairy cattle at label dosage may, but does not consistently result in the amplification of fecal coliform bacteria resistant to ceftriaxone in the intestinal tract of treated cattle. This implies that the amplification of resistant microorganisms may be a result of factors in addition to presence of the resistance gene in the intestinal flora and the administration of antimicrobial drugs providing selection pressure. These additional factors may include the ability of the flora to express or rapidly exchange resistance genes, or could include characteristics of the host animals or their environment that allow or prevent the amplification of resistant organisms. Additional work to elucidate these important factors may be warranted.

The results from Trial 1 using mature beef cows found that ceftiofur use resulted in an amplification of fecal coliform bacteria resistant to ceftriaxone and a decrease in susceptible organisms in the GI tract of treated animals. PFGE analysis of selected isolates from this trial (data not shown) suggested that the increased frequency with which these organisms were recovered may have been the result of amplification of a relatively few clones that were resistant to ceftriaxone that were
shared among individuals. Interestingly, we were able to recover clonal strains of fecal coliform bacteria resistant to ceftriaxone from both treated and control animals which suggests that there is widespread and rapid fecal contamination of the environment by treated animals which causes a shift in the microbiological flora of untreated penmates.

Previous studies have also reported that administration of ceftiofur resulted in the amplification of *E. coli* with resistance to various 3rd generation cephalosporins. Jiang *et al.* treated 3 calves with ceftiofur and monitored fecal concentrations of both susceptible and ceftiofur-resistant *E. coli.* They initially observed a rapid decline in the number of susceptible *E. coli* and a concurrent amplification of resistant *E. coli* which persisted until 17 days post injection at which time the levels of resistant and susceptible *E. coli* returned to baseline levels. Lowrance *et al.* found similar results when ceftiofur was injected into beef animals in a feedlot environment. They found that treating beef animals with ceftiofur resulted in the amplification of *E. coli* with resistance to multiple antimicrobial agents, including third generation cephalosporins.

Prior to beginning Trials 2 and 3 we were able to recover fecal coliform bacteria resistant to ceftriaxone from individuals in the study populations. However, we did not observe amplification of fecal coliform bacteria with resistant to third generation cephalosporins in either trial, although we were able to detect them. This result is similar to Singer *et al.* who reported that after administration of ceftiofur to dairy cattle, susceptible fecal *E. coli* decreased in number to low enough levels that allowed for the detection of rare ceftiofur resistant *E. coli.* Others have observed
that rare resistant strains are not amplified, but become detectable when the
predominant susceptible flora are reduced by antimicrobial therapy. Others have
reported no association between ceftiofur treatment and the isolation of resistant fecal
coliform bacteria, including *E. coli* at the individual cow level.  

Our results support the hypothesis that factors other than just the presence of
resistant organisms and antimicrobial selection pressure may be important in the
selection, amplification, and dissemination of antimicrobial resistant organisms in
food animals. Further work will be required in order to identify these additional
factors. These may be important in our efforts to understand the effect of agricultural
antimicrobial use on the zoonotic food-borne transmission of resistant organisms.
Figure 6.1. MPN total fecal coliform bacteria (A) and MPN total fecal coliform bacteria resistant to ceftriaxone recovered from fecal samples from beef calves treated with ceftiofur and control calves from the time of administration of ceftiofur (0hr) until 120 hr
Figure 6.2. MPN total fecal coliform bacteria recovered from fecal samples from beef calves treated with ceftiofur and control calves from the time of administration of ceftiofur (0hr) until 120 hr
Figure 6.3. MPN total fecal coliform bacteria recovered from fecal samples from dairy cows treated with ceftiofur and control cows from the time of administration of ceftiofur (0hr) until 120 hr
Chapter 7: Genetic and phenotypic characterization of the blaCMY gene from Escherichia coli and Salmonella enterica isolated from food-producing animals, humans, the environment and retail meat

Abstract

The $bla_{CMY-2}$ family of ampC $\beta$-lactamase genes confers broad spectrum resistance to $\beta$-lactam antimicrobials, including ceftriaxone and ceftiofur, as well as to $\beta$-lactamase inhibitors, such as clavulanic acid. Organisms with the $bla_{CMY-2}$ phenotype have been recovered from the environment and from retail meat products, posing a potential public health risk. The objectives of this study were to sequence the $bla_{CMY-2}$ gene from Escherichia coli and Salmonella enterica from multiple sources that had a reduced susceptibility to ceftriaxone and to determine the effect of observed mutations in the $bla_{CMY-2}$ gene on the antimicrobial resistance phenotype (spectrum and MIC/susceptibility patterns) of the isolates. The $bla_{CMY-2}$ genes from 52 bacterial isolates were sequenced for this study. Sixty-two percent (32/52) were E. coli and
38% (20/52) were *S. enterica*. Of the 32 *E. coli* isolates, 30 were found to carry a β-lactamase gene which was 100% homologous to *bla*<sub>CMY-2</sub>. One of the *E. coli* isolates was found to contain a gene that was 90% homologous to *bla*<sub>CMY-2</sub>. This isolate also had lower MICs to tetracyclines, streptomycin, and the sulfonamide antimicrobials than are commonly expected for isolates containing the *bla*<sub>CMY-2</sub>. Of the 20 genes obtained from *Salmonella* isolates, eight (40%) were found to be homologous to *bla*<sub>CMY-2</sub>, with no altered susceptibility phenotypes observed.
Introduction

Resistance to the third generation cephalosporin drugs in *Salmonella enterica* is frequently mediated by the β-lactamase gene *bla*$_{CMY-2}^7$. The *bla*$_{CMY-2}$ is a plasmid mediated ampC gene that is related to the chromosomal ampC gene of *Citrobacter freundii*. AmpC genes, including *bla*$_{CMY-2}$, confer reduced susceptibility or resistance to 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ generation cephalosporin drugs, as well as potentiated penicillins and the β-lactamase inhibitors such as clavulanic acid. Third generation cephalosporin antimicrobial drugs such as ceftriaxone are commonly used to treat invasive Gram-negative bacterial infections in humans and are the treatment of choice for pediatric cases of salmonellosis $^{72}$. It has been suggested that the agricultural use of the 3$^{rd}$ generation drug ceftiofur in food producing animals has provided selection pressure resulting in increased zoonotic food-borne transmission of the *bla*$_{CMY-2}$ gene that has resulted in increasing resistance to ceftriaxone in *S. enterica* infecting humans $^{73,121,180,181}$. Thus far, the *bla*$_{CMY-2}$ gene has not been reported to confer resistance to the 4$^{th}$ generation cephalosporin drugs.

Pathogens such as *Salmonella* species are less frequently recovered from the fecal flora of animals than are commensal *Escherichia coli*. The recovery of *S. enterica* with the *bla*$_{CMY-2}$ genotype is more likely to occur from clinically ill animals compared to clinically normal animals $^{182}$. Commensal *E. coli* may serve as a reservoir of resistance genes $^{138}$ including the *bla*$_{CMY-2}$, because they are able to share
genetic material via conjugation with potential pathogens such as *Salmonella* and *Klebsiella*. It has been reported that treating cattle with ceftiofur causes a transient shift in the commensal *E. coli* in the intestinal flora from a population that is predominantly susceptible to ceftiofur and other 3rd generation cephalosporins to a predominantly resistant population of bacteria\(^{160,161}\). Conversely, we have observed that this phenomenon occurs inconsistently and we have hypothesized that there are additional unknown factors that are required for the transient shift to *E. coli* with reduced susceptibility to 3rd generation cephalosporin drugs after administration of ceftiofur in agricultural animals.

The possibility that commensal flora may serve as a reservoir of resistance genes raises important questions regarding the similarities between the *bla*\(^{\text{CMY-2}}\) gene found in *E. coli* and *S. enterica*. Few epidemiologic studies of the *bla*\(^{\text{CMY-2}}\) gene have been reported. Reports from previous studies found that *bla*\(^{\text{CMY}}\) genes from *S. enterica* plasmids were at least 95% homologous to the *bla*\(^{\text{CMY-2}}\) genes reported to GenBank (Koeck, et al., 1997; Zhao, et al., 2001; Alcaine, et al., 2005).

Based on this information we hypothesized that the *bla*\(^{\text{CMY-2}}\) gene found in *E. coli* and *S. enterica* may be different and that the *bla*\(^{\text{CMY-2}}\) gene found in both *E. coli* and *S. enterica* may be evolving to acquire a wider spectrum of activity against β-lactam antimicrobial drugs, such as cefepime. To test our hypotheses, we designed a study with the following objectives: to determine if the *bla*\(^{\text{CMY-2}}\) ampC β-lactamase genes from *E. coli* and *S. enterica* are genotypically and phenotypically similar, and to determine if any identified alleles of the *bla*\(^{\text{CMY-2}}\) gene have an expanded spectrum of activity that might include the 4\(^{\text{th}}\) generation cephalosporin drugs.
Materials and Methods

Source of the isolates

A total of 52 E. coli and Salmonella isolates were included in this study (Table 7.1). The isolates were originally collected and archived as part of other research and surveillance projects conducted by the investigators, and were known to have a $bla_{CMY-2}$ phenotype.

Bacteria isolation and identification

Salmonella isolates were recovered using a variety of selective processes that were routinely used in the different laboratories in the different states. All Salmonella culture protocols utilized selective enrichment followed by differentiation agar and biochemical confirmation of suspected colonies. All E. coli isolates were recovered using the same selective process in our laboratory at The Ohio State University. E. coli were isolated by inoculating 90ml of nutrient broth, which contained 4μg/ml of the cephemycin drug cefoxitin, with 10g of feces and incubating overnight at 37ºC. The next day, sterile cotton tipped swabs were used to inoculate MacConkey Agar that contained 8μg/ml of the third generation cephalosporin drug, ceftriaxone which were incubated for 18-24 hours at 37ºC. Isolates were presumptively considered to be E. coli if they utilized lactose as indicated by pink colonies on MacConkey agar, and if they were able to metabolize tryptophan via the Indole test. To confirm reduced susceptibility to ceftriaxone, frozen Salmonella isolates were recovered following the procedures used for the recovery of E. coli isolates described above.
PCR amplification, DNA sequencing and cloning

For further molecular characterization purposes, an isolated colony was selected from MacConkey agar containing 8μg/ml of ceftriaxone and was inoculated onto 5ml of Lauria-Bertani broth which contained 8μg/ml of ceftriaxone and incubated at 39°C for 24 hours. Plasmid DNA was extracted from the 5ml of Lauria-Bertani broth using an alkaline lysis method following procedures recommended by a commercial kit.

PCR was performed using consensus primers previously published to determine if the plasmid mediated \( \text{bla}_{\text{CMY-2}} \) gene was present in the plasmid DNA.\(^{138, 145}\) Primers used were F: ATGATGAAAAAATCGTTATGC; R: TTGCAGCTTTTCAAGAATGCGC. This primer pair is known to amplify the following genes: \( \text{bla}_{\text{CMY-2}}, \text{bla}_{\text{BIL-1}}, \text{bla}_{\text{LAT-1}}, \text{bla}_{\text{LAT-2}}, \) and the \( \text{ampC} \) gene of \( \text{Citrobacter freundii} \), and produce a PCR product of 1146 bp in size in all these genes. The PCR product was cloned into a commercial vector and competent cells were transformed following manufacturers guidelines.

Plasmid vectors which contained the \( \text{bla}_{\text{CMY-2}} \) gene were submitted to a sequencing laboratory and sequence data was analyzed using commercial software. Consensus sequences were built using a minimum of three forward and three reverse sequences. The consensus sequences were compared to the \( \text{bla}_{\text{CMY-2}} \) gene sequence from NCBI using BLAST and \( \text{Klebsiella pneumoniae} \) described by Baurenfiend et al.\(^{139}\) which was used as the standard in this study. If a mutation was detected, a different clone was selected and a new consensus sequence was created to confirm the initial results. This was done to ensure that the mutation observed was not a
sequencing error or due to a DNA copy error by the selected clone. Completed consensus sequences were then compared to sequences found in GenBank (NCBI). Any consensus sequences which were different from the standard \( \text{bla}_{\text{CMY-2}} \) sequence were then subjected to antimicrobial susceptibility testing using commercial microbroth dilution methods to determine if these alleles produced changes in minimum inhibitory concentration (MIC) levels compared to the standard \( \text{bla}_{\text{CMY-2}} \) organisms.

MICs to a variety of antimicrobial drugs were determined for each isolate that carried an allele of \( \text{bla}_{\text{CMY-2}} \) gene and for 6 isolates that carried the \( \text{bla}_{\text{CMY-2}} \) genetic element. The MICs were determined using a Sensititre semi-automated broth micro-dilution system following manufacturer’s guidelines. The ATTC \( \text{E. coli} \) 25922, \( \text{Enterococcus faecalis} \) 29212, and \( \text{Pseudomonas aeruginosa} \) 27853 were used as quality control organisms as recommended by CLSI \(^{183}\). The MIC values for each antimicrobial from each isolate that carried an allele of \( \text{bla}_{\text{CMY-2}} \) were compared to the MICs of isolates which carried \( \text{bla}_{\text{CMY-2}} \) and evaluated to identify potential changes in antimicrobial spectrum.

Results

Of the original 52 \( \text{Salmonella} \) and \( \text{E. coli} \) isolates with the \( \text{bla}_{\text{CMY-2}} \) phenotype that we analyzed, 73\% (38/52) were confirmed to contain the \( \text{bla}_{\text{CMY-2}} \) genetic element.
We recovered the \textit{bla}\textsubscript{CMY-2} gene from nearly all (94%, 30/32) of the \textit{E.coli} isolates with only two (6%, 2/32) containing genes other than \textit{bla}\textsubscript{CMY-2}. One of the two genes, which was recovered from a swine fecal sample collected in Ohio, had only a single nucleotide change at position 209. This nucleotide change resulted in an amino acid change at position 70. The other gene was recovered from a retail package of ground pork purchased in Ohio and was only 90% homologous to \textit{bla}\textsubscript{CMY-2} and did not match any known sequences in GenBank. This allele is unusual in that it maintains the elevated second and third generation cephalosporin MICs that are characteristic of the \textit{bla}\textsubscript{CMY-2} phenotype, however, it has markedly lower MICs to other drugs, such as tetracyclines, streptomycin and sulfonamides, against which organisms with the \textit{bla}\textsubscript{CMY-2} phenotype are commonly resistant.

Unlike the \textit{E.coli} isolates, the majority (60%, 12/20) of the \textit{S. enterica} isolates were found to be alleles of the \textit{bla}\textsubscript{CMY-2}. These alleles had single or multiple point mutations throughout the length of the gene. In contrast, only eight (40%, 8/20) were found to be homologous to \textit{bla}\textsubscript{CMY-2}. The alleles were from isolates from a variety of sources from multiple states including N. Dakota, Ohio, Colorado and Pennsylvania. The majority of these genes (7 of 12) had single non-synonymous nucleotide substitutions that resulted in amino acid changes (Table 7.1). A single \textit{Salmonella} Typhimurium isolate from Colorado had a single nucleotide change, which did not result in any change in the amino acid sequence of the protein. There was a single \textit{S. Newport} isolate recovered from a human from N. Dakota which had five nucleotide changes, resulting in four amino acid substitutions. Additionally there were three different serovars from differing sources that were found to have the identical
nucleotide substitution at position 461, which resulted in the same amino acid substitution at position 154. These isolates were an S Agona isolate from a human source, a S. Newport isolate from a sample of retail ground turkey, and an S. Typhimurium isolate from a human source.

Although we detected many alleles of bla\textsubscript{CMY-2}, all the alleles recovered from the Salmonella isolates maintained the typical ampC/bla\textsubscript{CMY-2} phenotype and were found to have reduced susceptibility or were resistant to the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generation cephalosporins. All isolates were sensitive to the 4\textsuperscript{th} generation cephalosporin drug cefepime and also to imipenem and meropenem.

Discussion

It has been previously reported that ampC β-lactamase genes carried by S. enterica isolates that have the bla\textsubscript{CMY-2} phenotype are 95-100% homologous with the bla\textsubscript{CMY-2} in GenBank\textsuperscript{144,145,158}, which agrees with our results. Others have shown that the bla\textsubscript{CMY-2} gene is carried by 4 different types of plasmids \textsuperscript{73,93,138,146}. These plasmids are known to be found in both E. coli and S. enterica in low copy numbers \textsuperscript{147}. It has been reported that E. coli are more likely than Salmonella to possess plasmids from different species, and also that the plasmids recovered from S. enterica are likely to come from other Salmonella\textsuperscript{184}. This suggests that E. coli are more promiscuous and are able to collect genes from a greater variety of sources compared
to *Salmonella* and that there may be more mutations found in *bla*<sub>CMY-2</sub> from *E. coli* rather than *Salmonella*.

It has also been shown that *E. coli* typically have plasmids derived from both *S. enterica* and *E. coli* while plasmids found in *Salmonella* typically have been derived from *Salmonella*<sup>184</sup>. These authors hypothesized that the transmission and the epidemiology of the *bla*<sub>CMY-2</sub> gene may be different among *E. coli* and *S. enterica*. Our work on the genetics of the *bla*<sub>CMY-2</sub> shows that there are more alleles of *bla*<sub>CMY-2</sub> found in *Salmonella* compared to *E. coli* which supports this hypothesis.

Barlow and Hall studied the plasticity of the *bla*<sub>CMY-2</sub> gene *in vitro* and compared it to the *bla*<sub>TEM-1</sub> gene in its ability to change to confer resistance to the 4<sup>th</sup> generation cephalosporin drug cefepime<sup>185, 186</sup>. They found that *bla*<sub>CMY-2</sub> does have the ability to mutate and evolve to confer this expanded resistance. Additionally, in their study of *bla*<sub>CMY-1</sub>, which is also an *ampC* β-lactamase, Bauernfeind et al. found that a single amino acid substitution in the catalytic area of *bla*<sub>CMY-1</sub> caused the MIC to ceftazidime to shift from the susceptible breakpoint to the resistant breakpoint<sup>135</sup>. The catalytic area of active site serine β-lactamases, such as *bla*<sub>CMY-1</sub> and *bla*<sub>CMY-2</sub> is at position 64. Based on our sequence data, we did not find any amino acid substitutions at this position; however, we felt that substitutions in other areas of the protein may have affected the tertiary structure of the protein enough to alter the antimicrobial spectrum of the β-lactamase. To determine if this was true the alleles of *bla*<sub>CMY-2</sub> were subjected to antimicrobial microbroth dilution testing. We were unable to detect any difference in the antimicrobial spectrum of activity from any of the alleles of *bla*<sub>CMY-2</sub> sequenced in this study. However, we did find that the *bla*<sub>CMY-2</sub>
gene is evolving in vivo but that this evolution appears to be occurring more frequently in *S. enterica* than in *E. coli*. Based on these results, we postulate that if the *bla*$_{CMY-2}$ gene widens its spectrum of activity it will first occur in *S. enterica* rather than *E. coli*.

Analysis of our data revealed that one of the *bla*$_{CMY-2}$ alleles was found in three different *Salmonella* serovars from North Dakota. This finding may have at least two different meanings. The first is that this may provide evidence that an allele of *bla*$_{CMY-2}$ is being transferred between different serovars of *S. enterica*. The isolates were *S. Typhimurium* and *S. Agona* from separate human diagnostic samples and *S. Newport* isolated from retail turkey meat. These three isolates all have the same amino acid substitution at position 154. The mechanism by which the isolates are sharing this allele is not known at this time, but could be via conjugation or by a transposable element such as an integron or transposon. Another possibility for finding the same allele in differing serovars of *Salmonella enterica* is that the allele may confer a selective advantage and may have evolved independently in response to a similar but unknown selective pressure. Further characterization of these isolates may be needed to determine if this allele of *bla*$_{CMY-2}$ is transferable and able to be passed between bacterial organisms.

Following analysis of the consensus sequences, there is broad diversity in the *bla*$_{CMY-2}$ gene and this diversity is not concentrated in any particular area of the gene, especially in *S. enterica*. The difference in the number of alleles of the *bla*$_{CMY-2}$ gene in *S. enterica* compared to *E. coli* may be due to the differing stress responses in these organisms. These stress responses in *Salmonella* may increase the mutation rate in
the genetic code of the bacteria when it is exposed to a toxic substance and allow for survival of the clone with the mutation that confers a selective advantage.\textsuperscript{187, 188} We hypothesize that because \textit{S. enterica} are pathogens and that many infections are frequently treated with antimicrobial agents that they have evolved to have inaccurate DNA polymerases, which increase the rates of mutation when compared to \textit{E. coli}.

Other reasons why we may have detected differences in the genetic sequences of \textit{bla}_{CMY-2} from \textit{E. coli} and \textit{S. enterica} may have been due to the effect of clustering. The isolates in our study were recovered as part of research projects in 7 U.S. states. Isolates that were recovered from the same source are not necessarily independent and may be more alike than isolates from other sources. Our study design does not allow us to account for the potential clustering of isolates but we believe it had a minimal effect on our conclusions.

In addition to the effect of clustering our results may have been influenced by the use of selective media used in our bacterial culture procedures. All \textit{E. coli} isolates with reduced susceptibility to ceftriaxone included in this study were recovered in a single laboratory using the same selective methods. These methods may only select for \textit{bla}_{CMY-2} and not for alleles of \textit{bla}_{CMY-2}. The media used for the isolation of \textit{S. enterica} from the various samples did not contain the 3\textsuperscript{rd} generation drug, ceftriaxone and may allow alleles of \textit{bla}_{CMY-2} to remain. All \textit{S. enterica} isolates were subjected to the same selective media that had been used for recovery of the \textit{E. coli}, and all were successfully recovered. However, the recovery of a single isolate removed from its original source may be different than the recovery of an isolate from among the complex matrix of organic substances and flora found on or in the original
source. The actual effect of our different isolation procedures for *E. coli* and *Salmonella* is not known, but may have influenced our results and warrants further investigation.
<table>
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<tr>
<th>State</th>
<th>Microorganism</th>
<th>Source</th>
<th>N</th>
<th>% bla&lt;sub&gt;CMY-2&lt;/sub&gt; Homology</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
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</tr>
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<td></td>
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<td>154</td>
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<td><em>Salmonella Dublin</em></td>
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<td><em>Salmonella Newport</em></td>
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<td>0</td>
</tr>
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<td></td>
<td></td>
<td>Human feces</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Turkey Breast</td>
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<tr>
<td></td>
<td></td>
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Table 7.1. Results of sequence analysis of the bla<sub>CMY-2</sub> from 20 *Salmonella enterica* isolates and 32 *E. coli* isolates with either reduced susceptibility or resistance to ceftriaxone recovered from a variety of sources in 6 states.
Table 7.1 continued

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<th>State</th>
<th>Microorganism</th>
<th>Source</th>
<th>N</th>
<th>% bla&lt;sub&gt;CMY-2&lt;/sub&gt; Homology</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
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References


11. Smith BP, Oliver DG, Singh P, Dilling G, Martin PA, Ram BP, Jang LS, Sharkov N, Orsborn JS. Detection of *Salmonella* Dublin mammary gland infection in carrier...


61. R. A. Smith, editor. Decreased fecal shedding of *Salmonella* Newport through vaccination. 36th annual convention proceedings; September; Stillwater, OK: American Association of Bovine Practitioners; 2003. 185 p.


163. McKellar QA. Antimicrobial resistance: A veterinary perspective. antimicrobials are important for animal welfare but need to be used prudently. BMJ 1998 Sep 5;317(7159):610-1.


166. [Anonymous](2003) Rockville, MD 152.


