ABSTRACT

Title of Document: ANTIMICROBIAL RESISTANCE OF *SALMONELLA* AND *E. COLI* FROM PENNSYLVANIA DAIRY HERDS

Huilin Cao, Master of Science, 2015

Directed By: Abani K. Pradhan, Ph.D., Department of Nutrition and Food Science

The emergence and dissemination of bacterial antimicrobial resistance has become a major public health concern. A total of 444 manure composite samples were collected from 80 dairy farms in Pennsylvania, representing pre-weaned calves, post-weaned calves, dry cows, and lactating cows. *E. coli* and *Salmonella* were isolated, and tested for antimicrobial susceptibility. *Salmonella* was isolated from at least one sample from 51 (64%) farms and was more prevalent in adult animals than young animals. The predominant serotypes were Cerro, Montevideo and Kentucky. *Salmonella* isolates were mostly susceptible to all antimicrobials. *E. coli* were commonly resistant to tetracycline, streptomycin, sulfisoxazole and ampicillin. Resistance of up to 8 classes of antibiotics was observed in *E. coli* isolated from young animals. The bla_{CMY}- and bla_{CTX-M}-carrying *E. coli* were detected in 35% and 5% of the farms, respectively. The presence of multi-drug resistant *E. coli* suggested potential risks to human health associated with dairy farming.

ANTIMICROBIAL RESISTANCE OF *SALMONELLA* AND *E. COLI* FROM PENNSYLVANIA DAIRY HERDS

By

Huilin Cao

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2015

Advisory Committee:
Dr. Abani K. Pradhan, Chair
Dr. Jo Ann Van Kessel
Dr. Seong-Ho Lee

© Copyright by
Huilin Cao
2015

Acknowledgments

I would like to gratefully and sincerely thank Dr. Abani Pradhan for his guidance, support, caring, and most importantly, his friendship as my academic advisor during my graduate studies at the University of Maryland. His mentorship was paramount in providing a well-rounded experience and encourages me to grow as a researcher and an independent thinker. I would like to thank Dr. JoAnn Van Kessel for everything she has done for me, especially the mentorship and patience through the study, and also unique opportunity she provided in Agriculture Research Service at the U.S. Department of Agriculture (USDA). Moreover, it would not be possible to complete my research without the help from many other people, including the scientists and technicians from the USDA and the Pennsylvania State University.

I would like to thank Dr. Seong-Ho Lee for serving as my committee member and giving me invaluable advices to my research. I would like to thank Dr. Jeffery Karns and Dr. Elisabetta Lambertini for the precious discussions and suggestions during my research throughout my program. I thank my colleagues and friends, Jakeitha Sonnier, Laura Del Collo, Abhinav Mishra, Hao Pang, Miao Guo, and Miao Wang, for their encouragement and support. In particular, I would like to thank Brendan Cone and Carole Cone. Their love and faith were undeniably the bedrock upon which the past two years my life have been built. Finally, I would like to express my gratitude to my parents Zhiling Chen and Xiaodong Cao for their unwavering love that allowing me to be as ambitious as I wanted.

Table of Contents

List of Tables

the panel (n=285) for each type of samples. Prevalence were also calculated for young and adult animal samples, respectively, and on farm-level.

List of Figures

on NARMS GN Panel (n=285) and young animal data were pooled from results of pre-weaned and post-weaned samples, adult animal data were pooled from results of dry cow and lactating cow samples.

Figure A3. Line graph showing accumulative prevalence of multiple-classes drug resistance *E. coli* for pre-weaned calves, post-weaned calves, dry cows and lactating cows.

Figure A4. Resistance to various numbers of classes of antibiotics among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel suggesting that higher prevalence of resistance in lactating cows might partially contributed by the elevated sample size and increased detection limit.

Figure A5. Minimum inhibitory concentrations (MICs) distribution for ceftiofur among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel (n=285). Regression was generated using excel (R^2=0.99) suggesting CLSI cut-off is not suited for distinguishing wild and non-wild type in this study.

Chapter 1: Introduction

The emergence and dissemination of antimicrobial resistance in bacteria has become a major public health concern (WHO, 2014). In clinical settings, pathogens resistant to multiple classes of important antibiotics could complicate the treatment by significantly limiting therapeutic options. Infections caused by drug resistant bacteria can lead to failure of treatment, prolonged hospital stays, higher health care expenditures, and increased morbidity and mortality (ECDC, 2013). β-lactams, including cephalosporins, are a class of antibiotics of critical importance in human medicine (WHO, 2014). Ceftriaxone, one of the third-generation cephalosporins, is used for treatment of severe salmonellosis in children (Rabsch et al., 2001). The increasing prevalence of resistance to first-, second-, and third-generation cephalosporins has been reported worldwide in isolates from food-producing animals. Resistance to cephalosporins in Enterobacteriaceae, including *E. coli* and *Salmonella*, is mainly caused by production of AmpC-type β-lactamases and Extended spectrum β-lactamases (ESBLs) (Bonnet, 2004; Zhao and Hu, 2012). The ESBL/AmpC genes are often located on plasmids of Enterobacteriaceae that able to transfer intra-species and inter-species.

The objective of this study was to conduct a cross–sectional survey of dairy farms in Pennsylvania to investigate the scope of resistance problem. Rather than focusing on either lactating cows or pre-weaned calves exclusively, four different animal groups were examined on each farm, including pre-weaned calves, post-weaned calves, lactating cows and dry cows.

Chapter 2: Literature Review

2.1 Burden of antimicrobial resistance

The emergence and dissemination of antibiotic resistance (AR), including multidrug resistance (MDR), is an increasing problem around the world. Antimicrobial resistance is the phenomenon when a microorganism survives exposure to an antimicrobial agent at a concentration to which wild-type forms are normally susceptible. MDR is commonly defined as resistance to three or more classes of antibiotics. When infection occurs in community, MDR human pathogens are able to withstand attack by several classes of antibiotics. MDR can then greatly limit the choices of antibiotic therapy, resulting in substantial economic burden to the society. Infections associated with AR cost an estimated $20 billion in excess health care expenses and $35 billion in other societal costs annually in the U.S. (CDC), 2013) and cost €1.5 billion annually the European Union (EPHA, 2012).

In clinical settings, antibiotic resistance is also implicated in failure to respond to the standard treatment, prolonged illness, and a greater risk of death, in addition to the increased treatment costs (WHO, 2014). It is estimated that in the U.S. more than 2 million people are infected with AR bacteria annually, with 23,000 deaths as a direct result (CDC, 2013). In Europe 25,000 people die each year as a result of MDR bacterial infection (European Commssion, 2011).

When infections persist due to the ineffectiveness of antibiotics, the chance of spread of the resistant bacteria would increase. The global emergence and spread of bacteria with new resistance mechanisms threaten our ability to treat

common infectious diseases. It has become very difficult to stay ahead of the rapid acquisition of AR by some important pathogens (Doyle, 2015). For example, resistance to one of the most widely used antibacterial drugs for the oral treatment of urinary tract infections, fluoroquinolones, caused by *E. coli* is very widespread. Moreover, resistance to the treatment of last resort for life-threatening infections, carbapenem antibiotics, caused by gastrointestinal pathogens has spread to all regions of the world: key tools to tackle the resistance problem are tracking and monitoring resistance to reveal information gaps (WHO, 2014).

2.2 Acquired resistance mechanisms to major antibiotic classes

Although much attention has been focused on AR in pathogens, the development of resistance to antibiotics is a natural ecological phenomenon and genes for resistance to antibiotics, like the antibiotics themselves, are ancient (D'Costa et al., 2011). Ancient resistance genes in a microorganism could be a product of a series of spontaneous or induced genetic mutations. Exposure to an antibiotic naturally selects the strain carrying the corresponding resistance gene. The reservoir of resistance genes can be mobilized and can transfer into human pathogens (Blair et al., 2011). For example, many antibiotic resistance genes reside on transmissible genetic elements, such as plasmids and transposons, facilitating them to transfer inter-species. Horizontal transfer of resistance genes could also occur through transduction or transformation. It has been observed that plasmids and transposons sometimes contain genes conferring resistance to several different antibiotics, enabling co-selection of the resistance genes.

Researchers have been trying to understand the mechanisms by which bacteria successfully defend themselves against actions of antibiotics (Lin et al., 2015). Acquired resistance mechanisms play critical roles in the emergence and dissemination of resistance in the "post-antibiotic" era. Acquired resistance mechanisms can be categorized into three groups: elimination of the intracellular concentrations of the antibiotic, modification of the antibiotic target, and inactivation of the antibiotic. A good example of the first mechanism is efflux pump. The multidrug efflux systems contribute significantly to the increased resistance to multiple antibiotics in bacteria (Lin, 2015).

Mutation of the target site is another important mechanism, and usually results in a functional target with reduced affinity for the antibiotic, which does not bind efficiently and therefore has a reduced effect (Blair, 2015). A good example is mutations in topoisomerase genes in many species that confer fluoroquinolone resistance. Fluoroquinolones are regarded as critically important antimicrobial agents for human medicine according to WHO criteria, and cross-resistance to fluoroquinolones such as ciprofloxacin (a metabolite of enrofloxacin which is approved for treatment of food-producing animals) poses a formidable threat to public health (Collignon et al., 2009). In gram-negative bacteria, such as *E. coli*, high levels of quinolone resistance are mainly due to mutation of the genes encoding for the gyrase subunits gyrA.

In addition, bacteria can destroy or modify antibiotics, thus resisting their action in the form of hydrolyzing the antibiotic or addition of a chemical group (Blair, 2015). Thousands of enzymes have been identified that can degrade and

modify antibiotics of different classes, including β-lactams, aminoglycosides, phenicols and macrolides. β-lactam antibiotics are the most widely available antibiotics used to treat a number of bacterial infections and the subclasses include cephalosporins, penicillins, carbapenems, monobactams and clavams. Members of β-lactam antibiotics contain a β-lactam ring and take effect by inhibiting proper cross-linking of bacterial cell walls (Lin, 2015). Resistance to β-lactam antibiotics is mainly due to production of β-lactamase, an enzyme that inactivates the drug.

The early β-lactamases, such as TEM-1 and SHV-1 β-lactamases, which were active against first-generation β- lactamases were followed by extended-spectrum β-lactamases (ESBLs) which become active against third-generation cephalosporins. ESBLs have emerged in parallel and disseminated through enteric bacteria of both humans and animals. In most cases, ESBLs are not capable of hydrolyzing cephamycins e.g., cefoxitin, and are readily inhibited by clavulanic acid e.g., Augmentin (amoxicillin/clavulanic acid) (Bell and Fisher, 2009). ESBLs are found in many members of Enterobacteriaceae and one type of ESBL that is increasingly detected is the CTX-M family, which is plasmid mediated and notable for greater activity against cefotaxime. The original source of the gene encoding the CTX-M ESBLs is the chromosome of the enteric bacterium, *Kluyvera ascorbate*, that was originally isolated from humans (Humeniuk et al., 2002). *K. ascorbata* is a commensal bacterium of both humans and animals, and selective pressure led to the mobilization of its beta-lactamase onto a plasmid, which was then shared among commensal bacteria such as *E. coli* (Humeniuk et al., 2002). By 2007 in the U.S., 80% of 15 geographically dispersed medical

centers reported *E. coli* or *Klebsiella pneumonia* infections with strain carrying associated $bla_{\text{CTX-M}}$ genes (Castanheira et al., 2008).

Another type of plasmid-mediated cephalosporinase has arisen through transferring of genes coding for the chromosomal AmpC β-lactamases, which contribute greatly to cephalosporin resistance in *E. coli, K. pneumonia* and *Salmonella* species (Bell and Fisher, 2009). This type of β-lactamases cannot be inhibited by clavulanic acid. Other newly emerged type of β-lactamases are carbapenemases, including the IMP (imipenemase), VIM (Verona integrin encoded metallo β-lactamase), KPC (*K. pneumonia* carbapenemase), and OXA (oxacillinase). They have serious implications in hospital settings, but have rarely been detected in isolates from food-producing animals.

Aminoglycosides are another class of clinically important antibiotics for treating various bacterial pathogens. Examples of aminoglycosides are gentamicin, tobramycin, streptomycin and kanamycin. The increasing resistance of clinical isolates against aminoglycosides, however, has compromised the effectiveness of this class of antibiotics. Aminoglycosides act by binding to the 30S subunit of the prokaryotic ribosome and interrupting the translation process. Aminoglycoside antibiotics are particularly susceptible to modification as they tend to be large molecules with many exposed hydroxyl and amide groups. Production of aminoglycoside-modifying enzymes was considered as the major mechanisms for aminoglycoside resistance (Lin, 2015).

2.3 Impact of antibiotic use in food-producing animals

Antibiotics are used in food-producing animals for treatment of disease and are critical for animal welfare and food safety. Regardless of the benefits of using antimicrobial agents in food-producing animals, concerns from public health, food safety, and regulatory perspectives arise from the potential for development of antimicrobial resistance (Oliver et al., 2011). Research has been conducted to understand the role of agricultural antibiotic usage in the global AR emergence and dissemination problem. Recent analyses of metagenomics sequences from beef cattle feces, chicken ceca, and swine feces all reveal an abundance of resistance genes regardless of antibiotic treatment. In a study of conventionally raised beef cattle with no exposure to therapeutic antibiotics, sequence-based metagenomics predicted that 3.7% of the sequences encoded resistance to antibiotic and toxic compounds; around 50% of genes harbor multi-drug resistance efflux pumps (Durso et al., 2011).

Evidence supports agricultural usage of antibiotics was linked to the increasing prevalence of resistance. Among seven European countries for whom antibiotic use and antibiotic resistance data were available, a clear correlation was seen between antibiotic use and resistance gene prevalence in food animals (Chantziaras et al., 2014). However, knowledge gap exists in data supporting the direct link of antibiotic use and emergence of resistance. Little is known about the distribution of the resistance bacteria in different reservoirs on farm and how the reservoirs assist its persistence. Another knowledge gap lies in data on the dynamics of resistance gene transfer between commensal microorganisms and

human pathogens (Oliver et al., 2011). Understanding the dynamics is important because increased antibiotic resistance in human pathogens can seriously threaten public health. Humans may potentially be exposed to antimicrobial resistant pathogens through a variety of routes, including foods from livestock carrying resistant bacteria, direct contact with farm animals, farm environments, fresh produce fertilized by contaminated manure, and irrigation water carrying resistant pathogens. Li et al. (2014) have shown that groundwater can be potentially contaminated by antibiotic resistant bacteria originated from dairy farm. Survival of drug-resistant bacteria in manure and waste lagoons was observed in two dairy farms in California resulting spread from these sources to ground water (Li et al., 2014). River water downstream from concentrated animal feeding operations in the U.S. contained much higher levels of MDR bacteria than the reference sites (West et al., 2011).

2.4 Monitoring resistance in livestock and food

In the U.S., the National Antimicrobial Resistance Monitoring System (NARMS), a collaborative effort among the U.S. Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC) and U.S. Department of Agriculture (USDA), tracks changes in the susceptibility of bacteria to antimicrobial agents of importance. Bacteria monitored in NARMS are not only clinical human isolates, but also isolates from retail meats and food-producing animals. According to NARMS 2011 Retail Meat Report, MDR *Salmonella* and MDR *Escherichia coli* were detected in 11.1% and 6%, respectively, among all the

Salmonella and *E. coli* isolated from the ground beef samples (NARMS, 2013). Moreover, MDR *Salmonella* was recovered from 28.7% of cattle carcass swabs obtained at federally inspected slaughter and processing plants. Prevalence of MDR *E. coli* was 38.3% in chicken carcass, and 37.5% in retail chicken; prevalence of MDR *Salmonella* was 27.9% in swine, and 28.6% in pork chop (NARMS, 2013).

European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) also publish a yearly report on antimicrobial resistance in bacterial isolates from humans, livestock and food. The MDR *Salmonella* spp., in cattle was reported to be 34.2% in 2012, which was lower than *Salmonella* prevalence in poultry and swine, respectively. MDR *Salmonella* isolates from retail chicken (64.2%) and pork (50.9%). In livestock, MDR *Salmonella* were isolated from 46.4% of the poultry isolates and 73.5% of the swine isolates. MDR *E. coli* was 1.1% in broilers and 30.9% in pigs, and data are not available for *E. coli* in retail meat (EFSA, 2014).

2.4.1 *Salmonella* serotype and resistance in retail meat

The latest NARMS 2013 Retail Meat Interim Data revealed the top serotypes among *Salmonella* isolates from retail ground beef samples collected in 2013. Fifteen *Salmonella* isolates from 1663 ground beef samples belong to serotype Dublin (26.7% 4 isolates), Montevideo (26.7% 4 isolates), Infantis (13.3% 2 isolates), Kentucky (6.7% 1 isolates), and others. Among the 15 isolates, 46.7% (7) were pan-susceptible on NARMS GN Panel and none was resistant to

quinolones or macrolides (azithromycin) (FDA, 2015). However when compared with retail chicken (19.7%), ground turkey (9.4%) and pork chop (0%), prevalence of ceftriaxone resistant *Salmonella* (26.7%) among *Salmonella* isolates from ground beef was higher. Multidrug resistant *Salmonella* (5) was detected among 33.3% of isolates, which was comparable to pork chop prevalence, but higher than retail chicken prevalence (26%), and lower than ground turkey prevalence (39.6%). The 2012 Retail Meat Report showed the top serotypes among *Salmonella* isolates from retail ground beef (n=13 N=1300 1.0%) were Dublin (30.8 % 4 isolates), Cerro (15.4%, 2 isolates), Newport (7.7%, 1 isolate), Kentucky (7.7%, 1 isolate), Typhimurium (7.7%, 1 isolate), Montevideo (7.7%, 1 isolate), Anatum (7.7%, 1 isolate) and Agona (7.7%, 1 isolate). Among these isolates, all of the Cerro, Montevideo, Newport and Kentucky were pan-susceptible, and 3 of the 4 *S.* Dublin were multidrug resistant (FDA, 2013). In comparison, *Salmonella* Kentucky isolated from retail chicken were mostly (88.7%) resistant to at least one antibiotic, with 22.6% of them being multidrug resistant and 21.0% exhibiting resistance to ceftriaxone.

2.5 Emergence of multi-drug resistant Salmonella

In the mid-1990s, widespread reports of *Salmonella* Typhimurium DT104 in meats and livestock were the first indication of the emerging problem of resistance (Doyle, 2015). DT104 is resistant to amplicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, a resistance pattern designated as ACSSuT. Multi-drug resistance has increased in other *Salmonella* serovars,

including *Salmonella* Newport and *Salmonella* Heidelberg. The ACSSuT pattern has been found among different serotypes in human isolates and was identified in 17% of *Salmonella* Typhimurium, 4% of *Salmonella* Newport, and 88% of *Salmonella* Dublin isolates according to NARMS 2012 report (CDC, 2014). Resistance to ceftriaxone and ciprofloxacin also increased during this time (EPHA, 2012; WHO, 2014).

MDR *Salmonella* Kentucky is another common serotype that may pose a threat to food safety. Highly drug-resistant *Salmonella* Kentucky ST198-X1 strain was recently detected in poultry flocks and turkey meat in Europe and Canada (Le Hello et al., 2013; Mulvey et al., 2013). In France during 2000-2008, about 40% of *Salmonella* Kentucky isolates were resistant to ciprofloxacin; in 2009-2011, the percentage increased to 83%. Some *Salmonella* strains were also observed to be resistant to carbapenems, fluoroquinolones, trimethoprim-sulfamethoxazole, and azithromycin (Le Hello et al., 2013). Based on the 2012 CDC NARMS Human Isolates Report, Enteriditis was the most common serotype among nalidixic acid-resistant non-typhoidal *Salmonella* isolates, and the most common serotypes among ceftriaxone-resistant isolates were Newport (7%), Typhimurium (5%), Heidelberg (22%), and Dublin (75%). CDC (2014) reported that the resistance (to one or more Clinical and Laboratory Standards Institute antibiotic classes) among non-typhoidal *Salmonella* human isolates has decreased from 20% in 2003-2007 to 15% in 2012; multidrug resistance (to three or more classes) decreased from 12% to 9% in the same time period, but this was likely due to a reduction in numbers of *Salmonella* Typhimurium.

2.6 Antibiotic use on dairy farm and development of resistance

Antimicrobial agents that are currently licensed for use in dairy cattle in the U.S. include enrofloxacin, florfenicol, and various penicillins, cephalosporins, macrolides, sulfonamides, and tetracyclines; extralabel use of some additional drugs is also permitted under certain circumstances (APHIS, 2008). National data shows that the degree of prophylactic and therapeutic antimicrobial use on dairy operations across the U.S. remained essentially unchanged between the 2002 and 2007 NAHMS Dairy studies (APHIS, 2008). Common uses for antimicrobial agents on dairy farms include feeding of medicated milk replacer to pre-weaned calves, treatment of respiratory and gastrointestinal disease in pre-weaned calves, treatment of respiratory disease in weaned calves, prevention and treatment of mastitis in cows, and treatment of respiratory disease, reproductive disorders, and lameness in cows (APHIS, 2008).

The most common disease in calves that results in the use of antimicrobial drugs is diarrhea, followed by pneumonia (APHIS, 2008). According to the last published NAHMS data in 2007, the most common drugs used to treat diarrhea belonged to the tetracycline (16%) and β-lactam (9%) classes (APHIS, 2008). In the same report, the 2 antimicrobial drugs most commonly used for treatment of respiratory disease were florfenicol (18%) and drugs belonging to the macrolide class (15%). Clinical laboratories routinely culture bovine fecal and gastrointestinal tract samples for *E. coli* only when whose samples have been obtained from calves, because *E. coli* enteritis and septicemia are important clinical problems in calves rather than adult cattle (Cummings et al., 2014). In a

U.S. study, >81% of *E. coli* isolated from calves with diarrhea were MDR, whereas in Australia, 72.4% of *Salmonella* isolates associated with diarrhea in calves were susceptible to all drugs tested (Doyle, 2015).

In addition to these drugs, two fluoroquinolone drugs (danofloxacin and enrofloxacin) are approved to use for food producing animals in the U.S., including cattle and swine. Enrofloxacin is approved to treat dairy cattle less than 20 months of age for respiratory disease and control. Extra-label use of fluoroquinolones is strictly prohibited. Study has shown that intramuscular enrofloxacin administered to cattle and swine is partly metabolized to ciprofloxacin, and results in measurable concentrations of ciprofloxacin and enrofloxacin in intestinal contents (Wiuff et al., 2002). The presence of quinolones in the feces could pose selective pressure on intestinal bacteria. Because all quinolones have common mechanisms of resistance, resistance to one quinolone will usually result in resistance to all other quinolones and selection pressure from enrofloxacin treatment could result in the selection of resistance to cirprofoxacin (Hopkins et al., 2005). Even though the concentration might be nonlethal, the exposure to antimicrobial drugs can enrich pre-existing resistant mutants with very small fitness costs (Hughes and Andersson, 2012). Other studies showed increased resistance to quinolones in dairy cattle *E. coli* isolates in northeastern region (Cummings, 2014). These observations suggest a review of continuous judicious use of quinolones in veterinary medicine.

In the U.S. an increasing trend of aminoglycoside resistance has been observed *in E. coli* isolates from calves, because dairy calves are frequently

exposed to neomycin. Cross-resistance between gentamycin and other aminoglycosides such as neomycin could be an explanation. According to the 2007 NAHMS Dairy study, 50% of U.S. dairy operations used neomycin and oxytetrcycline in medicated milk replacer for claves (APHIS, 2008). Alternatively, co-selection caused by gene linkage could cause resistance in the absence of selection pressure from a specific drug.

Resistance of *E. coli* from calves in other countries has also been monitored. *E. coli* isolated from calves younger than one year of age in Austria, Germany and the Netherlands showed moderate to high resistance to ampicillin, streptomycin, sulfonamides and tetracyclines (EFSA, 2014). Resistance to chloramphenicol and gentamicin was reported remaining at relatively low levels. The occurrence of resistance to fluoroquinolones and third-generation cephalosporins was less common and resistance to cefotaxime was also very low. The reported resistance in *E. coli* isolates from calves of less than one year was higher than young cattle and adult cattle in Austria.

2.7 Salmonella and resistance on dairy farms

Dairy cattle are known reservoirs of *Salmonella* spp. and asymptomatic shedders pose substantial risk to food safety. The virulence and antimicrobial resistance of *Salmonella* spp. can vary greatly among serotypes. *Salmonella* Dublin, Newport, Typhimurium, etc., can cause clinical salmonellosis in both humans and cattle, and were frequently reported to carry multi-resistance genes. Some other serotypes, i.e. Kentucky, Cerro, Muenster, Infantis, etc., may persist

on farms without showing clinical symptoms in the animals. However, all *Salmonella* serotypes are considered human pathogens, and monitoring prevalence and antimicrobial resistance in *Salmonella* is significant for food safety. Dairy cattle can serve as a source of *Salmonella* transmission to people through contaminated ground beef, dairy products, produce, and water, as well as through direct contact. In the U.S., dairy cattle are an important source of lean or extra-lean ground beef and therefore dairy cattle might be a source of *Salmonella* infections in humans, because both whole cuts and ground beef derived from market dairy cows are at risk for contamination with *Salmonella*. Milk and dairy products are at risk of contamination prior to leaving the farm, usually as a result of inadvertent fecal contamination during the milking process (Van Kessel et al., 2013). Outbreaks of MDR serotype Newport and Typhimurium strains have been frequently associated with consumption of unpasteurized cheeses and undercooked retail meats.

Antimicrobial susceptibility of bacteria isolates from dairy farm is also monitored by NAHMS Dairy studies that have been conducted by the U.S. Department of Agriculture every 5 to 6 years since 1996 (APHIS, 2009). In NAHMS Dairy 2002-2007 Survey, 176 *Salmonella* isolates representing 26 serotypes were recovered from bulk tank milk and milk filters. MDR-AmpC type resistance was observed in all 14 *Salmonella* Newport exhibited, as well as Dublin (3 of 7), Typhimurium (2 of 5) and Infantis (1 of 2), Kentucky (4 of 22) and Anatum (1 of 13) (Van Kessel et al. 2013). A longitudinal study of the acquisition of new MDR *Salmonella* strains by dairy herds in the U.S. found that this was a

fairly common event. On-farm practices such as herd size and off-farm heifer raising were found significantly correlated with the introduction of new MDR salmonellae (Doyle, 2015).

2.8 Judicious use of antibiotic on dairy farm and alternative approach

A new proposed rule, Veterinary Feed Directive, encourages judicious use of antibiotics in animal agriculture, particularly for drugs that are important in human medicine. FDA (2013) published Guidance for Industry #213 in December 2013, which announced a specific strategy for animal drug companies to voluntarily revise the labeling of their medically important antimicrobials used in the feed and water of food-producing animals to withdraw approved production uses and place the remaining therapeutic uses of these products under veterinary oversight by December 2016.

Several approaches may be utilized for reducing antibiotic usage in livestock. These include use of immunomodulators that increase immune function and disease resistance of animals; timely inspections to identify and treat sick animals before disease spreads; maintenance of a hygienic and healthy living environment; and use of laboratory tests to detect animals at risk of developing disease (USDA, 2014). Farm practice approaches were shown to be affective to limit food animal morbidity and mortality while reducing the use of antimicrobial drugs (Pereira et al., 2014). A study by Berge et al. (2009) observed that calves in a conventional therapy had 70% more days with diarrhea than calves in the targeted therapy group. The use of preventive-measures has been shown to result

in lower occurrence of disease and could subsequently reduce use of antimicrobial drugs.

Chapter 3: Antimicrobial Resistance of *Salmonella* and *E. coli* from Pennsylvania Dairy Herds

3.1 Introduction

Antibiotics are used in food-producing animals in many countries for treatment of diseases, but sometimes also for growth promotion and prevention of infections. In the U.S., antibiotic use in dairy operations is highly regulated. According to 2007 National Animal Health Monitoring System (NAHMS) Dairy Survey conducted by the U.S. Department of Agriculture, antibiotics are commonly used to treat respiratory disorders and diarrhea in pre-weaned calves, respiratory disease in weaned calves, and mastitis in cows. Other antibiotic exposures include medicated milk replacer used by more than half of the U.S. dairy operations in pre-weaned calves. In addition on 90 % of dairy operations, intramammary antibiotics are used in dry cows to prevent mastitis (APHIS, 2008). It has been suggested that the agricultural use and misuse of antibiotics in food-producing animals has provided selective pressure in the farm environment resulting in increased prevalence of antibiotic resistance (Economou and Gousia, 2015). Fecal carriage of resistant bacteria in food producing animals, including dairy cattle, has been reported. Even though resistance genes are sometimes present in the absence of anthropogenic selective pressure, antibiotic use in farm animals may aggravate the resistance problem by accelerating the transfer of resistance genes among bacterial species (including human pathogens), or assisting the clonal spread of resistant strains (Allen, 2014; Stokes and Gillings, 2011).

Salmonella is a leading foodborne pathogen in the U.S. (Varma et al., 2005) and can be transferred to humans through contaminated food, water, or direct contact with infected animals. Human salmonellosis usually results in self-limiting diarrhea and does not require antibiotic treatment. However, in severe cases of invasive infections, antimicrobial therapy is required and thus, the spread of resistant *Salmonella* is a concern. Drug resistant non-typhoidal *Salmonella* is listed as a "serious" pathogen to combat in Antibiotic Resistance Threats in the U.S. reported by the CDC (2013). Dairy cattle are a well-documented reservoir for *Salmonella*. *Salmonella* can also cause disease in cattle, and sometimes even death. Clinical symptoms of salmonellosis in cattle include fever, diarrhea, anorexia, abortion, and decreased milk production. Cattle can also asymptomatically shed *Salmonella* in their feces for extended period of time without any apparent impact on health or production (Van Kessel et al., 2012).

USDA Animal and Plant Health Inspection Service (APHIS) have been examining *Salmonella* occurrence and antibiotic resistance in the U.S. dairy operations via the NAHMS program. An increasing trend of *Salmonella* prevalence was observed in fecal samples of healthy cows based on results of NAHMS Dairy Studies. *Salmonella* prevalence increased from 21% in 1996 to 40% in 2007 on U.S. dairy operations (APHIS, 2009). The common *Salmonella* serotypes were Cerro, Kentucky, Montevideo, Muenster, Meleagridis, Mbandaka, and Newport. When tested for antimicrobial susceptibility, *Salmonella* isolates from the three NAHMS Dairy Studies showed relatively little resistance, with 89%, 83%, and 93% of all the isolates being pan-susceptible in 1996, 2002, and 2007, respectively (APHIS, 2009).

In 2007, resistance to ceftriaxone in a single *Salmonella* isolate and multidrug-resistance in *S.* Montevideo were observed for the first time in the NAHMS Dairy Study. Milk is inevitably contaminated by feces during the milking process, and thus serves as a good indicator for bacteria shedding by the lactating cows, including *Salmonella*. Van Kessel et. al. (2013) detected AmpC-type *Salmonella* from bulk tank milk and milk filters samples in NAHMS milk study. Cephalosporin resistant *Salmonella* was also isolated from beef cattle, dairy cattle, and milk samples. The National Antimicrobial Resistance Monitoring System (NARMS) in the U.S. have identified ceftiofur resistant *Salmonella* in beef cattle (Zhao, 2003).

Generic *E. coli* are ubiquitous in the environment and play a dynamic role in the ecology of intestinal microflora. Most *E. coli* are nonpathogenic, however, their genome exhibits a high degree of heterogeneity and readily acquires genetic elements. Resistant *E. coli* have been commonly found in food-producing animals and could be a useful indicator organism to evaluate the presence of antimicrobial resistance in the general bacterial population. *E. coli* from dairy cattle may serve as a reservoir of resistance genes. The mobile genetic elements in dairy commensal *E. coli* could potentially transfer to human pathogens, such as *Salmonella* and *Klebsiella*.

Enterobacteriaceae-associated $bla_{\text{CTX-M}}$ genes have become globally widespread within the past 30 years. In 2007, 48% of extended-spectrum cephalosporin-resistant *E. coli* from a clinical laboratory in Philadelphia, Pennsylvania were CTX-M type (McGettigan et al. 2009). CTX-M-type *E. coli* has already been identified in livestock samples in different regions of the world, but the

first isolation in the U.S. was by Wittum et al. (2010) from sick and healthy dairy cattle samples collected in 2009 in Ohio. More recently, researchers from Washington State University detected the emergence of *bla*$_{\text{CTX-M}}$ *E. coli* in dairy cattle when testing fecal samples collected in 2011(Davis et al., 2015).

Many research have shown the presence of resistant bacteria in dairy cattle. However, an in-depth characterization of bacterial resistance is needed to further understand the dynamics of antibiotic resistance on dairy farms. The objective was to determine the prevalence of antimicrobial resistance among *Salmonella enterica* and commensal *Escherichia coli* isolates from different animal groups on dairy farms in Pennsylvania.

3.2 Materials and methods

3.2.1 Sample collection

Manure composite samples were collected from 80 dairy farms in Pennsylvania from November, 2013 to February, 2015. Up to 6 samples representing 4 different age groups were obtained from each farm. One sample was collected from pre-weaned calves, one from post-weaned calves, one from dry cows, and up to three from lactating cows. The samples were placed into sterile vials, packed on ice, and shipped overnight to the USDA-ARS Environmental Microbial and Food Safety Laboratory, Beltsville, MD for processing.

3.2.2 Bacterial isolation

To isolate *Escherichia coli*, 45 ml of buffered peptone water was added to 5 grams of sample and vortexed until well mixed. Approximately 30-40 µl was streaked onto CHROMagar EC plates (Hardy Diagnostics, Santa Maria, CA). Plates were incubated at 37°C for 18 to 24 hours and 5 presumptive *E. coli* isolates (blue colonies) from each sample were selected for further confirmation. When multiple phenotypes were present, at least one colony of each phenotype was selected. The presumptive *E. coli* colonies were transferred from Chromogenic EC plates onto Simmons Citrate Agar, MacConkey Agar, Sorbitol-MacConkey Agar and L-Agar plates (Lennox Broth base with 1.5% agar; Gibco Laboratories , Long Island, NY), and incubated at 37°C for 18-24h. Colonies that exhibited the *E. coli* phenotype (negative on Citrate agar, positive on MacConkey, positive or negative on Sorbitol-MacConkey agar) were preserved for future analysis.

For isolation of *Salmonella* spp., 5 gram of each sample was added to 45 ml Tetrathionate broth (BD Diagnostics, Sparks, MD) and then incubated at 37°C for 24h, after which 30-40 µl of the dilution was streaked onto XLT4 agar plates (XLT4 agar base with XLT4 supplement, BD Diagnostics, Sparks, MD). Plates were incubated at 37°C and examined at 24 to 48 h for presumptive *Salmonella* (black colonies). When multiple phenotypes present, at least one isolate of each phenotype was selected. Presumptive *Salmonella* colonies (at least five randomly chosen isolates per sample) were transferred from XLT4 plates onto Brilliant Green, and L-Agar plates (Lennox Broth base with 1.5% agar; Gibco Laboratories, Long Island, NY) and

incubated at 37°C for 24 h. Colonies that exhibited the *Salmonella* phenotype (pink on brilliant green) were preserved and stored at – 80°C for future analysis.

Two *Salmonella* isolates per sample, when present, were classified into serogroups using a PCR method described by Karns et al. (2015). The method classifies *Salmonella enterica* subsp. *enterica* into serogroup B, C1, C2, C and E, which accounts for the majority of the isolates associated with human foodborne outbreaks. DNA was extracted using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's directions and extracted DNA samples were stored at -20°C. Not all serotypes fall within the 5 serogroups identified by this PCR analysis and isolates that were not classified into one of the 5 groups were categorized as Group Unknown (U). Serogroup K comprising Cerro, a serotype that is commonly isolated from northeastern U.S. dairy farms, is not detected by this method and thus was categorized as Group U. One *Salmonella* isolate per serogoup was selected for each farm, and sent to the National Veterinary Services Laboratories (U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Ames, IA) for serotype classification.

3.2.3 Antimicrobial susceptibility testing

Selected *Salmonella* and *E. coli* isolates were replicated on Mueller Hinton agar supplemented with NARMS breakpoint concentrations of ampicillin (32 μg/ml), cefoxitin (32 μg/ml), chloramphenicol (32 μg/ml), cefotaxime (*E. coli* only) (4 μg/ml), tetracycline (16 μg/ml), streptomycin (64 μg/ml), kanamycin (64 μg/ml), and

ciprofloxacin (4 μg/ml for *E. coli* and 1 μg/ml for *Salmonella*), incubated 18 h at 37°C, and scored for growth. An isolate representing each unique resistance pattern per sample was then further assayed for susceptibility to a panel of 14 antibiotics on NARMS GN Panel via the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH). The breakpoint values used for each antibiotic on NARMS Panel are listed in Table 1. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards where available. In the absence of a CLSI value, minimum inhibitory concentrations (MICs) were interpreted using the breakpoints as described by the NARMS (FDA, 2012). Isolates were classified as being resistant or susceptible to each agent; those few isolates with intermediate susceptibility were categorized as being susceptible. *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms to ensure the validity of the susceptibility testing.

Table 1. Antimicrobials used to test resistance of *E. coli* isolates in this study with broth microdilution method. Fourteen antimicrobial agents on NARMS GN Panel can be categorized into 9 classes according to Clinical and Laboratory Standards Institute (CLSI), and each corresponding abbreviation was used in this study

Antimicrobial Classes	*Sub-classes*	*Antimicrobial Agent*	*Abbrev.*	*Breakpoint Concentration (μg/ml)*
β-lactam: Penicillins	Penicillins	Ampicillin	AMP	≥ 32
Penicillins + β-lactamase Inhibitors		Amoxicillin-clavulanic acid	AUG	≥ 32
β-lactam: Cephems	Cephamycins	Cefoxitin	FOX	≥ 32
	Third-generation Cephalosporins	Ceftiofur	TIO	≥ 8
		Ceftriaxone	AXO	≥ 4
Aminoglycosides		Gentamycin	GEN	≥ 16
		Streptomycin	STR	≥ 64
Folate Pathway Inhibitors		Sulfisoxazole	FIS	>256
		Trimethoprim-Sulfamethoxazole	SXT	>4
Tetracyclines		Tetracycline	TET	≥ 16
Phenicols		Chloramphenicol	CHL	≥ 32
Macrolides		Azithromycin	AZI	>16
Quinolones		Ciprofloxacin	CIP	>4 *E. coli* ≥ 1 *Salmonella*
		Nalidixic acid	NAL	≥ 32

3.2.4 Analysis for *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes

DNA was isolated from bacterial biomass using InstaGene Matrix (Bio-Rad, Hercules, California) following the manufacturer's instructions. Isolates were analyzed for the presence of the plasmid mediated AmpC β-lactamase gene, *bla*$_{CMY,}$ using a PCR method developed by Zhao et al. (2003) and modified as described previously (Van Kessel et al., 2013; Zhao et al., 2003). The master mix consisted of 50 pmol of each primer (cmyF and cmyR or CS5' and CS3'), 200 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, and 1.5 U of Ampli Taq Gold enzyme (Applied Biosystems, Foster City, CA). Each 25-μl reaction mixture consisted of 24 μl of master mix and 1 μl of template DNA. The cycle included a 10-min enzyme activation step at 94°C and 30 cycles of 94°C for 1 min, 50°C for 90 s, and 72°C for 90 s, followed by a 10-min final extension step at 72°C. Two strains of *Salmonella enterica* serotype Typhimurium were used as positive (CVM 1290) and negative (CVM 785) controls.

The presence of the extended-spectrum β-lactamase gene, *bla*$_{CTX-M,}$ was determined using a multiplex PCR method developed by Woodford et al. (2005) with a few modifications. The master mix consisted of 50 pmol of each primer (Group 1 F, Group 1 R, Group 2 F, Group 2 R, Group 9 F, Group 9 R, Group 8/25 F, and Group 8/25 R). The amplification conditions included 5-min initial enzyme activation at 94°C and 30 cycles of 94°C for 25 s, 52°C for 40 s, and 72°C for 50 s, followed by a 6-min elongation step at 72°C. Four in-house strains were used as positive controls: CC8767, CC8770 for CTX-M Group 1, and CC8768, CC8769 for CTX-M Group 9.

3.2.5 Statistical Analysis

A farm was considered *Salmonella* positive when *Salmonella* was isolated from at least one fecal composite samples from the farm. A farm was considered AR *E. coli* positive when resistant *E. coli* was isolated from at least one fecal composite samples from the farm. PROC FREQ procedure in SAS® software (version 9.4; SAS Institute, Cary, NC) was used for data analysis.

3.3 Results

From November, 2013 to February, 2015 a total of 444 composite manure samples were collected from 80 Pennsylvania dairy herds, including samples of pre-weaned calves from 77 farms, post-weaned calves from 75 farms, dry cows from 72 farms, and 219 samples of lactating cows from 80 farms. As anticipated, *E. coli* was isolated from all the samples. At least 5 *E. coli* isolates (n=2370) were selected from each sample for antibiotic resistance prescreening and analysis. *Salmonella* was isolated from 13% (10/77) of pre-weaned calf samples, 25% (19/75) of post-weaned calf samples, 61% (44/72) of dry cow samples, and from 66% (145/219) lactating cow samples representing 64% (51/80) of the farms (Table 2). *Salmonella* was isolated from at least one sample from 64% of the farms. When present, 5 *Salmonella* isolates (n=2370) were selected from each sample for further characterization and antibiotic resistance analysis.

Table 2. Prevalence and serogroup distribution of *Salmonella* isolates from composite samples of pre-weaned calves, post-weaned calves, dry cows, and lactating cows on Pennsylvania dairy farms

			Serogroup (No. of farms)				
	No. of farms	*Sal* $^{+}$ *% (n)*	*C1*	*C2*	*E*	*B*	*U*
Pre-weaned calves	77	13.0 (10)	1	2	1	0	6
Post-weaned calves[1]	75	25.3 (19)	3	6	0	0	14
Dry cows[2]	72	61.1 (44)	16	8	0	0	30
Lactating cows[3]	80	63.8 (51)	35	13	0	1	39
Total	80	63.8 (51)	25	15	1	1	40

[1] post-weaned calf samples from 2 farms had both C2 and U; samples from 1 farm had both C1 and C2;
[2] dry cow samples from 2 farms had both C2 and U; samples from 8 farms had both C1 and U;
[3] lactating cow samples from 25 farms had two or three different serogroups: 17 farms had serogroup C1 and U, 4 farms had serogroup C2 and U, 1 farm had serogroup C2 and B, and 2 farms had all of the three most common serogroups, C1, C2, and U.

When isolates were classified into serogroups, serogroup C1 was detected from 25 (31%) farms, serogroup C2 from 25 (19%) farms (Table 2). Isolated from 40 farms could not be classified and were therefore grouped into serogroup U. Serogroup U was predominant in samples from both young and adult animal groups, seroroup C1 was more prevalent in adult cow samples, while seroroup C2 was more prevalent in calf samples. Serogroup B and E were each isolated from a single farm. When isolates representing each unique serogroup from each farm were analyzed, the serotypes were highly clustered within each serogroup. All 25 (100%) isolates

classified from serogroup C1 were Montevideo, 14 (93.3%) of the 15 isolates in seroroup C2 were Kentucky, and 39 (97.5%) of the 40 isolates in seroroup U were Cerro (Table 3). One of each following serotypes were observed: Newport (serogroup C2), Rough (serogroup U), Muenster (serogroup E) and Paratyphi_B_var._L-tartrate^{+} (serogroup B). Our results concurred with the previous studies, indicating the temporal stability of *Salmonella* serotype distribution in this study region (Van Kessel et al., 2013, 2007).

Table 3. *Salmonella* serogoup-prevalence in composite manure samples from Pennsylvania dairy farms, representative isolates and their serotypes, and percentage of serotypes within their corresponding serogroups

Serogroup	*No. Farms (No. of isolates)*	*% farms (n=80)*	*Serotype*	*Percent of serotype within the serogroup (No. of isolates)*
C1	25	31.3	Montevideo	100% (25)
C2	15	18.8	Kentucky	93.3% (14)
			Newport	6.7% (1)
E	1	1.3	Muenster	100% (1)
B	1	1.3	Paratyphi_B_var._L-tartrate^{+}	100% (1)
U	40	50.0	Cerro	97.5% (39)
			Rough_O:z4,z23:-	2.5% (1)

Due to financial restraints, a prescreening for resistance was conducted for all isolates. At least five *E. coli* isolates (n=2370) and five *Salmonella* isolates (n=1095) from each sample, when present, were screened for antimicrobial resistance via replica plating on to antibiotic-supplemented agar. A few *Salmonella* isolates showed

reduced susceptibility to ampicillin and tetracycline, and the rest were inclusively susceptible to cefoxitin, chloramphenicol, streptomycin, kanamycin, and ciprofloxacin (data not shown). Based on the pre-screening results, resistant *E. coli* isolates were observed in manure composite samples from pre-weaned calves on 88% of farms, post-weaned calves on 81% of farms, dry cows on 46% of farms, and lactating cows on 64% of farms. Among the 8 antimicrobials, commensal *E. coli* were mostly frequently resistant to tetracycline, streptomycin, ampicillin, and kanamycin and the prevalence of resistant isolates from calves (detected in samples from 84%, 68%, 62%, and 61% of the farms, respectively) were higher than those from adult cows (51%, 30%, 15%, and 7% of farms, respectively).

Previous work with *E. coli* isolated from dairy animals indicated that isolates identified as pan-susceptible via the pre-screening method were also pan-susceptible when tested for resistance to the NARMS GN Panel for antimicrobial resistance via broth microdilution method (data not shown). In the present study, 30 pan-susceptible isolates based on the replica plating (presreening) results were randomly selected, and then confirmed pan-susceptible on the NARMS GN Panel.

Based on the pre-screening results, 376 *E. coli* isolates were selected to represent unique resistance profiles from each sample, and the resistant phenotypes were further characterized for MICs by the broth microdilution test. *E. coli* isolates that exhibited resistance to at least one antimicrobial on the NARMS GN Panel (n=285) were identified in 42.34% (188/444) of samples from 97.5% (78/80) of farms. Additionally, 91 (23.94% n=376) of the isolates identified as potentially

resistant in pre-screening were pan-susceptible, indicating that the replica plating yielded an appreciable number of false positive results, and therefore was a relatively conservative method for selecting resistant bacteria isolates.

Among the *E. coli* isolates showing resistance to at least one antibiotic, 93.3% were resistant to tetracycline, and the other common resistances were to sulfisoxazole (56.1%), streptomycin (53.0%) and ampicillin (41.8%). *E. coli* was rarely resistant to ciprofloxacin (1.4%), azithromycin (1.8%), nalidixic acid (4.2%), or gentamycin (5.3%) (Figure 1) (Table 4). Even though resistance was infrequent to some of the antimicrobials, at least one *E. coli* isolate was identified as resistant to each of the antibiotics on the panel. Prevalence of resistance to individual antibiotic differed among the four animal groups and the highest level of resistance was observed in *E. coli* isolated from pre-weaned calf samples (Figure 1). On more than 30% of the farms, pre-weaned calf *E. coli* isolates were detected resistant to each of the following agents: amoxicillin-clavulanic acid (Augmentin), ampicillin, cefoxitin, ceftiofur, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline. The prevalence of resistant *E. coli* from the adult animal groups was much lower than in the calf samples, and no isolates were identified with resistance to azithromycin, ciprofloxacin, gentamycin, or nalidixic acid.

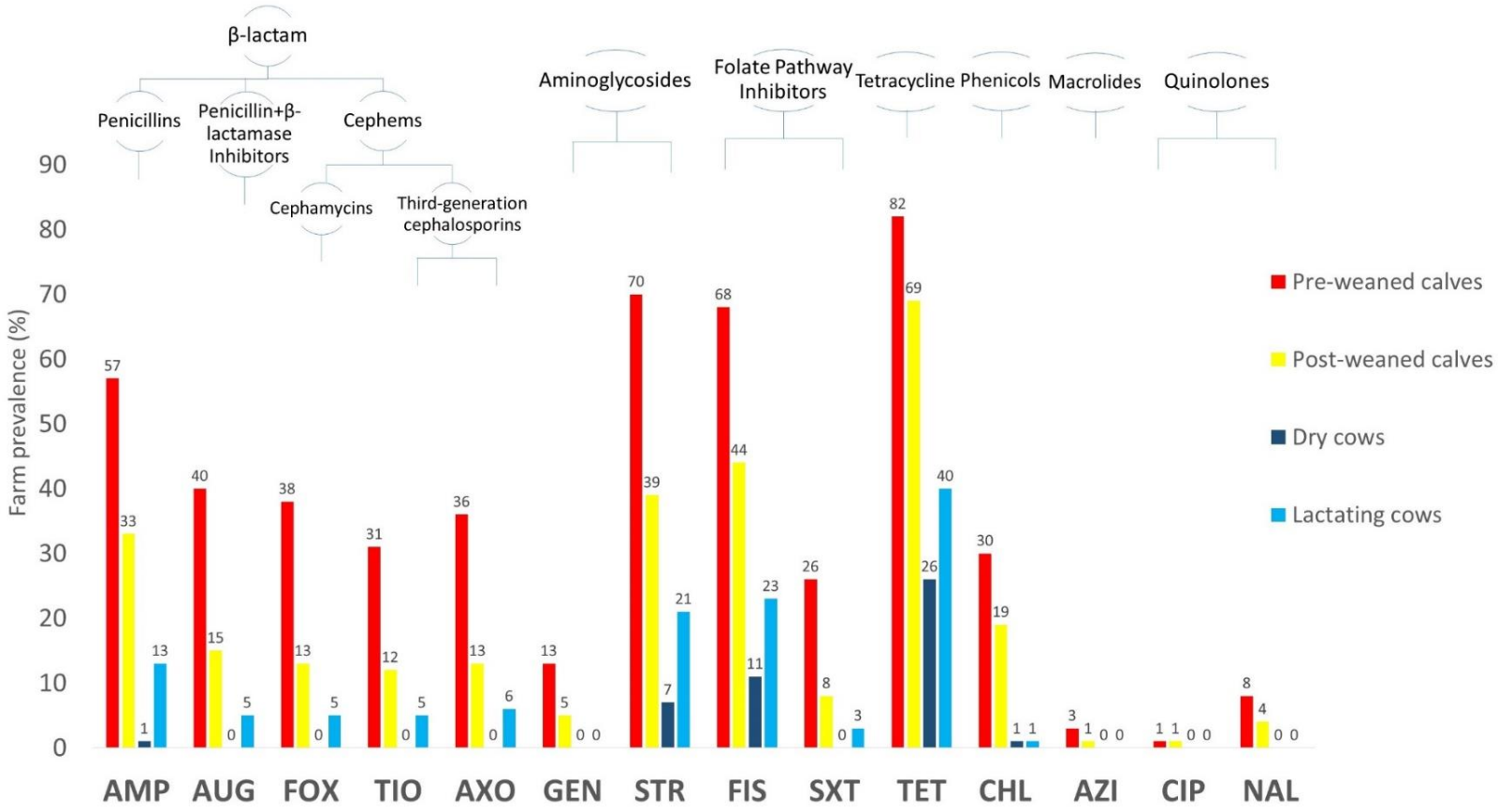


Figure 1. Resistant to individual antimicrobial agents among selected *Escherichia coli* isolates from four animal groups in Pennsylvania dairy farms: pre-weaned calves, post-weaned calves, drycows, and lactating cows.

Table 4. Prevalence of antimicrobial-resistant *E. coli* isolates (n=285) from manure samples of pre-weaned calves, post-weaned calves, dry cows, and lactating cows

Antimicrobial Agents	*Farm Prevalence (%)*			
	Pre-weaned calves (n=77)	*Post-weaned calves (n=75)*	*Dry cows (n=72)*	*Lactating cow (n=80)*
AUG	40.3 (31)	14.7 (11)	0.0 (0)	5.0 (4)
AMP	57.1 (44)	33.3 (25)	1.4 (1)	12.5 (10)
AZI	2.6 (2)	1.3 (1)	0.0 (0)	0.0 (0)
FOX	37.7 (29)	13.3 (10)	0.0 (0)	5.0 (4)
TIO	31.2 (24)	12.0 (9)	0.0 (0)	5.0 (4)
AXO	36.4 (28)	13.3 (10)	0.0 (0)	6.3 (5)
CHL	29.9 (23)	18.7 (14)	1.4 (1)	1.3 (1)
CIP	1.3 (1)	1.3 (1)	0.0 (0)	0.0 (0)
GEN	13.0 (10)	5.3 (4)	0.0 (0)	0.0 (0)
NAL	7.8 (6)	4.0 (3)	0.0 (0)	0.0 (0)
STR	70.1 (54)	38.7 (29)	6.9 (5)	21.3 (17)
FIS	67.5 (52)	44.0 (33)	11.1 (8)	22.5 (18)
TET	81.8 (63)	69.3 (52)	26.4 (19)	40.0 (32)
SXT	26.0 (20)	8.0 (6)	0.0 (0)	2.5 (2)

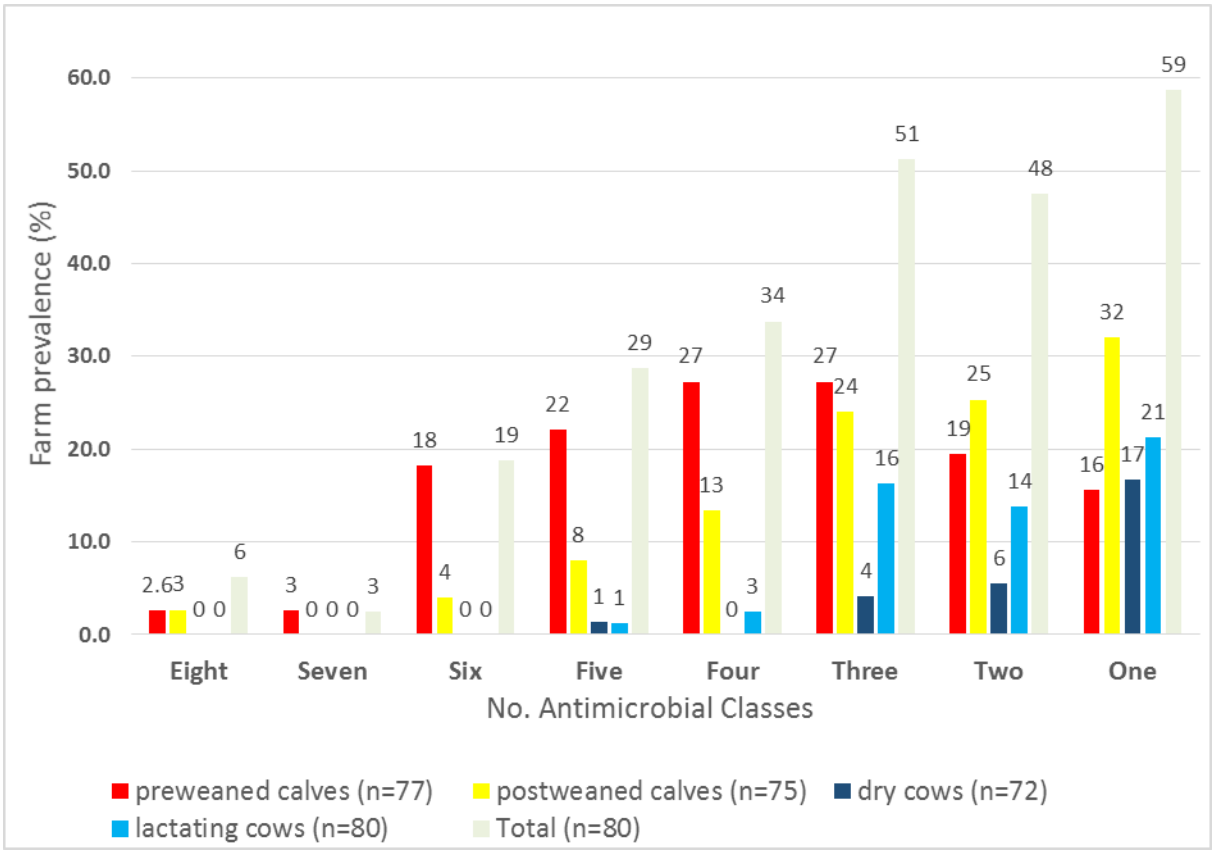


Figure 2. Resistance to each number of antibiotic classes among selected (n=285) *E. coli* tested for antimicrobial susceptibility using broth microdilution method on NARMS GN Panel. *E. coli* isolates were from manure samples of pre-weaned calves, post-weaned calves, dry cows, and lactating cows in 80 Pennsylvania dairy farms.

Among the isolates that were characterized as resistant to at least one antibiotic, the most common resistance patterns were TET only (68 isolates from 56.3% of farms), STR-FIS-TET (36 isolates from 33.8% of farms), FIS-TET (20 isolates from 22.5% of farms), CHL-STR-FIS-TET (14 isolates from 16.5% of farms) and AMP-TET (14 isolates from 17.5% of farms) (Table 5). The most common pattern of resistance to extended-spectrum beta-lactams was AUG-AMP-FOX-TIO-AXO- STR-FIS-TET and was observed in 8 isolates from 8 (10%) different farms.

TABLE 5. Resistance patterns among 285 *E. coli* isolates tested for antimicrobial susceptibility using broth microdilution method on NARMS GN Panel

	Resistant Pattern	*No. of Isolates*	*Isolate %*	*No. of farms*	*Farm % (n=80)*
1	TET	68	23.9	45	56.3
2	STR-FIS-TET	36	12.6	27	33.8
3	FIS-TET	20	7.0	18	22.5
4	CHL- STR-FIS-TET	14	4.9	13	16.3
5	AMP-TET	14	4.9	14	17.5
6	STR-TET	12	4.2	10	12.5
7	AUG-AMP-FOX-TIO-AXO- STR-FIS-TET	9	3.2	8	10.0
8	AUG-AMP-FOX-TIO-AXO-CHL- STR-FIS-TET	7	2.5	6	7.5
9	AMP-FIS-TET	6	2.1	6	7.5
11	AUG-AMP-FOX-TIO-AXO	6	2.1	4	5.0
12	AUG-AMP-FOX-TIO-AXO-TET	6	2.1	6	7.5
13	AUG-AMP-FOX-TIO-AXO-CHL-GEN-STR-FIS-TET	6	2.1	4	5.0
14	AMP-STR-TET	4	1.4	4	5.0
15	AMP- STR-FIS-TET	4	1.4	4	5.0
16	AUG-AMP-FOX-AXO- STR-FIS-TET	4	1.4	4	5.0
17	AUG-AMP-FOX-TIO-AXO- STR-FIS-TET-SXT	4	1.4	4	5.0
18	AUG-AMP-FOX-TIO-AXO-CHL STR-FIS-TET-SXT	4	1.4	3	3.8
19	AMP-STR-FIS-TET-SXT	3	1.1	1	1.3
20	AUG-AMP-FOX-TIO-AXO- STR-TET	3	1.1	3	3.8

The 14 antibiotics on the NARMS GN Panel were grouped into 9 classes: penicillins, penicillins and β-lactamase inhibitors, cephems, aminoglycosides, folate pathway inhibitors, tetracyclines, phenicols, macrolides, and quinolones. Multidrug

resistance (MDR), here defined as resistance to three or more classes of antimicrobial agents, was observed in isolates from 70% of farms in pre-weaned calf samples, 39% of farms in post-weaned calf samples, 6% of farms in dry cow samples, and 20% of farms in lactating cow samples (Figure 3) (Table 6). Resistance to up to 8 antibiotic classes was observed in *E. coli* isolates from pre-weaned and post-weaned calves. The highest observed resistance in adult animals was to 5 drug classes which was observed in *E. coli* isolated from one dry cow sample and one lactating cow sample.

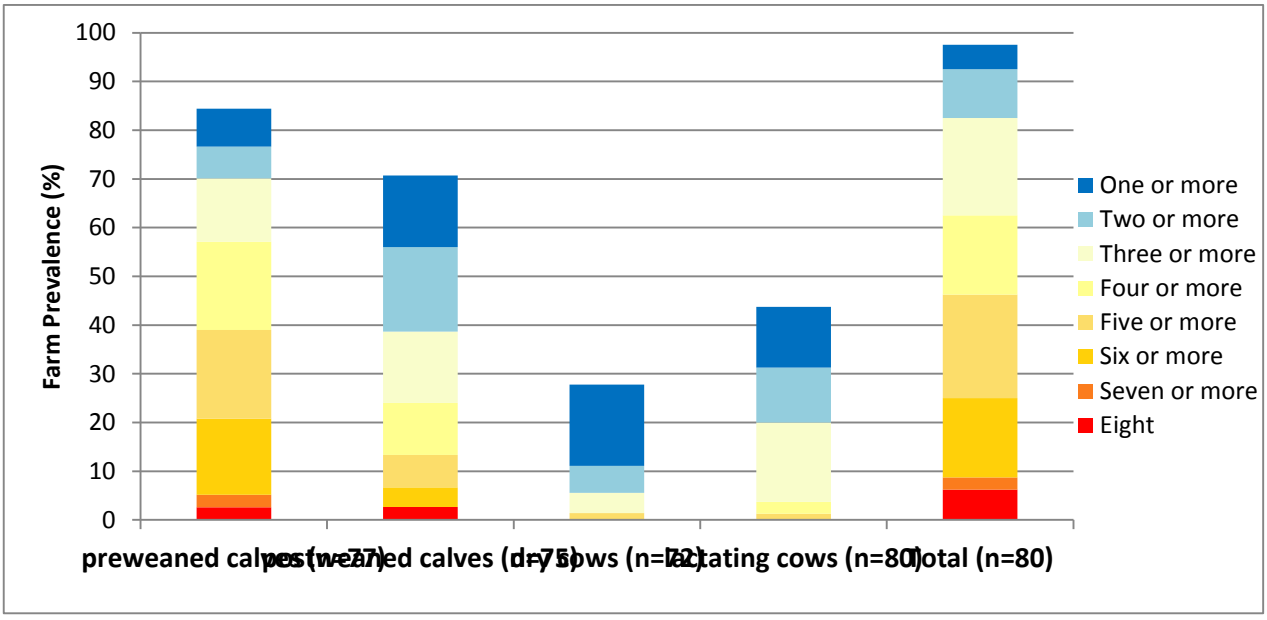


Figure 3. Cumulative prevalence of multiple-classes of drug resistance *E. coli* for pre-weaned calves, post-weaned calves, dry cows and lactating cows.

Table 6. Cumulative prevalence of multiple-classes of drug resistance *E. coli* for pre-weaned calves, post-weaned calves, dry cows and lactating cows

Classes of Antibiotics	*Farm Prevalence (%)*				
	Pre-weaned Calves (n=77)	*Post-weaned Calves (n=75)*	*Dry Cows (n=72)*	*Lactating Cows (n=80)*	*Total (n=80)*
8	2.6 (2)	2.7 (2)	0.0 (0)	0.0 (0)	6.3 (5)
≥ 7	5.2 (4)	2.7 (2)	0.0 (0)	0.0 (0)	8.8 (7)
≥ 6	20.8 (16)	6.7 (5)	0.0 (0)	0.0 (0)	25.0 (20)
≥ 5	39.0 (30)	13.3 (10)	1.4 (1)	1.3 (1)	46.3 (37)
≥ 4	57.1 (44)	24.0 (18)	1.4 (1)	3.8 (3)	62.5 (50)
≥ 3	70.1 (54)	38.7 (29)	5.6 (4)	20.0 (16)	82.5 (66)
≥ 2	76.6 (59)	56.0 (42)	11.1 (8)	31.3 (25)	92.5 (74)
≥ 1	84.4 (65)	70.7 (53)	27.8 (20)	43.8 (35)	97.5 (78)

The AmpC phenotype was identified in *E. coli* isolates from 28 pre-weaned calf samples, 10 post-weaned calf samples, and 4 lactating cow samples from 40% (32/80) of farms, while this phenotype was never identified in dry cow isolates (Table 7). Based on the PCR results, *bla*$_{\text{CMY-2}}$ genes were detected in AmpC phenotype *E. coli* isolates from 37 samples on 35% (28/80) of farms. The prevalence of *bla*$_{\text{CMY-2}}$ -carrying *E. coli* in pre-weaned calf, post-weaned calf, and lactating cow samples was 31%, 13%, and 4%, respectively (Table 8). On one (1% 1/80) farm, *bla*$_{CMY-2}$ was found in *E. coli* isolates from pre-weaned calves, post-weaned calves, lactating cows, but not dry cows. The *bla*$_{\text{CMY-2}}$ genes were detected in both pre-weaned calf and post-weaned calf samples from 7 (9%) farms. In addition, *E. coli* encoding the *bla*$_{\text{CTX-M}}$ gene were isolated from 4 samples in 4 (5%) different farms, one from pre-weaned calf, one from post-weaned calf, one from lactating cow, and another from an unknown young stock sample (Table 8).

Table 7. Prevalence of MDR AmpC phenotype *E. coli* and *bla*$_{CMY-2}$ gene-carrying *E. coli* for pre-weaned calves, post-weaned calves, dry cows and lactating cows

FOX^{+}*TIO*$^{+}$*AXO*$^{+}$	*No. of farms candidate based on pattern*	*Co-detection in younger animal group(s) (No. of farms) based on pattern*	*No. of farms detected (showed PCR band)*	*Farm % based on PCR*	*Co-detection in younger animals (No. of farms) based on PCR*
Pre-weaned calves	28	-	24	31.2	-
Post-weaned calves	10	8	10	13.3	7
Dry cows	0	0	0	0.0	0
Lactating cows	4	3	3	4.2	2
Total	32 (42 samples)	10	28 (37 samples)	35.0	8

Table 8. Prevalence of *E. coli* containing *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$ in pre-weaned calves, post-weaned calves, dry cows and lactating cows

	bla$_{CMY-2}$			*bla*$_{CTX-M}$		
	No. of Farms (+)	*%*	*Co-detection in younger animal group(s) (No. of farms)*	*No. of Farms (+)*	*%*	*Co-detection in younger animal group(s) (No. of farms)*
Pre-weaned calves	24	31.2	-	1	1.3	-
Post-weaned calves	10	13.3	7	1	1.3	0
Dry cows	0	0.0	0	0	0.0	0
Lactating cows	3	4.2	2	1	1.4	0
Total	28 (37 samples)	35.0	8	4 (4 samples)	5.0	0

Figure 4. PCR assay for genes encoding acquired cephalosporins-resistant genes in AmpC-/ESBL- phenotype *E. coli* isolated from Pennsylvania dairy herds.

3.4 Discussion

The use of antimicrobial drugs in agriculture is believed to contribute to the emergence of antimicrobial resistance, but which role in the selection for resistant bacteria has not been completely described (USDA, 2014). In the present study, a cross-sectional survey of dairy farms was conducted in Pennsylvania to determine the prevalence and profile of antibiotic resistant bacteria on dairy farms. Within-farm comparisons were made of prevalence among pre-weaned calves, post-weaned calves, dry cows and lactating cows.

Salmonella is one of the leading foodborne pathogens in the U.S. and emergence of antibiotic resistant *Salmonella* in human infections is particularly serious due to increased morbidity and mortality (Varma et al., 2005). Dairy cows are an important reservoir of *Salmonella enterica*. This organism can cause mild to

severe illness in calves and cows, resulting in loss of productivity and impairment on animal health. In addition, subclinical shedding of *Salmonella* is commonly observed in dairy cows and may be quite extensive (Van Kessel et al., 2012; Van Kessel et al., 2007). Even though the asymptomatic persistence of salmonellosis may not affect herd health and production, it presents a significant food safety and public health issue. Human are at risk of salmonellosis when consuming raw milk or unpasteurized dairy products. In United Sates, 60% of the states permit sales of raw milk in some form according to 2011 Raw Milk Survey (National Association of State Departments of Agriculture, 2011). Also, cull dairy cows contribute significantly to the ground beef supply in the U.S, and can cause human salmonellosis when ground beef is improperly prepared (Troutt, 2001). Thus, it is importance to control asymptomatic shedding of *Salmonella* on dairy farms.

Consecutive cross-sectional studies by the National Animal Health Monitoring System (NAHMS) coordinated by USDA have shown an increase in *Salmonella* prevalence on U.S. dairy operations from 21% in 1996 to 40% in 2007 (APHIS, 2009). In another cross-sectional survey in 2002, *Salmonella* was detected in 56% of 16 farms from 4 states (Callaway et al., 2005). In the present study, the prevalence was observed to be 64% at the premise level which is higher than the results of previous surveys. Results from each of these studies were based on one-time sampling and may cause underestimation of the prevalence, because *Salmonella* shedding can be intermitant: on one Pennsylvania dairy farm, the shedding prevalence ranged from 8 to 97% in a 6-year time frame (Van Kessel et al., 2012). Factors associated with *Salmonella* shedding include season, region, herd size, manure

management etc. (Habing et al., 2012; Wells et al., 2001). Despite the influencing factors, an increasing trend of *Salmonella* prevalence is clear for the past two decades. In addition, it was observed that *Salmonella* prevalence is lower in pre-weaned calf and post-weaned calf samples compared with adult dairy cattle samples, which concurred with other studies (Berge et al., 2006).

Antimicrobial susceptibility testing results suggest low levels of resistance in *Salmonella* spp. derived from healthy calves and cows. This result is not surprising given the serotype distribution data: Cerro, Montevideo and Kentucky made up the majority *Salmonella* population in the region of the current study, and resistance has been historically uncommon among these serotypes (APHIS, 2009; Blau et al., 2005; Wells et al., 2001). Serotype distribution results in the present study concurred with previous studies, indicating the temporal stability of *Salmonella* serotype distribution in this study region (Van Kessel et al., 2013, 2007). Cummings et al. (2013) observed *Salmonella* Cerro from clinical samples were frequently pan-susceptible. Isolates form cattle that have subclinical infections are more likely to be pan-susceptible than isolates from dairy cattle with salmonellosis (Wells 2001; Ray 2007). In a study examining clinical *Salmonella* in Northeastern U.S. from 2004 to 2011, 56% of isolates were resistant to at least one antimicrobial on NARMS GN Panel, with extended-spectrum cephalosporins resistance being the most common resistance phenotype (Cummings, 2013). In this study, none of the tested isolates were found to be resistant to any quinolones or cephalosporins, classes of antibiotics that are of critical importance to human medicine. Van Kessel et al. (2013) examined antimicrobial susceptibility of *Salmonella enterica* isolated from bulk tank milk and

milk filters in the NAHMS 2002 and 2007 surveys, and found serotypes Newport, Dublin, and Typhimurium were commonly multi-drug resistant. The single *S.* Newport isolated from one of the farms in the present study was pan-susceptible, despite the fact that MDR *S.* Newport is frequently detected in bovine isolates.

Use of antibiotics in dairy cattle are highly regulated in the U.S., although extra-label use of some drugs is permitted under certain circumstances (APHIS, 2008). Sawant et al. (2005) conducted a survey of antibiotic usage on dairy herds in Pennsylvania. Comprehensive records from 33 farms indicated that antibiotic usage was greatest for calves with enteritis (36%) followed by pneumonia in calves (23%) and foot rot in cattle (16%). Antibiotics including beta-lactams, spectinomycin, florfenicol, and tetracyclines were used on these farms for both therapeutic and prophylactic purposes. On 70% of the farms, calves were fed medicated milk replacers containing oxytetracycline and neomycin and in 18% of the herds, ceftiofur was used in an extra-label manner to treat mastitis in lactating cattle. Beta-lactam antibiotics were used mostly for dry cow therapy, for clinical mastitis and sometimes pneumonia and metritis. The results of the study by Sawant et al. (2005) suggest that the use of antibiotics at sub-therapeutic levels in dairy cattle could pose selective pressure and result in selection of resistant strains.

Recent studies have shown that commensal bacteria, including generic *E. coli*, serve as good indicators of antimicrobial resistance and reveal the resistance genes that may emerge in pathogens. In the present study, even though resistance of *E. coli* isolates was infrequent to some of the antimicrobials tested, at least one *E. coli* isolate

was resistant to each of the 14 antibiotics on the NARMS GN Panel. Overall, *E. coli* were most commonly resistant to tetracycline, streptomycin, trimethoprim-sulfamethoxazole and ampicillin. High prevalence of *E. coli* resistant to tetracycline and streptomycin could be the result of feeding pre-weaned calves with antibiotic-supplemented milk replacer. Use of neomycin could cross-select other aminoglycosides such as streptomycin due to the similarities of their resistance mechanisms (Lin, 2015). Resistance to ciprofloxacin and azithromycin were the lowest among all antimicrobials tested on NARMS GN Panel, which correlates with absence of macrolides and quinolones usage on the farms. Resistance to sulfisoxazole was prevalent even though usage of folate-pathway antibiotics was not reported in Sawant et al.'s survey. One explanation is the co-selection of sulfisoxazole genes by the presence of other antibiotics or chemicals.

Prevalence of *E. coli* resistant to each antibiotic was always highest in pre-weaned calf samples, followed by post-weaned calf, and was relatively low in lactating cows and dry cows. The prevalence of multi-drug resistant *E. coli* was also the highest compared with the other animal groups. Among *E. coli* resistant to at least one antibiotic on the NARMS GN Panel, isolates from pre-weaned calf samples were most commonly resistant to 4 of 9 classes of antibiotics, whereas isolates from other animal groups were more likely to exhibit resistance to just 1 of 9 classes of antibiotic. These results point to a potential selective pressure in calf gastrointestinal environment. Enteritis and septicemia are important clinical problems in calves and not generally adult cattle (Cummings et al., 2014), thus calves are frequently exposed to antimicrobial therapy for digestive problems. In addition, pre-weaned calves are

given antibiotic-supplemented milk replacer as a preventative measure. According to the 2007 NAHMS Dairy study, 50% U.S. dairy operations used medicated milk replacer for claves (APHIS, 2008). Pneumonia in pre-weaned and weaned calves also requires antibiotic treatment (APHIS, 2008). The presence and spread of multi-drug resistant *E. coli* isolated from healthy calves is worthy of further consideration. Increased number of multidrug-resistant *E. coli* could serve as a reservoir for genes that encode antimicrobial resistance and facilitate the exchange of antimicrobial genetic determinants with other species in the environment.

Another factor allowing the amplification of bacteria that may have antimicrobial resistance mechanisms that result in a high fitness cost could be the lack of a developed microbiota in young calves, as observed in metagenomics studies (Oikonomou et al., 2013). Previous study has associated increased levels of MDR with calves of 2 to 4 wk of age (Berge et al., 2006). Hoyle et al. (2004) observed that calves were rapidly colonized by ampicillin resistant *E. coli*, with peak prevalence in the 4 month-old calf group. Consistent decline of ampicillin resistant *E. coli* to low levels with increasing age of the calves was observed ($p<0.001$).

Cephalosporins belong to β-lactam antibiotic family which is an important class of antibiotics in human medicine (WHO, 2014). Ceftriaxone, one of the third-generation cephalosporins, is used for treatment of severe salmonellosis in children (Rabsch et al., 2001). In the present study, high prevalence of resistance to cephalosporins was observed in *E. coli*, especially in young animals. In the U.S., ceftiofur is the only cephalosporin approved for food production animals: it was

initially allowed to treat bovine respiratory disease and subsequently approved for other species, such as swine, sheep and poultry (Bradford et al., 1999). Many isolates resistant to ceftiofur also exhibit decreased susceptibility to cephamycins and extended-spectrum cephalosporins, therefore the use of ceftiofur in food animals has come under increasing scrutiny as a selective factor responsible for the emergence and dissemination of ceftriaxone-resistant enteric pathogens such as *Salmonella* (Zhao et al., 2003). Ceftiofur treatment in calves was observed to be associated with reduced susceptibility to ceftriaxone (Pereira, 2014). However, a causal relationship between ceftiofur use and occurrence or dissemination of cephalosporin-resistant bacteria has not been established (Daniels et al., 2009; Singer et al., 2008). Quinolones, including ciprofloxacin, is another class of antibiotic of critical importance in human medicine. Enrofloxacin is the only quinolone drug approved for use in food producing animals: in 2008 enrofloxacin was approved for use in nonlactating cows less than 20 months of age for the treatment of bovine respiratory disease. Because all quinolones have a common mechanisms of resistance, resistance to one quinolone will usually result in resistance to all other quinolones and selection pressure from enrofloxacin treatment could result in the selection of resistance to ciprofoxacin (Hopkins et al., 2005). However, it appears that in the present study the resistance to quinolones was low in dairy related *E. coli*.

In bovine *E. coli* isolates, resistance to third-generation cephalosporins is mainly conferred by the plasmid-encoded AmpC-like CMY β-lactamases and by the plasmid-encoded CTX-M β-lactamases. The $bla_{\text{CMY-2}}$ gene is responsible for resistance to cefoxitin and reduced susceptibility to ceftiofur and ceftriaxone. CMY

β-lactamases are not inhibited by clavulanic acid. The AmpC-like resistance phenotype *E. coli* isolates were also mostly resistant to tetracycline, sulfisoxazole, and trimethoprim-sulfamethoxazole, and also commonly resistant to chloramphenicol and streptomycin. These links of resistance suggest that there is a possible co-selection of resistance genes against commonly used antibiotics and extended-spectrum β-lactamase producing genes. Zhao et al. (2001) have shown the presence of CMY gene in *E. coli* and *Salmonella* from food animals and ground meat and the $bla_{\text{CMY-2}}$ gene was transferable from *Salmonella* to recipient *E. coli* through conjugation.

The prevalence of $bla_{\text{CTX-M}}$ type *E.coli* was lower than the prevalence of bla_{CMY} type *E. coli*. Enterobacteriaceae-associated $bla_{\text{CTX-M}}$ genes have become globally widespread within the past 30 years. Since they were first detected in the late 1980s in Europe, various alleles of $bla_{\text{CTX-M}}$ have become the predominant genes encoding ESBL phenotype isolated from human clinical isolates of *E. coli* and *Klebsiella* spp. in many parts of the world. In 2007, 48% of extended-spectrum cephalosporin-resistant *E. coli* from a clinical laboratory in Philadelphia, Pennsylvania were CTX-M type (McGettigan et al. 2009). CTX-M-type *E. coli* has been identified in livestock samples in different regions of the world, but the first isolation in the U.S. was by Wittum et al. (2010) from both sick and healthy dairy cattle in Ohio. Three clonal strains were isolated from fecal samples that carried two distinguishable plasmids encoding $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-79}}$. One of the samples was from a calf that had recently received ceftiofur treatment. Based on a non-selective isolation method, Davis et al. (2015) concluded an overall prevalence of CTX-M *E.*

coli of 4.4% and an overall prevalence of *bla*$_{CMY}$-positive *E. coli* of 32.1% in Washington State dairy farms. Our results also further demonstrated high prevalence *bla*$_{CMY\text{-}2}^{+}$ *E. coli* in pre-weaned calves across all animal groups, which was also observed in the Washington State survey.

In spite of the co-existence of *Salmonella* and multi-resistant enteric *E. coli*, *Salmonella* remained pan-susceptible. Especially in young animal groups, the prevalence of multidrug resistant *E. coli* was as high as 70% and 39% for pre-weaned calves and post-weaned calves, respectively, and the prevalence of *Salmonella* was 13% and 25%, respectively. It was observed that *Salmonella* strains did not exhibit any similar resistance patterns with the *E. coli* strains present on the same farms. Resistance genes weren't readily transmitted despite *E. coli*'s perception as a supposed resistance gene pool. Further genetic characterization is needed to understand this phenomenon.

3.5 Conclusions

Salmonella was isolated from 64% dairy farms and *Salmonella* was more frequently detected from cows than from calves. The majority *Salmonella* isolates belong to serogroup C1, C2, U. Serogroup C1 were mostly *S.* Montevideo, C2 *S.* Kentucky, and U *S.* Cerro. *Salmonella* spp. isolates were mostly pan-susceptible. *E. coli* isolates were commonly resistant to tetracycline, sulfonamides, aminoglycosides and β-lactams. *E. coli* isolated from calves were more resistant than isolates from cows. *E. coli* resistant to up to 12 antibiotics (9 classes) on NARMS GN Panel was observed. The *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}9}$ genes were detected in 4 *E. coli* isolates from

4 different farms (5%). The *bla*$_{CMY}$ gene was found in 35% of the farms surveyed. The results of this study indicate that resistant *E. coli* are more prevalent in calves than in adult cows within the same herd. Higher prevalence of resistant *E. coli* in calves may be due to the selective pressures associated with higher exposure to antimicrobials. The presence of MDR *E. coli* on dairy farms poses potential risks to human health.

Chapter 4: Pulsed-field Gel Electrophoresis Characterization of AmpC-/ESBL-type *E. coli* from Dairy Herds

4.1 Further characterization of ESBLs/AmpC-type E. coli

Antibiotic susceptibility test is useful to identify resistant and multidrug-resistant microorganisms. Resistance phenotype is especially helpful to speculate the resistance mechanisms of interest, including extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases.

ESBLs are typically inhibitor-susceptible β-lactamases that hydrolyze penicillins, cephalosporins, and aztreonam, and also are usually multi-drug resistant (Thomson, 2010). ESBLs are encoded by mobile genes and therefore ESBL genes are transmissible. The most frequently encountered ESBLs belong to the CTX-M, SHV, and TEM families. Most ESBL detection tests are growth based, with confirmatory tests based on a β-lactamase inhibitor potentiating (enhancing) the activity of a cephalosporin or aztreonam in the presence of an ESBL (Thomson, 2010). In the present study, ESBLs candidates were selected based on the following criteria: AMP^{+}, FOX^{-}, TIO^{+}, AXO^{+}, and resistance to cefotaxime/ceftazidime are significantly inhibited by clavulanic acid (confirmed by NARMS ESBL Panel).

AmpC β-lactamases preferentially hydrolyze narrow-, broad-, and expanded-spectrum cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam and tazobactam (Thomson, 2010). Transmissible AmpC β-lactamases, also referred as plasmid-mediated AmpC β-lactamases, were originated from chromosomally mediated AmpC gram-negative bacilli. The most commonly

encountered plasmid-mediate β-lactamases belong to the CMY, FOX, and DHA families, and are typically associated with multidrug resistance. Phenotypic insusceptibility to cephamycin i.e. cefoxitin, will distinguish AmpC β-lactamases from ESBLs. Due to the fact that phenotypic tests do not differentiate between chromosomal and plasmid-mediated AmpC β-lactamases, plasmid-mediated AmpC β-lactamases are most accurately detected with the PCR test. In the present study, selected AmpC-type candidates exhibiting phenotype AMP^+, FOX^+, TIO^+, and AXO^+ were tested for the presence of *bla*$_{CMY}$ gene by PCR.

In the study described in Chapter 3, the AmpC-type extended spectrum cephalosporin resistant *E. coli* were isolated from 32 farms. AmpC-type *E. coli* were isolated from more than one animal groups in 10 farms. *bla*$_{CMY}^+$ *E. coli* were detected in 35% of the farms and *bla*$_{CTX\text{-}M}^+$ *E. coli* on 5% of the farms. However, little is known about how the resistance spread – both within farms and between farms. For example, on a given farm, is there a specific resistant *E. coli* strain isolated from both young and adult animals? Whether an *E. coli* strain was obtained from different farms? Pulsed-field gel electrophoresis (PFGE) subtyping is one of the laboratory techniques used to examine the epidemiological relatedness of *E. coli*.

PFGE technique is used generate DNA fingerprints of bacterial isolates. PFGE used molecular scissors, called restriction enzymes, to cut bacterial DNA at certain locations known as restriction sites. These molecular scissors are selected to generate a small number of DNA pieces that can be separated based on size. Firstly, the bacteria are loaded into an agarose suspension then the bacterial cell is opened to release the DNA. Then the agarose and DNA suspension, also called plug, is treated

with restriction enzymes and loaded onto an agarose gel. PFGE is able to separate large restriction fragments because an electric field that constantly changes direction is applied.

4.2 Materials and methods for PFGE

Seven farms having at least one young animal group and one adult animal group harboring AmpC/ESBL phenotype *E. coli* were selected in this preliminary study. Some of the *E. coli* isolates included in this study were from the random isolation described in Chapter 3. The other *E. coli* were isolated from the manure composite samples using MacConkey agar supplemented with breakpoint concentrations of cefotaxime or cefepime.

PFGE was performed following the standardized PulseNet *E. coli* protocol (Ribot et al., 2006) with a few modifications as described previously (Van Kessel et al., 2012). Cultures were streaked onto tryptic soy agar supplemented with 0.6% yeast extract (BD, Sparks, MD) and incubated overnight at 37°C, and the biomass was used for agarose plug preparation. The DNA in plug slices was digested with 50 U XbaI for 4h at 37°C. Thiourea (50 μM) was added to both the gel, composed of 1% Seakem Gold agarose in 0.5 Tris-borater-EDTA buffer, and the electrophoresis running buffer, and the gels were run on a CHEF-DR II and CHEF-DR III system (Bio-Rad, Hercules, California). The gels were stained with 1 μg/ml ethidium bromide, and images were obtained using a ChemiDoc XRS gel documentation system (Bio-Rad, Hercules, California). Bands were assigned manually, and PFGE profiles were analyzed using BioNumerics software (Applied Maths, Austin, TX). Dendrograms

were derived using the individual XbaI experiments with arithmetic average cluster analysis.

4.3 Preliminary results and discussion

The Xbal enzyme PFGE restriction digest patterns of the cephalosporin-resistant *E. coli* isolates showed high level of diversity (Figure 4). Indistinguishable strain clusters were observed on 4 farms, 144, 111, 108 and 142. Based on the dendrogram (Figure 4), all three isolates from pre-weaned calves, dry cows, and lactating cows on farm 144 were indistinguishable (≥96% similarity, cluster B1). Three isolates shared identical resistance phenotype profile, except isolate R#665 had a MIC of 16 μg/ml for FOX (cefoxitin), which was lower than the CLSI breakpoint concentration for FOX (32 μg/ml), and thus was considered as "intermediate". In the present study, for the convenience of data analysis, "susceptible" and "intermediate" were categorized as "susceptible". It is important to point out that most FOX "susceptible" *E. coli* isolates have MIC value around 4 μg/ml (data not shown), which is greatly lower than 16 μg/ml. Therefore, this specific isolates exhibiting "intermediate" resistance to FOX should be considered as AmpC-type as the other two isolates from the same farm.

On farm 111, four AmpC-type isolates from pre-weaned calves and lactating cows were indistinguishable (≥96% similarity, cluster B2). The 7 AmpC phenotype *E. coli* isolates from farm 111 and 144 in cluster B1 and B2 shared 90% similarity, and none of the isolates harbored bla_{CMY} gene when tested with PCR. It could be hypothesized that an unknown plasmid-mediated AmpC-like gene was acquired by two closely related *E. coli* strains on these two farms. In addition, farm 111 wasn't

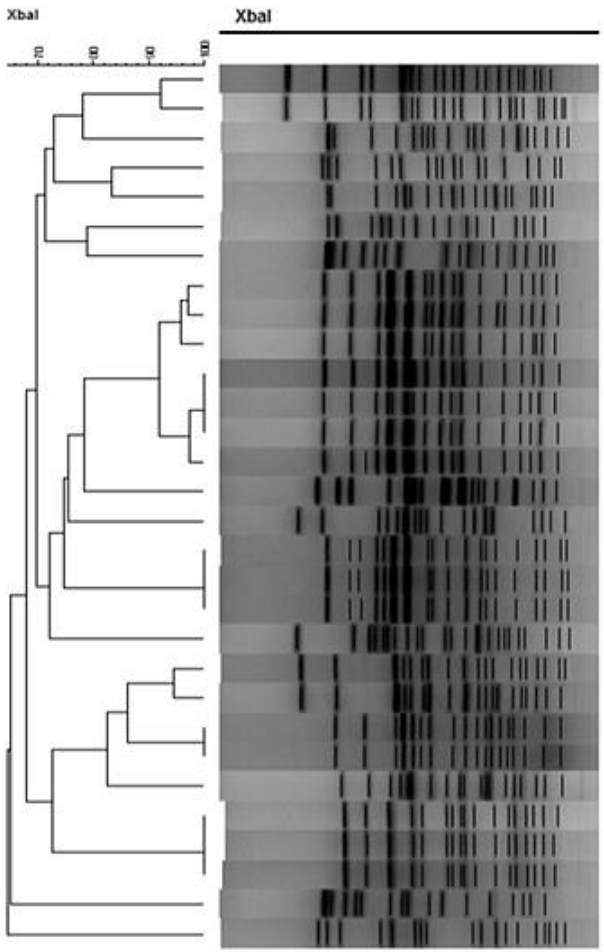
completely dominated by one cephalosporin resistant *E. coli* strain. The other cephalosporin resistant *E. coli* exhibiting AmpC phenotype was isolated from post-weaned calves, with bla_{CMY} gene detected by PCR.

Isolates from farm 108 can be categorized into two distinct clusters B3 and C2. Identical AmpC-type *E. coli* (100% similarity) in cluster B3 were isolated from post-weaned calves, dry cows, and lactating cows. All of the isolates in cluster B3 were carrying bla_{CMY} genes, and only resistant to β-lactam but not any other classes of antibiotics. Isolates in C2 were TIO^{+}, AXO^{+}, FOX-, clavulanic acid inhibit CTX and TAZ inactivation, which are characteristic for ESBL CTX-M type except being resistant to Augmentin. This uncommon resistance phenotype implicated the presence of multiple β-lactamase genes, possibly including ESBL gene. Overall, the dendrogram of farm 108 (Figure 4) showed clonal within-farm-spread of cephalosporin-resistant *E. coli* strains, and co-existence of two different cephalosporin-resistant strains on the same farm.

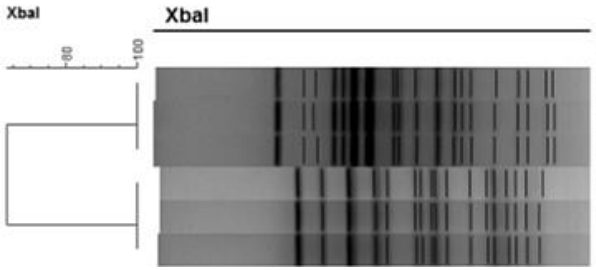
Cluster C1 included two identical isolates from farm 142, one from post-weaned calves and one from dry cows. Another isolate from pre-weaned calves on farm 142 exhibited ~87% similarity with the two isolates in cluster C1. The other two farm 142 isolates belonged to clusters A and, B, well removed from cluster C. All of the isolates from farm 142, as well as farm 35, 80, and 82, carried bla_{CMY} genes. The heterogeneity of these *E. coli* isolates suggested that bla_{CMY} genes were likely obtained through multiple independent acquisitions by different *E. coli* strains, or that several introductions of resistant *E. coli* occurred on these farms.

Within-farm cephalosporin-resistant *E. coli*, isolated from different animal groups, were commonly observed to be identical strains. However, resistant *E. coli* isolates sampled from different farms were not found to be closely related. The highest between-farm similarity observed was between the pre-weaned calf isolates from farms 80 and 142, sharing 94% similarity (cluster C). In cluster A, a pre-weaned calf isolate from farm 142 and a lactating cow isolate from farm 35 exhibited ~92% similarity. The majority of *E. coli* strains from separate farms were distinct (<80% similarity).

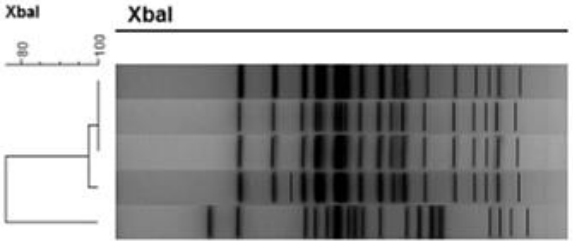
Even though only 7 farms were examined in this preliminary study, the clonal spread of ESBL-/AmpC-type *E. coli* was commonly observed (57%, 4 out of 7) among young and adult animal groups within individual farms. In general, a high degree of heterogeneity was observed in AmpC-type *E. coli* between different farms.



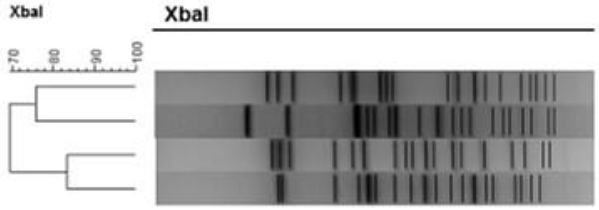
R #	Plate Set#	Farm	Sample Source	AR Profile	CMY
250	11/21/13-3 # 38	142	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
613	01/16/14-1A # 27	35	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
642	04/17/14-2A # 21	82	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET	+
608	11/22/13-1ABX # 27	80	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	+
244	11/21/13-3 # 05	80	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, TET	+
643	04/17/14-2A # 23	82	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET	+
644	04/17/14-2A # 25	82	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	+
662	04/28/14-1A # 04	144	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
664	04/28/14-1A # 10	144	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
665	04/28/14-1A # 12	144	Lactating cattle	AMP, CTX, TIO, AXO, AUG, STR, FIS, TET	-
617	05/19/14-1A # 13	111	Lactating cattle	AMP, FOX, CTX, AXO, AUG, STR, FIS, TET	-
463	05/08/14-1 # 31	111	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
469	05/08/14-1 # 49	111	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
618	05/19/14-1A # 16	111	Lactating cattle	AMP, FOX, CTX, AXO, AUG, STR, FIS, TET	-
256	11/21/13-4 # 07	142	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, SXT	+
619	05/19/14-1A # 33	111	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET, SXT	+
695	06/26/14-2A # 04	108	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG	+
694	06/26/14-1A # 46	108	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG	+
696	06/26/14-3A # 01	108	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG	+
645	04/18/14-1A # 02	82	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET	+
239	11/21/13-2 # 28	80	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG	+
249	11/21/13-3 # 40	142	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, SXT	+
624	11/22/13-1 ABX # 41	142	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
623	11/22/13-1 ABX # 37	142	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
313	01/16/14-2 # 15	35	Pre-weaned calves	AMP, FOX, TIO, AXO, AUG, AZI, CHL, STR, FIS, TET, SXT	+
667	06/26/14-1A # 39	108	Pre-weaned calves	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-
668	06/26/14-1A # 41	108	Post-weaned calves	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-
669	06/26/14-3A # 03	108	Lactating cattle	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-
443	04/17/14-2 # 10	82	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET	+
610	11/22/13-1ABX # 32	80	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG	+



R #	Plate Set#	Farm	Sample Source	AR Profile	CMY
695	06/26/14-2A # 04	108	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG	+
694	06/26/14-1A # 46	108	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG	+
696	06/26/14-3A # 01	108	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG	+
667	06/26/14-1A # 39	108	Pre-weaned calves	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-
668	06/26/14-1A # 41	108	Post-weaned calves	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-
669	06/26/14-3A # 03	108	Lactating cattle	AMP, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	-



R #	Plate Set#	Farm	Sample Source	AR Profile	CMY
617	05/19/14-1A # 13	111	Lactating cattle	AMP, FOX, CTX, AXO, AUG, STR, FIS, TET	-
463	05/08/14-1 # 31	111	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
469	05/08/14-1 # 49	111	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	-
618	05/19/14-1A # 16	111	Lactating cattle	AMP, FOX, CTX, AXO, AUG, STR, FIS, TET	-
619	05/19/14-1A # 33	111	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET, SXT	+



R #	Plate Set#	Farm	Sample Source	AR Profile	CMY
610	11/22/13-1ABX # 32	80	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG	+
239	11/21/13-2 # 28	80	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG	+
608	11/22/13-1ABX # 27	80	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, TET	+
244	11/21/13-3 # 05	80	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, TET	+

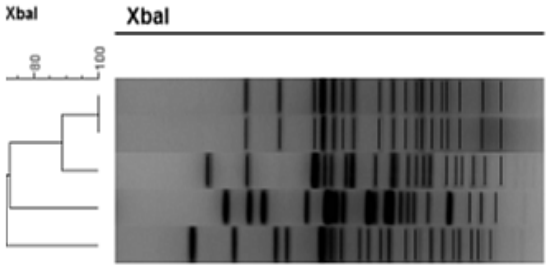


Plate Set#	R #	Farm	Sample Source	AR Profile	CMY
11/22/13-1 ABX # 41	624	142	Post-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
11/22/13-1 ABX # 37	623	142	Dry cows	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+
11/21/13-3 # 40	249	142	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, SXT	+
11/21/13-4 # 07	256	142	Lactating cattle	AMP, FOX, CTX, TIO, AXO, AUG, STR, FIS, SXT	+
11/21/13-3 # 38	250	142	Pre-weaned calves	AMP, FOX, CTX, TIO, AXO, AUG, CHL, STR, FIS, TET, SXT	+

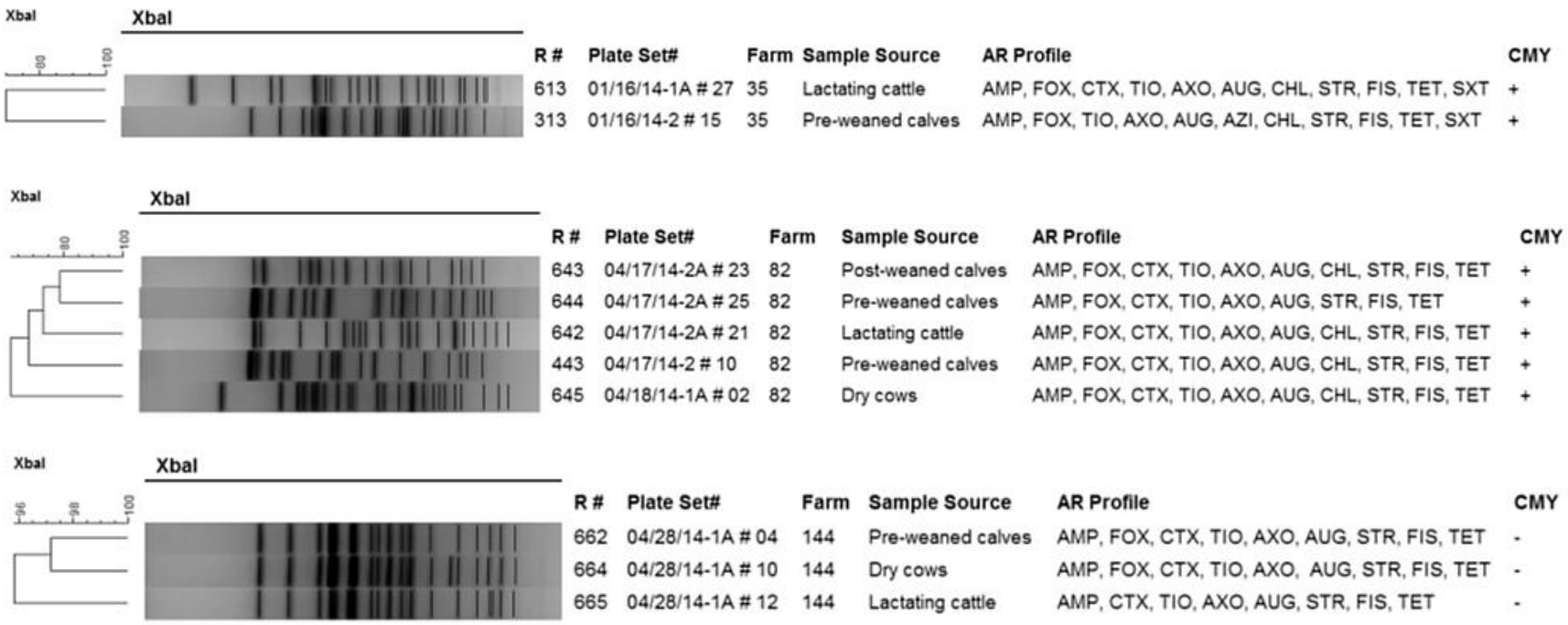


Figure 5. Dendrogram of Xbal pulsed-filed electrophoresis of selected cephalosporins resistant *E. coli* isolates from manure composite samples from pre-weaned calf, post-weaned calf, dry cow and lactating cow on 6 farms in Pennsylvania. Isolates were obtained through random isolation or direct isolation through spiral plating on MacConkey Agar supplemented with breakpoint concentration of cefotaxime or cefepime.

Chapter 5: Suggestions for Future Research

Antibiotics are a primary defense against many bacterial diseases in both human and veterinary medicine. Thus, efforts to promote the appropriate use of antimicrobials in both humans and animals and to enhance surveillance are essential for controlling multidrug resistance of bacterial pathogens. Antibiotics need to be used more prudently in both human and veterinary medicine in order to slow down resistance gene distribution and prevent the emergence of new resistance genes (Allen, 2014). In this study, farmers from each farm were asked to fill out a short survey about farm demographics and practices, including antibiotic use. Analysis of these survey results in the future would be helpful to evaluate the correlation of antibiotic use and prevalence of resistant bacteria.

The high prevalence of *bla*$_{\text{CMY}}$-type *E. coli* on dairy farms is a public health concern. The resistant *E. coli* in calves might serve as a reservoir for antimicrobial-resistance genes on dairy operations. Additional research is needed on the mechanisms of how the resistant *E. coli* strains persist in calves. In the current study, the PFGE subtyping of ESBL-/AmpC-type *E. coli* has been successfully applied to identify epidemiological relatedness within a farm at a single point in time, but one limitation is that the level of resolution provided by PFGE allows only limited phylogenetic inferences. Thus, further characterization of mobile elements and genome sequencing of *E. coli* will allow a higher resolution epidemiological investigation into patterns of dissemination over a larger geographical area and longer periods of time. Metagenomics which enables the study of community genomics

would be useful in the future for more detailed analyses of antibiotic resistant organisms and resistance genes directly in samples.

Appendix

Table A1. *Salmonella* serogroup combinations in lactating cow samples on *Salmonella* positive farms

Serotype Combinations				*No. of farms*	*Farm percentage (%)*
C1	*C2*	*U*	*B*		
1	0	1	0	17	21.3
0	0	1	0	16	20
0	1	0	0	5	6.3
1	0	0	0	5	6.3
0	1	1	0	4	5
1	1	1	0	2	2.5
0	1	0	1	1	1.3
1	1	0	0	1	1.3
0	0	0	0	29	36.3

Table A2. Resistance to each antibiotic tested on NARMS GN Panel among *E. coli* exhibiting resistance to at least one antibiotic on the panel (n=285)

Antimicrobial Agents	*Resistant Breakpoint (μg/ml)*	*No. of Isolates*	*% (n=285)*
AUG	32	74	26.0
AMP	32	119	41.8
AZI	>16	5	1.8
FOX	32	69	24.2
TIO	8	61	21.4
AXO	4	70	24.6
CHL	32	49	17.2
CIP	>4	4	1.4
GEN	16	15	5.3
NAL	32	12	4.2
STR	64	151	53.0
FIS	>256	160	56.1
TET	16	266	93.3
SXT	4	34	11.9

Table A3. Number of *E. coli* resistant to each total number of antibiotics on NARMS GN Panel among *E. coli* exhibiting resistance to at least one antibiotic on the panel (n=285) isolated from each type of samples

No. of Antimicrobials to which the isolates showed resistance	*Total*	*Percent (n=285)*	*Pre-weaned claves*	*Post-weaned claves*	*Dry cows*	*Lactating cows 1*	*Lactating cows 1*	*Lactating cows 1*
1	73	25.6	14	27	13	7	6	6
2	48	16.8	13	19	4	3	7	2
3	47	16.5	15	17	3	6	2	4
4	24	8.4	15	6	0	1	1	1
5	25	8.8	15	8	1	1	0	0
6	14	4.9	8	5	0	0	0	1
7	10	3.5	6	3	0	1	0	0
8	12	4.2	10	0	0	2	0	0
9	14	4.9	10	4	0	0	0	0
10	14	4.9	10	4	0	0	0	0
11	3	1.1	2	0	0	0	0	0
12	1	0.4	1	0	0	0	0	0

Table A4. Number and percentage of *E. coli* exhibiting different levels of resistance on NARMS GN Panel among *E. coli* exhibiting resistance to at least one antibiotic on the panel (n=285) isolated

Resistance Level	*Frequency*	*Percent*	*Cumulative Frequency*	*Cumulative Percent*
Resistant to 1-3 antibiotics	168	59.0	168	59.0
Resistant to 4-8 antibiotics	85	29.8	253	88.8
Resistant to 9-12 antibiotics	32	11.2	285	100.00

Table A5. Prevalence of *E. coli* resistant to each total number of antibiotics on NARMS GN Panel among *E. coli* exhibiting resistance to at least one antibiotic on the panel (n=285) for each type of samples. Prevalence were also calculated for young and adult animal samples, respectively, and on farm-level

No. of Antimicrobials to which the isolates showed resistance	*Pre-weaned claves*	*Post-weaned calves*	*Young animals*	*Dry cows*	*Lactating cows*	*Adult animals*	*Overall prevalence*
1	16.0	32.0	38.0	17.0	21.0	33.0	59.0
2	14.0	24.0	33.0	6.0	13.0	16.0	43.0
3	17.0	21.0	29.0	4.0	14.0	18.0	41.0
4	18.0	8.0	25.0	0.0	4.0	4.0	29.0
5	18.0	9.0	22.0	1.0	1.0	3.0	24.0
6	10.0	7.0	15.0	0.0	1.0	1.0	16.0
7	8.0	4.0	10.0	0.0	1.0	1.0	11.0
8	13.0	0.0	13.0	0.0	3.0	3.0	13.0
9	12.0	5.0	15.0	0.0	0.0	0.0	15.0
10	12.0	4.0	14.0	0.0	0.0	0.0	14.0
11	3.0	0.0	4.0	0.0	0.0	0.0	4.0
12	1.0	0.0	1.0	0.0	0.0	0.0	1.0

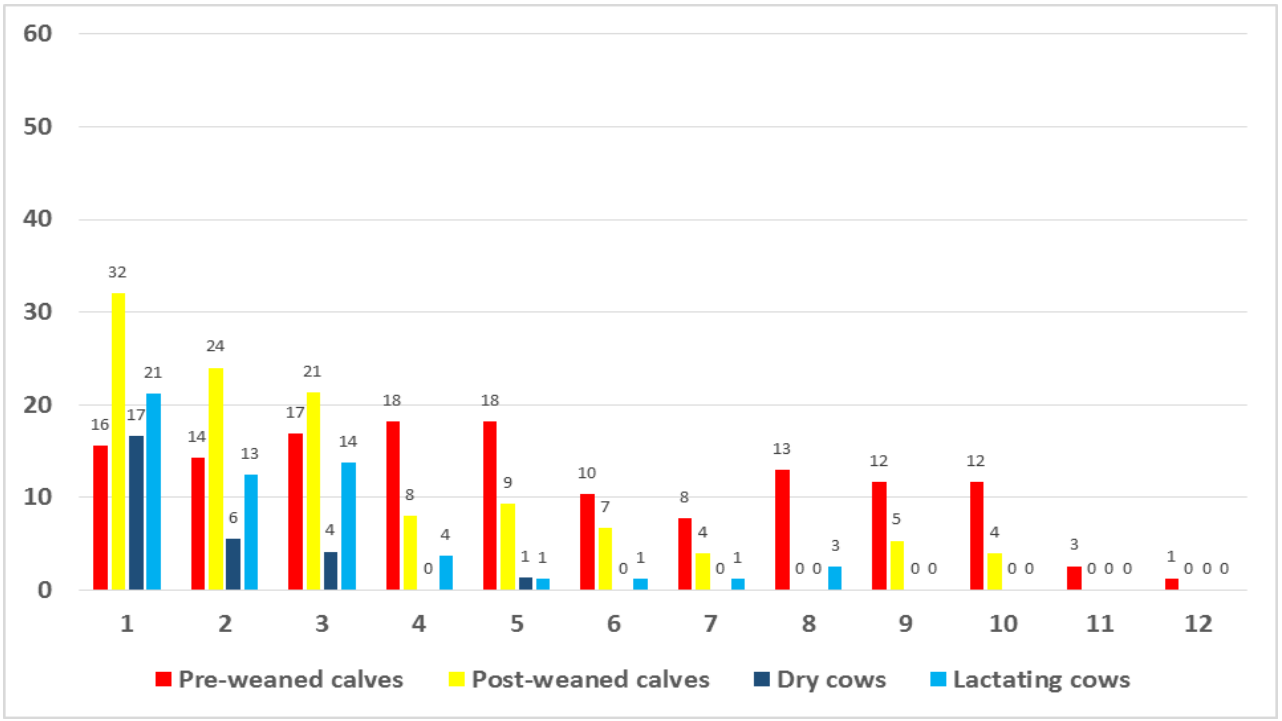


Figure A1. Resistance to various number of antibiotics among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel (n=285). *E. coli* isolates were from manure samples of pre-weaned calves, post-weaned calves, dry cows, and lactating cows in 80 Pennsylvania dairy farms.

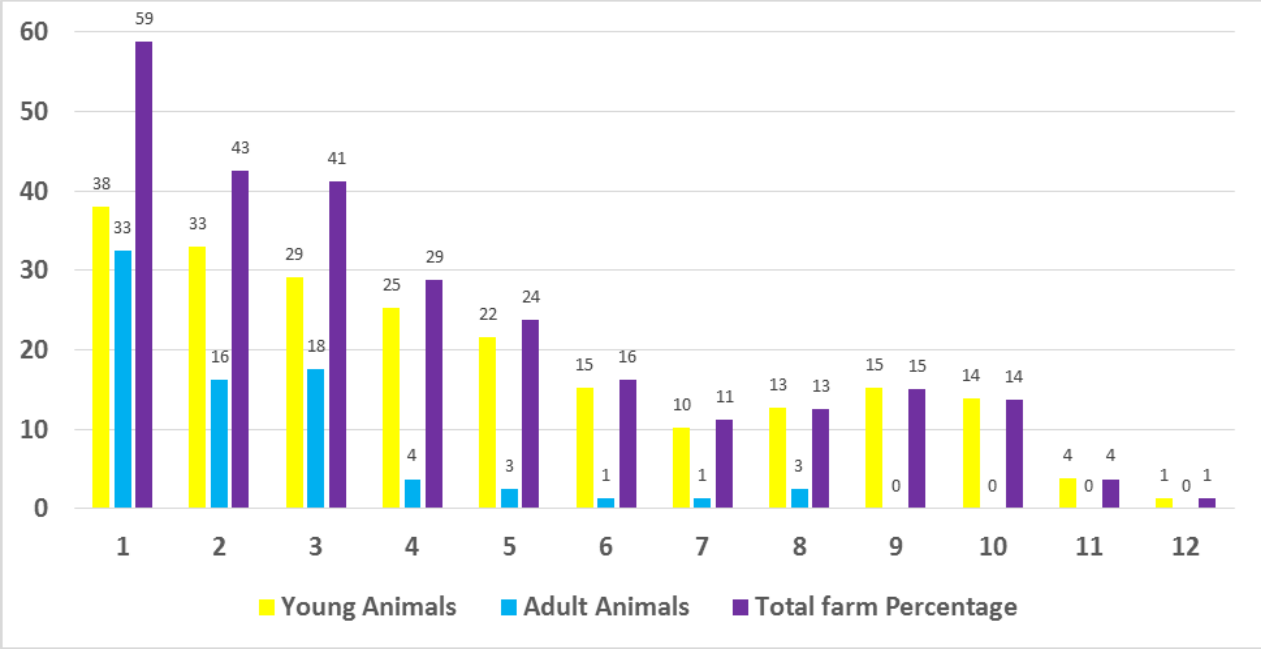


Figure A2. Resistance to various number of antibiotics among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel (n=285). *E. coli* isolates were from manure samples of pre-weaned calves, post-weaned calves, dry cows, and lactating cows in 80 Pennsylvania dairy farms. Young animal data were pooled from results of pre-weaned and post-weaned samples, and adult animal data were pooled from results of dry cow and lactating cow samples.

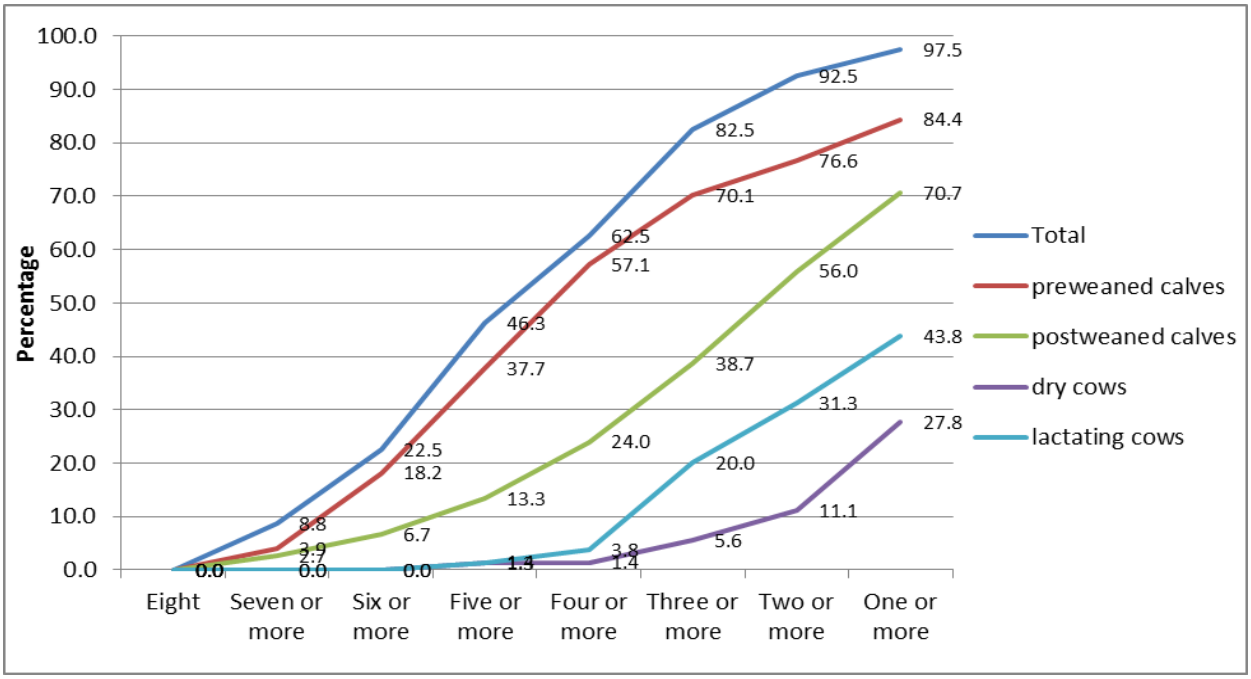


Figure A3. Trend lines showing accumulative prevalence of multiple-classes drug resistance *E. coli* for pre-weaned calves, post-weaned calves, dry cows and lactating cows. Results were based on broth microdilution method with NARMS GN Panel antimicrobials. Fourteen antimicrobials tested were categorized into 9 drug classes.

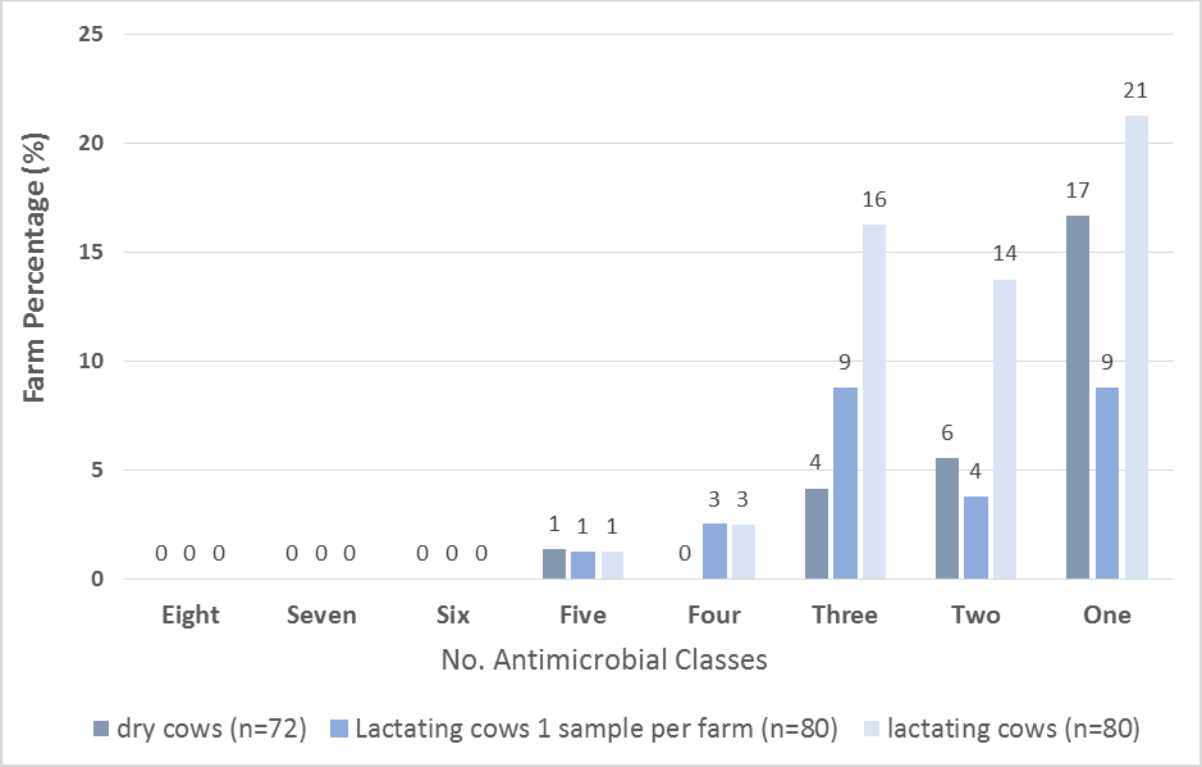


Figure A4. Resistance to various number of classes of antibiotics among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel. *E. coli* isolates were from manure samples of dry cows, one lactating cow sample, and pooled results of up to three lactating cow samples. Graph suggesting that higher prevalence of resistance in lactating cows might partially contributed by the elevated sample size and increased detection limit.

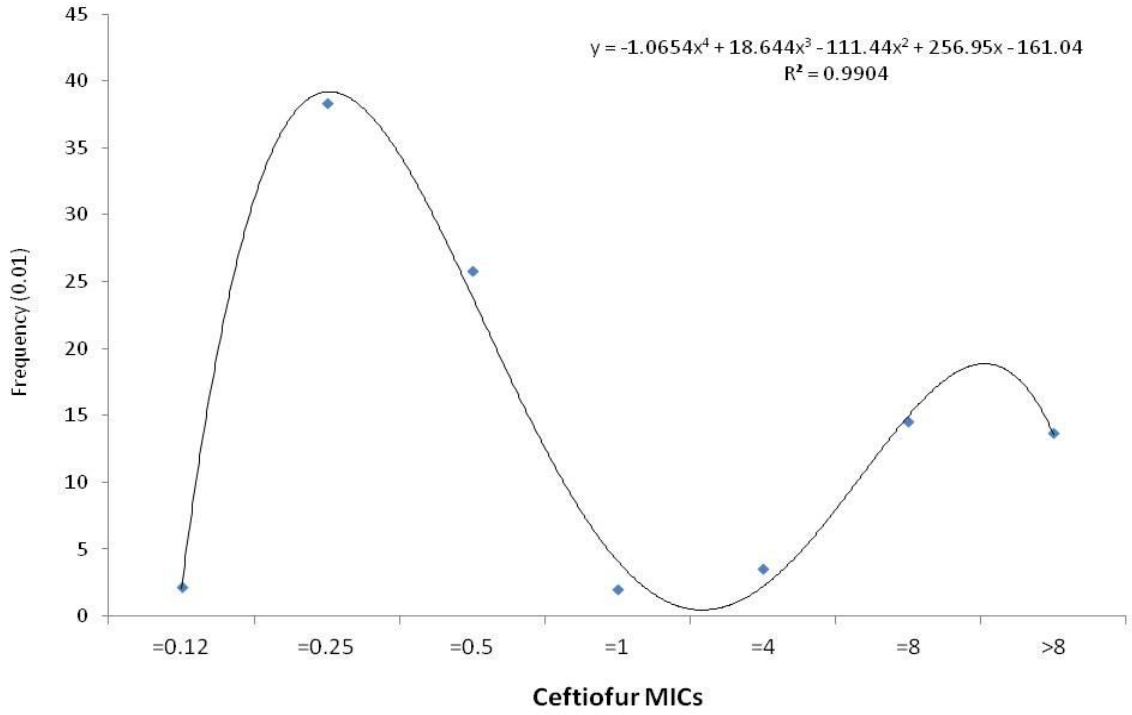


Figure A5. MICs distribution for ceftiofur among *E. coli* resistant to at least one antibiotic when tested for susceptibility using broth microdilution method on NARMS GN Panel (n=285). Multinomial regression was generated using Excel (R^2=0.99). Results suggesting CLSI cut-off is not suited for distinguishing wild and non-wild type in this study.

References

1. Allen, H. K. 2014. Antibiotic resistance gene discovery in food-producing animals. Current Opinion in Microbiology, 19(1), 25–29. doi:10.1016/j.mib.2014.06.001
2. APHIS. 2008. Antibiotic Use on U.S. Dairy Operations, 2002 and 2007. U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Centers for Epidemiology and Animal Health.
3. APHIS. 2009. *Salmonella* and *Campylobacter* on U.S. Dairy Operations, 1996–2007, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Centers for Epidemiology and Animal Health.
4. Bell, S. M., J. N. Pham, P. J. Newton, and T. T. Nguyen. 2009. Antibiotic Susceptibility Testing By the CDS Method.
5. Berge, C. B., D. Moore, T. E. Besser, and W. M. Sischo. 2009. Targeting therapy to minimize antimicrobial use in pre-weaned calves: effects on health, growth, and treatment costs. Journal of Dairy Science, 92(9), 4707–4714. doi:10.3168/jds.2009-2199
6. Berge, A. C. B., D. A. Moore, and W. M. Sischo. 2006. Prevalence and antimicrobial resistance patterns of *Salmonella enterica* in pre-weaned calves from dairies and calf ranches. American Journal of Veterinary Research, 67(9), 1580–1588. doi:10.2460/ajvr.67.9.1580
7. Blair, J. M. A., M. A. Webber, A. J. Baylay, D. O. Ogbolu, and L. J. V. Piddock. 2011. Molecular mechanisms of antibiotic resistance. Chemical Communications (Cambridge, England), 47(14), 4055–4061. doi:10.1039/c0cc05111j

8. Blau, D. M., B. J. McCluskey, S. R. Ladely, D. A. Dargatz, P. J. Fedorka-Cray, K. E. Ferris, and M. L. Headrick. 2005. *Salmonella* in dairy operations in the United States: prevalence and antimicrobial drug susceptibility. Journal of Food Protection, 68(4), 696–702.

9. Bonnet, R. 2004. Growing group of extended-spectrum beta-lactamases: The CTX-M enzymes. Antimicrobial Agents and Chemotherapy, 48(1), 1–14. doi:10.1128/AAC.48.1.1-14.2004

10. Bradford, P., P. J. Petersen, I. M. Fingerman, and D. G. White. 1999. Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrheal disease. Journal of Antimicrobial Chemotherapy, 44(5), 607–610. doi:10.1093/jac/44.5.607

11. Callaway, T. R., J. E. Keen, T. S. Edrington, L. H. Baumgard, L. Spicer, E. S. Fonda, K. E. Griswold, T. R. Overton, M. E. VanAmburgh, R. C. Anderson, K. J. Genoverse, T. L. Poole, R. B. Harvey, and D. J. Nisbet. 2005. Fecal prevalence and diversity of *Salmonella* species in lactating dairy cattle in four states. Journal of Dairy Science, 88(10), 3603–3608. doi:10.3168/jds.S0022-0302(05)73045-9

12. Castanheira, M., R. E. Mendes, P. R, Rhomberg, and R. N. Jones. 2008. Rapid emergence of *bla*$_{\text{CTX-M}}$ among Enterobacteriaceae in U.S. medical centers: Molecular evaluation from the MYSTIC program (2007). Microbial Drug Resistance (Larchmont, N.Y.), 14(3), 211–216. doi:10.1089/mdr.2008.0827

13. Centers for Disease Control and Prevention. 2013. National Antimicrobial Resistance Monitoring System: Enteric Bacteria, 2012 Human Isolates Final Report. doi:10.1016/S0022-3913(12)00047-9

14. Centers for Disease Control and Prevention. 2013. Antibiotic Resistance Threats in the United States. doi:CS239559-B

15. Chantziaras, I., F. Boyen, B. Callens, and J. Dewulf. 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: A report on seven countries. Journal of Antimicrobial Chemotherapy, 69(3), 827–834. doi:10.1093/jac/dkt443

16. Collignon, P., J. H. Powers, T. M. Chiller, A. Aidara-Kane, and F. M. Aarestrup. 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 49(1), 132–141. doi:10.1086/599374

17. Cummings, K. 2013. Antimicrobial resistance trends among *Salmonella* isolates obtained from dairy cattle in the Northeastern United States, 2004–2011. Foodborne Pathogens and Disease, 10(4), 353–361. doi:10.1089/fpd.2012.1285

18. Cummings, K. J., V. A. Aprea., and C. Altier. 2014. Antimicrobial resistance trends among *Escherichia coli* isolates obtained from dairy cattle in the Northeastern United States, 2004-2011. Foodborne Pathogens and Diseases, 11(1), 61–67. doi:10.1089/fpd.2013.1605

19. D'Costa, V. M., C. E. King, L. Kalan, M. Morar, W. W. L. Sung, C. Schwarz, E. Froese, G. Zazula, F. Galmels, R. Debruyne, G. B. Golding, H. N. Poinar, and G. D. Wright. 2011. Antibiotic resistance is ancient. Nature, 477(7365), 457–461. doi:10.1038/nature10388

20. Daniels, J. B., D. R. Call, D. Hancock, W. M. Sischo, K. Baker, and T. E. Besser. 2009. Role of ceftiofur in selection and dissemination of *bla*$_{\text{CMY-2}}$- mediated cephalosporin

resistance in *Salmonella enterica* and commensal *Escherichia coli* isolates from cattle. Applied and Environmental Microbiology, 75(11), 3648–3655. doi:10.1128/AEM.02435-08

21. Davis, M. A., W. M. Sischo, L. P. Jones, D. A. Moore, S. Ahmed, D. M. Short, and T. E. Besser. 2015. Recent emergence of *E. coli* carrying *bla*$_{\text{CTX-M}}$ encoded cephalosporin resistance on Washington State dairy farms. Applied and Environmental Microbiology, (April), AEM. 00463–15. doi:10.1128/AEM.00463-15
22. Doyle, M. E. 2015. Multidrug-resistant pathogens in the food supply. Foodborne Pathogens and Disease, 12(4), 261–279. doi:10.1089/fpd.2014.1865
23. Durso, L. M., G. P. Harhay, J. L. Bono, and T. P. L. Smith. 2011. Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach. Journal of Microbiological Methods, 84(2), 278–282. doi:10.1016/j.mimet.2010.12.008
24. European Center for Disease Prevention and Control. 2013. Summary of the Latest Data on Antibiotic Resistance in the European Union.
25. Economou, V., and P. Gousia. 2015. Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infection and Drug Resistance, 8:49–61.
26. European Public Health Alliance. 2012. European Public Health Alliance Briefing on Antimicrobial Resistance.
27. European Commission. 2011. Communication from the Commission to the European Parliament and the Council: Action plan against the rising threats from Antimicrobial Resistance.

28. European Food Safety Authority. 2014. Antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2012. EFSA Journal (12). doi:10.2903/j.efsa.2014.3590

29. Habing, G. G., J. E. Lombard, C. A. Kopral, D. A. Dargatz, and J. B. Kaneene. 2012. Farm-level associations with the shedding of *Salmonella* and antimicrobial-resistant *Salmonella* in U.S. dairy cattle. Foodborne Pathogens and Disease, 9(9), 815–821. doi:10.1089/fpd.2012.1149

30. Hopkins, K. L., R. H. Davies, and E. J. Threlfall. 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. International Journal of Antimicrobial Agents, 25(5), 358–373. doi:10.1016/j.ijantimicag.2005.02.006

31. Hoyle, D. V., D. J. Shaw, H. I. Knight, H. C. Davison, M. C. Pearce, C. Low, G. J. Gunn, and M. E. J. Woolhouse. 2004. Age-related decline in carriage of ampicillin-resistant *Escherichia coli* in young calves. Applied and Environmental Microbiology, 70(11), 6927–6930. doi:10.1128/AEM.70.11.6927-6930.2004

32. Hughes, D., and D. I. Andersson. 2012. Selection of resistance at lethal and non-lethal antibiotic concentrations. Current Opinion in Microbiology, 15(5), 555–560. doi:10.1016/j.mib.2012.07.005

33. Humeniuk, C., G. Arlet, V. Gautier, P. Grimont, R. Labia, and A. Philippon. 2002. β-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrobial Agents and Chemotherapy, 46(9), 3045–3049. doi:10.1128/AAC.46.9.3045-3049.2002

34. Le Hello, S., A. Bekhit, S. A. Granier, H. Barua, J. Beutlich, M. Zając, S. Munch, V. Sintchenko, B. Bouchrif, K. Fashae, J. L. Pinsard, L. Sontag, L. Fabre, M. Garnier, V.

Guibert, P. Howard, R. S. Hendriksen, J. P. Christensen, P. K. Biswas, A. Cloeckaert, W. Rabsch, D. Wasyl, B. Doublet, and F. X. Weill. 2013. The global establishment of a highly-fluoroquinolone resistant *Salmonella enterica* serotype Kentucky ST198 strain. Frontiers in Microbiology, 4(12), 1–10. doi:10.3389/fmicb.2013.00395

35. Li, X., N. Watanabe, C. Xiao, T. Harter, B. McCowan, Y. Liu, and E. R. Atwill. 2014. Antibiotic-resistant *E. coli* in surface water and groundwater in dairy operations in Northern California. Environmental Monitoring and Assessment, 186(2), 1253–1260. doi:10.1007/s10661-013-3454-2
36. Lin, J., K. Nishino, and M. C. Roberts. 2015. Mechanisms of antibiotic resistance. Frontiers in Microbiology, 6, 1–3. doi:10.1016/S0011-393X(96)80095-6
37. McGettigan, S. E., B. Hu, K. Andreacchio, I. Nachamkin, and P. H. Edelstein. 2009. Prevalence of CTX-M β-lactamases in Philadelphia, Pennsylvania. Journal of Clinical Microbiology, 47(9), 2970–2974. doi:10.1128/JCM.00319-09
38. Mulvey, M. R., D. A. Boyd, R. Finley, K. Fakharuddin, S. Langner, V. Allen, L. Ang, S. Bekal, S. E. Bailey, D. Haldane, L. Hoang, G. G. Horsman, M. Louis, L. Robberts, and J. Wylie. 201). Ciprofloxacin-resistant *Salmonella enterica* serovar Kentucky in Canada. Emerging Infectious Diseases, 19(6), 999–1001. doi:10.3201/eid1906.121351
39. National Association of State Departments Agriculture. 2011. National Association of State Departments Agriculture Releases Raw Milk Survey.
40. Oikonomou, G., A. G. V. Teixeira, C. Foditsch, M. L. Bicalho, V. S. Machado, and R. C. Bicalho. 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA: Associations of faecalibacterium species with health and growth. PLoS ONE, 8(4). doi:10.1371/journal.pone.0063157

41. Oliver, S. P., S. E. Murinda, and B. M. Jayarao. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. Food Borne Pathogens and Disesase, 8(3), 337–355. doi:10.1089/fpd.2010.0730

42. Pereira, R. V., J. D. Siler, J. C. Ng, M. A. Davis, and L. D. Warnick. 2014. Effect of pre-weaned dairy calf housing system on antimicrobial resistance in commensal *Escherichia coli*. Journal of Dairy Science, 97, 7633–7643. doi:10.3168/jds.2014-8588

43. Rabsch, W., H. Tschäpe, A. J. Bäumler. 2001. Non-typhoidal salmonellosis: Emerging problems. Microbes and Infection, 3(3), 237–247. doi:10.1016/S1286-4579(01)01375-2

44. Ribot, E. M., M. A. Fair, R. Gautom, D. N. Cameron, S. B. Hunter, B. Swaminathan, and T. J. Barrett. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathogens and Disease, 3(1), 59–67. doi:10.1089/fpd.2006.3.59

45. Sawant, A. A, L. M. Sordillo, and B. M. Jayarao. 2005. A survey on antibiotic usage in dairy herds in Pennsylvania. Journal of Dairy Science, 88(8), 2991–2999. doi:10.3168/jds.S0022-0302(05)72979-9

46. Singer, R. S., S. K. Patterson, and R. L. Wallace. 2008. Effects of therapeutic ceftiofur administration to dairy cattle on *Escherichia coli* dynamics in the intestinal tract. Applied and Environmental Microbiology, 74(22), 6956–6962. doi:10.1128/AEM.01241-08

47. Stokes, H. W., and M. R. Gillings. 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiology Reviews, 35(5), 790–819. doi:10.1111/j.1574-6976.2011.00273.x

48. Thomson, K. S. 2010. Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. Journal of Clinical Microbiology, 48(4), 1019–1025. doi:10.1128/JCM.00219-10

49. U.S. Department of Agriculture. 2014. Antimicrobial Resistance Action Plan.

50. U.S. Food and Drug Administration. 2013. National Antimicrobial Resistance Monitoring System 2011 Executive Report.

51. U.S. Food and Drug Administration. 2014. National Antimicrobial Resistance Monitoring System 2012 Retail Meat Annual Report.

52. U.S. Food and Drug Administration. 2013. Guidance for Industry #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food- Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GIF #209.

53. U.S. Food and Drug Administration. 2015. National Antimicrobial Monitoring System (NARMS) 2013 Retail Meat Interim Report.

54. Van Kessel, J. A. S., J. S. Karns, D. R. Wolfgang, and E. Hovingh. 2013. Regional distribution of two dairy-associated *Salmonella enterica* serotypes. Foodborne Pathogens and Disease, 10(5), 448–452. doi:10.1089/fpd.2012.1380

55. Van Kessel, J. A. S., J. S. Karns, D. R. Wolfgang, E. Hovingh, and Y. H. Schukken. 2012. Serotype shifts in an endemically infected dairy herd. Foodborne Pathogens and Disease, 9(4), 319–324. doi:10.1089/fpd.2011.1054

56. Van Kessel, J. S., S. Karns, D. R. Wolfgang, E. Hovingh, and Y. H. Schukken. 2007. Longitudinal study of a clonal, subclinical outbreak of *Salmonella enterica* subsp. *enterica* serovar Cerro in a U.S. dairy herd. Foodborne Pathogens and Disease, 4(4), 449–461. doi:10.1089/fpd.2007.0033

57. Van Kessel, J. S., J. Sonnier, S. Zhao, and J. S. Karns. 2013. Antimicrobial resistance of *Salmonella enterica* isolates from bulk tank milk and milk filters in the United States. Journal of Food Protection, 76(1), 18–25. doi:10.4315/0362-028X.JFP-12-263

58. Varma, J. K., K. Molbak, T. J. Barrett, J. L. Beebe, T. F. Jones, T. Rabatsky-Ehr, K. E. Smith, D. J. Vugia, H. G. H. Chang, and F. J. Angulo. 2005. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. The Journal of Infectious Diseases, 191(4), 554–561. doi:10.1086/427263

59. Wells, S. J., P. J. Fedorka-Cray, D. A. Dargatz, K. Ferris, and A. Green. 2001. Fecal shedding of *Salmonella spp.* by dairy cows on farm and at cull cow markets. Journal of Food Protection, 64(1), 3–11.

60. West, B. M., P. Liggit, D. L. Clemans, and S. N. Francoeur. 2011. Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near CAFO farms and wastewater treatment facilities. Water, Air, and Soil Pollution, 217(1-4), 473–489. doi:10.1007/s11270-010-0602-y

61. Wittum, T. E., D. F. Mollenkopf, J. B. Daniels, A. E. Parkinson, J. L. Mathews, P. R. Fry, M. J. Abley, and W. A. Gebreyes. 2010. CTX-M-type extended-spectrum β-lactamases present in *Escherichia coli* from the feces of cattle in Ohio, United States. Food Borne Pathogens and Diseases, 7(12), 1575–1579.

62. Wiuff, C., J. Lykkesfeldt, F. M. Aarestrup, and O. Svendsen. 2002. Distribution of enrofloxacin in intestinal tissue and contents of healthy pigs after oral and intramuscular administrations. Journal of Veterinary Pharmacology and Therapeutics, 25(5), 335–342. doi:10.1046/j.1365-2885.2002.00430.x

63. World Health Organization. 2014. Antimicrobial Resistance Global Report on Surveillance.

64. Zhao, S., S. Qaiyumi, S. Friedman, R. Singh, S. L. Foley, D. G. White, P. F. McDermott, T. Donkar, C. Bolin, S. Munro, E. J. Baron, and R. D. Walker. 2003. Characterization of *Salmonella enterica* serotype Newport isolated from humans and food animals. Journal of Clinical Microbiology, 41(12), 5366–5371. doi:10.1128/JCM.41.12.5366

65. Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker. 2001. Identification and expression of cephamycinase *bla*$_{CMY}$ genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrobial Agents and Chemotherapy, 45(12), 3647–3650. doi:10.1128/AAC.45.12.3647

66. Zhao, W., and Hu, Z. 2013. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Critical Reviews in Microbiology, 39(1), 79–101. doi:10.3109/1040841X.2012.691460