CHANGES IN ANTIMICROBIAL SUSCEPTIBILITY OF FECAL *ESCHERICHIA COLI* RECOVERED FROM DAIRY CATTLE ON 16 FARMS IN OHIO 2001-2011

THESIS

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Abstract

It has been hypothesized that the use of veterinary antimicrobials in livestock populations may lead to an increase in bacterial resistance to these antimicrobials among both animals and humans. Additionally, the transfer of resistance genes among bacterial pathogens may result in increased risk of food-borne disease resulting from pathogens with reduced susceptibility to the antimicrobials commonly used to treat them. Our objective is to measure changes in antimicrobial susceptibility of fecal *Escherichia coli* between 2001 and 2011 on 16 Ohio dairy farms. We assessed the reduced susceptibility proportions (RSP) of the 17 select antimicrobials, excluding apramycin, included in the National Antimicrobial Resistance Monitoring System surveillance program. We also examined the difference in the reduced susceptibility index (RSI) for these 16 herds.

Over a 2 year period (2001-2002) 9,253 fecal samples were collected via rectal palpation from cows on 42 Ohio dairy farms. In 2011 we returned to 16 of these same 42 herds and collected a composite sample of 400 fecal samples (25 samples from each of the 16 herds) using the same methods of collection as in the previous study period. Results of the RSP data shows an increase in resistance to 4 antimicrobials (Ampicillin, Cephalothin, Chloramphenicol and Sulfamethoxazole) and no significant change in resistance to the remaining 12 antimicrobials included in our study.
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Chapter 1: Literature Review

The development of antimicrobial drugs represents one of the most important discoveries in medicine. Until the most recent decade, since their introduction, the number of antimicrobials available for use within the United States has been increasing steadily. Today more than 150 antimicrobial drugs comprising 20 different classes with 10 targets of action are utilized to treat and prevent both human and animal bacterial diseases of importance (Khachatourians, 1998). In its annually published summary report detailing approved antimicrobial use in food animal agriculture within the United States, the Food and Drug Administration’s Center for Veterinary Medicine estimated that between 15,000 to 16,000 tons of antibiotics were sold and distributed in 2009 and 2010 (FDA, 2009; FDA, 2010).

Antimicrobials may reduce the threat of disease in domesticated livestock. However, the use of veterinary antimicrobial agents to relieve pain and animal suffering and improve production efficiency does not come without criticism. Media attention and consumer awareness of the risks associated with antimicrobial usage within food animals, has created demand for further study on how antibiotics work and what their impact for human, animal and environmental health may be. As with other facets of the food production industry, antimicrobial usage and reduced susceptibility trends within the United States dairy industry continue to be a subject of staunch controversy and research. Some will argue the consumer and media concerns are sensationalized and warrant no investigation,
however, microbiologists have provided evidence that concerns surrounding the effects of selection pressures applied by antimicrobial use are theoretically valid. Once introduced into an environment, antimicrobials place selective pressure on the existing microbes which may foster the development of resistant phenotypes.

Additionally, of more recent concern, there is mounting evidence to show that these resistant microbes may have capabilities of spreading these resistance factors to other, previously susceptible microbes (McDermott, 2002; Tenover 2006).

Aside from the concerns regarding selection pressure, the risks associated with antimicrobial drug residues within the food supply are of equal importance. For example, consumption of dairy products containing antibiotics can lead to allergic reactions in susceptible consumers. The recognition of such risks prompted the Federal Food and Drug Administration in 1924 to establish the Standard Milk Ordinance now called the Pasteurized Milk Ordinance (USPHS, 2009). Under these established guidelines which were most recently updated in 1991 to include testing for beta-lactam antibiotics, chloramphenicol, and sulfonamides; all milk products are required to test negative for 6 of the most frequently used beta-lactam antibiotics including: Penicillin, Amoxicillin, Ampicillin, Cloxacillin, Cephapirin and Ceftiofur prior to processing for human consumption. Chloramphenicol administration to lactating dairy cattle is prohibited by law and sulfadimethoxine is the only approved sulfonamide for administration to lactating dairy cattle.

Fear of reduced drug efficacy and differing opinions on what factors may contribute to antimicrobial resistance, as well as if it’s occurring and how to prevent it, continues to polarize academicians, dairy industry leaders, consumers and regulatory
agencies alike. Recently, concerns regarding food safety have lead to demand for increased regulation of the antimicrobials used and more advanced methods of detection within the dairy and meat industries.

Reports of unprocessed milk products containing antibiotic residues are extremely rare, widely reported to be less than 0.5% (Schaik et al., 2002). When antibiotic residues are found the milk is destroyed and not sold for public consumption. However, after discovering increasing frequency of antibiotic residues in the meat of cull dairy cows, in order to ensure compliance of antibiotic free dairy products the FDA made an expanded milk testing proposal in 2010. Although this proposal is currently being contested, it would expand testing of milk products for residues from up to 27 different therapeutic agents, including non-antimicrobials. Understanding the types of antimicrobials used in the dairy industry, the purposes for their use, the primary risks associated with them, and resistance trends helps to better our understanding of how dairy populations can be managed to maximize animal welfare, production, and food safety. Monitoring reduced susceptibility trends and the changes of minimum inhibitory concentrations over time for common food pathogens such as E. coli is critical to determining the effects of using antimicrobial therapies in food producing animals.

**Antimicrobial Use in the Dairy Industry:**

Growing concern over how antimicrobial resistance in animals affects humans makes some uses of antimicrobials in the dairy industry controversial. Foods of animal origin can serve as vehicles of food-borne disease in humans. If antimicrobial use in animal populations causes reduced susceptibility among food-borne pathogens, such as
Salmonella and Escherichia coli, this may prove to be one avenue for the transfer of resistance genes between not only bacterial pathogens, but between animals and humans as well. Additionally, if common food-borne pathogens are resistant to antimicrobials used in food animal populations, this poses public health concerns as it may result in food-borne outbreaks that are increasingly more difficult to treat.

As antimicrobial use and exposure within the environment increases, concerns over increased antimicrobial resistance have come to a forefront. The risk of resistant microorganisms entering the food chain has caused some regulatory agencies to make recommendations for limiting the ways in which antibiotics are used. In January of 2010 the Food and Drug Administration submitted to the US Congress a proposal to ban the administration of antibiotics at subtherapeutic levels for growth promotion (Becker, 2010). The potential impact of such legislation to limit antimicrobial use in the United States is unclear. Following the European Union’s 1997 ban of avoparcin, which was used as an antimicrobial growth promoter, the prevalence of vancomycin-resistant enterococci recovered from both humans and food decreased (McDonald et al., 2001). In 1995 the US Office of Technology Assessment reported that 50% of the antibiotics created in the United States are used for production of food animals and it reported that 90% of agricultural use of antibiotics within the United States is for the purpose of growth promotion (US Assessment, 1995). To address this issue, in 2012 the United States Food and Drug Administration made recommendations to reduce the amount of antimicrobials being used for growth promotion or production (USDA, 2014).

In the United States, ionophores, are the only antimicrobial drug approved for subtherapeutic use in lactating dairy rations. These antimicrobial compounds, which are
of little significance to human medicine, alter the ruminal bacterial population and improve digestive efficiency (Beckett et al., 1998). Feeding ionophores to lactating dairy cows can improve milk production by more than 5% (Phipps et al., 2000). Aside from improved feed efficiency and milk production, ionophores are not known to offer any additional benefits to the health of dairy cows. The practice of feeding ionophores does not come without criticism as some question whether the administration of ionophores is fostering further antimicrobial resistance. Investigators have explored the antimicrobial susceptibility of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium isolates from the gastrointestinal tract of sheep fed a variety of dietary antimicrobials and determined these various fed antimicrobials had no effect on antimicrobial susceptibility of enteric bacteria (Callaway et al., 2003).

While ionophores are the only form of antimicrobial approved for use as a feed additive in lactating dairy cattle, there are a number of antimicrobials approved for use as additives to milk replacers used to prevent disease in pre-weaned calves and also non-lactating dairy heifers under 20 months of age. Recently, the Animal Drug Availability Act of 1996 was amended to implement the new animal drug regulation known as the Veterinary Feed Directive (USDA, 2015). This new regulation goes into effect December of 2016 and will make it illegal for antimicrobials such as Chlortetracycline to be added to feed rations for the purposes of growth promotion. Additionally, the rules mandate that the addition of antimicrobial feed additives shall be provided exclusively under the supervision of a veterinarian.

As full enforcement of the Veterinary Feed Directive has yet to go into effect at the writing of this paper, the results of the efforts to reduce the over the counter
availability of feed additive antimicrobials remain to be seen. While it is not yet clear how the changes will affect production agriculture, the popularity of feed additives on dairy operations will likely result in changes to feeding and disease prevention protocols on most dairy farms in the United States. To depict this point, one study detailing antibiotic usage in 113 Pennsylvania dairy herds recorded that 70% of herds routinely use medicated milk replacers containing oxytetracycline and neomycin (Sawant et al., 2005). The Animal and Plant Health Inspection Service reported that over half of dairy operations fed medicated milk replacer in cohort studies that evaluated antibiotic use trends in both 2002 (55.7%) and 2007 (57.5%) (APHIS, 2008). The same study also reported a 9 percent and 3 percent increase in the percentage of dairy operations that used antibiotics to treat pre-weaned heifers for respiratory and digestive problems respectively (APHIS, 2008). Despite a relatively higher prevalence of antibiotic usage in pre-weaned heifers, the same study reports that less than 20% of operations use antibiotics other than ionophores in feed rations for weaned heifers in both 2002 and 2007 and fewer than 8% of weaned heifers reportedly required antibiotic treatment (APHIS, 2008).

Aside from feed and milk replacer additives, antimicrobials are utilized for a number of other purposes in the dairy industry. Oxytetracycline, cephalosporins and penicillins are frequently used to treat infections in both heifers and lactating cows (McEwen et al., 2002). One study outlining antibiotic use among dairy producers in Washington State reported that penicillin, ceftiofur, and oxytetracycline were the most commonly used drugs for disease treatment, and that chlortetracycline and sulfamethazine drugs were the most commonly used for disease prevention (Raymond et al., 2006).
Another commonly prescribed use of antibiotics for lactating dairy cattle involves local intramammary infusion for both mastitis treatment and dry cow therapy (mastitis prevention).

Mastitis is the most costly animal health problem within the dairy industry and antibiotic administration resulting in milk withholds is the primary economic deterrent for their usage (Seegers et al., 2003). Due to the economic ramifications of antibiotic administration during lactation, 9 out of 10 operations elect to administer antibiotics to every quarter of every cow at the beginning of the dry period to cure ongoing infections and prevent the establishment of future infection in early lactation (APHIS, 2008). Dry cow therapy helps producers avoid costly withhold times, and the need to discard milk by treating animals when they are not lactating, thus, the risk of antibiotic entrance into the milk supply is further reduced. Studies aimed at determining the efficacy of dry cow therapy have been performed. One study which compared the effects of selective dry cow therapy with complete therapy indicated a 90% reduction in Strep tococcus agalactiae and a 40-70% reduction in Stapholoccus aureus infections (Rindsig et al., 1978). Another study detailing the prophylactic effects of dry cow therapy indicated quarters treated at dry off were 3 times less likely to develop mastitis at calving (Berry et al., 2003).

Despite the documented success of complete dry cow therapy programs, some have questioned the necessity of the practice in today’s management systems. Mounting concerns over the effects of prophylactic and metaphylactic antibiotic administration have prompted investigators to re-examine whether the benefits of complete dry cow therapy outweighs the inherent risks. In contrast to other prophylaxis therapies, which use
subtherapeutic dosages for long periods of time, it should be noted that dry cow therapies generally consist of long-term antibiotics being administered at therapeutic levels. However, despite therapeutic dosage, a study reporting the resistance trends among intramammary pathogens surviving a penicillin and dihydrostreptomycin based dry cow therapy (20%) showed that 70% and 80% of the previously sensitive isolates became resistant to dihydrostreptomycin and penicillin respectively (Schultze, 1983). Furthermore, the cure rate of *S. aureus* infections following dry cow therapy ranges from 40-70%. Additional research has shown that recovery rates among untreated cows may be as high as 38%, therefore the actual therapeutic effects of the antibiotics administered at dry off are not entirely clear (Osteras et al., 1991). Guidelines suggesting cows testing free of major udder pathogens should not be empirically treated with antibiotics at dry off have been proposed. Aside from the availability of relatively expensive laboratory culture tests, the lack of accurate, inexpensive diagnostic techniques available may hinder the effective implementation of this approach (Osteras et al., 1999).

Beta-lactam antibiotics used in combination or alone make up the most frequently used dry cow therapies (Berghash et al., 1983). In addition to being used to reduce the occurrence of clinical mastitis during high risk periods and eliminate subclinical infections within the udder, many of these same antibiotics are being used to treat mastitis throughout lactation. Extended-spectrum cephalosporins and other antibiotics such as lincosamides and non-cephalosporin beta-lactam antibiotics are administered through intramammary infusion and other routes. Ceftiofur, a beta-lactam antibiotic and the only third-generation cephalosporin approved for use in dairy production systems, is commonly used for the treatment of mastitis, pneumonia, and metritis (Daniels et al.,
This widespread use of extended-spectrum cephalosporin products within the dairy industry has caused speculation that increased resistance to this important antimicrobial class will increase and that reduced-susceptibility of *E. coli* organisms is attributable to ceftiofur use in dairy herds (Tragresser et al., 2006). While an increase in individual resistance may be occurring among some pathogens, a 7 year study following the resistance trends of *Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Serratia marcesens,* and *Pseudomonas aeruginosa* showed no change in the overall resistance patterns of any of these organisms to the antibiotics commonly used to treat them (Erskine et al., 2002). In addition, although a sizeable percentage of operations reported using antibiotics to treat respiratory diseases and other diseases unassociated with mastitis, only about 3 percent of cows received antibiotic treatments for these conditions (APHIS, 2008).

**Potential Public Health Implications**

While there may be no clear correlation between antibiotic administration and increased recovery of resistant *E. coli*, veterinarians’ ability to treat disease within the dairy population may be hindered if the prevalence of resistant pathogens increases. In addition, the presence of these resistant organisms increases the likelihood of transfer of resistance genes between commensal bacteria present in dairy cows, such as from *E. coli* to *Salmonella*. This is an important consideration when assessing the human health impacts that an increased reservoir of resistance within animal populations may have. Special consideration is given to *E. coli* and *Salmonella* because they are common agents of zoonotic food-borne illness in humans (Buzby et al., 1996). The United States Centers
for Disease Control and Prevention estimates 76 million food-borne illnesses contributing to 5,000 deaths occur annually, with *Salmonella* indicated as the primary bacterial etiology (Lynch et al., 2006). With 1.4 million people infected annually, *Salmonella* has consistently been indicated as a primary factor for food-borne disease (Varma, 2006). While most food-borne illnesses from pathogens like *Salmonella* are self-limiting, certain populations, such as: children under the age of 12, the elderly, and the immunocompromised are at greater risk of acquiring these infections and are more likely to require treatment to effectively recover. Some theorize that the use of antimicrobials, with medical importance to humans, within food animals such as dairy cattle populations could make treating these acquired infections more difficult. The extended spectrum cephalosporins are commonly used to treat a variety of important health conditions in dairy cows, in addition to being frequently employed to treat life-threatening foodborne-illness in people (Hohmann, 2001). The ability to effectively treat food-borne illness will be greatly reduced if the agents causing foodborne-sickness in humans are resistant to the third-generation cephalosporins. This is especially important in children because, florquinolones, an alternate front-line antimicrobial choice, are contraindicated in children under the age of 12 in the United States (Wain et al., 1997). Additionally, antibiotic resistance poses both a health risk and economic cost within both veterinary and human medicine. A 2-fold increase in human mortality, morbidity, and cost for patients with resistant versus susceptible infections has been reported (Cosgrove et al., 2003). Antimicrobial resistance among *Salmonella* strains has been increasing over the decades and it has created opportunity for outbreaks of food-borne diseases to resistant pathogens (Varma, 2006).
The potential for food-borne outbreaks due to antimicrobial resistant pathogens is very real and a primary concern for epidemiologists studying the effects of antibiotic resistance in the dairy cattle population. Dairy cows culled following their productive life often enter the food supply as fresh meat products. As a result, administration of cephalosporin antibiotics including dry cow therapy may lead to the introduction of cephalosporin-resistant bacteria into the food supply (Mollenkopf et al., 2010). In the 1990s, the emergence of a multidrug-resistant (MDR) strain of *Salmonella* Typhimurium definitive type 104 resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline was discovered in the United States (Glynn et al., 1998).

One decade later, the first emergence of an MDR *Salmonella* serotype Newport, resistant to the previously mentioned antibiotics as well as extended-spectrum cephalosporins was reported (Dunne et al., 2000). By the year 2003, *Salmonella* Newport infections had become increasingly more common; representing almost 12% of all reported *Salmonella* outbreaks, an increase of 7 percent from the previous decade (Varma, 2006). More recently, a study involving 97 dairy operations across 21 different states, found that 8 herds (8.20%) contained resistant strains of both *E. coli* and *Salmonella* while 85 percent of the *E. coli* and 87 percent of the *Salmonella* strains recovered were pan-susceptible to the antibiotics tested (Lundin et al., 2008).

A few years prior, one study detailing the antimicrobial drug susceptibility of *Salmonella* found 83 percent of the isolates to be pan-susceptible (Blau et al., 2005). These studies show that most *E. coli* and *Salmonella* recovered from dairy cows are pan-susceptible to the common antimicrobials used on the dairy farms and the resistance
patterns within the dairy population did not seem to change from 2005-2008. Despite the fact that the overall percentage of resistant *Salmonella* seems insignificant, and it is true that antimicrobials may not be directly responsible for food-borne disease, the use of antimicrobial agents in dairy cattle has been associated with the emergence of antimicrobial-resistant *Salmonella* strains and with the dissemination and transmission of these strains to humans (Threlfall et al., 2000).

Today, outbreaks of foodborne-illness linked to large livestock production facilities across the United States have sparked criticism of large dairy operations. While the economic advantages to larger scale operations are undeniable for today’s dairy producers, additional information supporting the views of critics may be equally convincing. The number of dairy herds and cows has been declining the past 3 decades, yet United States milk production continues to increase.

With increased demands of production being placed on each cow, stress on the animal increases and may predispose the animal to metabolic and infectious disease while making the animal subsequently more vulnerable to opportunistic pathogens such as *E. coli* and *Salmonella* (Hillman, 1982). Additionally, it has been reported that the size and population density of a dairy farm directly correlates to the amount of *Salmonella* shed in the feces of dairy cattle (Wells et al., 1998). If animals are more stressed and more susceptible to disease it is possible that more antibiotics may need to be administered to the animal population.

In addition, shedding of food-borne pathogens may increase if the animals are more likely to harbor them. Combining the effects of such shedding with the increased density of dairy cattle resulting in food from fewer sources may mean higher risk of food-
borne disease. Many have tested these and other theories only to find that trends related to the shedding of *Salmonella* continue to be variable (Fitzgerald et al., 2003). Others have shown the prevalence of resistant *Salmonella* strains in the United States has increased over the past 20 years (Varma, 2006).

**Antimicrobial Resistance Trends**

It has been reported that organic farms tend to be smaller and use fewer antibiotics than conventional dairy farms (Zwald et al., 2003). When comparing the resistance patterns of *Salmonella* between organic and conventional farms, the correlation between findings and management practices are much less clear. One study reported an association of increased *Salmonella* resistance on conventional farms for only 2 antibiotics: streptomycin and sulfamethoxazole (Ray et al., 2005). These studies highlight the differences in management practices of conventional and organic dairy farms and suggest that animals on smaller farms receive less antibiotics, possibly because of lower stress, yet the *Salmonella* shedding characteristics are still quite variable. It is also worth noting that while antibiotic usage is greater on conventional farms when correcting for herd size and location, for most of the antimicrobials tested, the *Salmonella* isolates from organic farms have similar resistance patterns to those from conventional farms (Ray et al., 2005).

While the changes in antimicrobial resistance may be similar regardless of the frequency of antimicrobial use on dairy operations, there are still some important trends being detected. Data from the National Antimicrobial Resistance Monitoring System (NARMS) shows the percentage of *Salmonella* isolates recovered from dairy cattle with resistance to extended spectrum cephalosporins to be increasing. From the beginning of
2000 through 2006 the recoveries of isolates resistant to Cefoxitin, Ceftiofur and Ceftriaxone have increased by over 150% (US FDA, 2010c). Somewhat paradoxically however, the overall prevalence of extended spectrum cephalosporin resistant *Salmonella* isolates recovered from ground beef products has decreased to 14.3% in 2009 from a reported high of almost 40% in 2003 (US FDA, 2010e). This may reflect improved sanitation and meat processing techniques. The prevalence of resistant *E. coli* isolates in ground beef for these same antibiotics during the same time period has never reached a level above 2.4%. (US FDA, 2010b). In addition, there is no apparent trend suggesting an increase in the number of resistant *E. coli* isolates recovered from ground beef during this time period (US FDA, 2010d).

Some studies indicate that the prevalence of resistant isolates within meat products may be much higher than 2.4% today. One study reported that the overall prevalence of *E. coli* resistant to extended-spectrum cephalosporins recovered from retail beef and pork products was 8% (Mollenkopf et al., 2011). The same study also reported the prevalence of *Salmonella* to be 4% in retail beef and pork, with 0.5% of these retail meat products containing *Salmonella* strains expressing extended-spectrum cephalosporin resistance. A study reported in 2009 assessing microbial resistance trends in the fecal flora of dairy cattle recovered extended-spectrum cephalosporin resistant *E. coli* from 92% of the 50 herds tested accounting for 60.9% of the 3,840 cows sampled (Heider et al., 2009). A second study performed for the same purposes in 2010 recovered third generation resistant *E. coli* from 100% of the 25 herds tested accounting for 94.8% of the 747 cows sampled (Mollenkopf et al, 2012). Within the same study, 20% of the farms representing 9.4% of the sampled cows contained *E. coli* expressing cefepime resistance.
which had not previously been reported in commercial US dairy herds. Cefepime, a fourth-generation cephalosporin has never been approved for use in food animals. *Salmonella* isolates were identified in 76% of the herds representing 37.9% of the cows sampled but, of importance none of the *Salmonella* isolates expressed extended-spectrum cephalosporin resistance (Mollenkopf et al, 2012).

**Discussion**

Despite the ongoing debate over antibiotic administration in food animal populations, it is unclear what the exact effects are on human, animal, and environmental health. Academic, producer, and consumer opinions vary as much as the interpretations of the data reported. It is clear that antibiotic administration places a selection pressure on bacteria present within the environment leading to increased opportunity of resistance development. What is not clear is how significant these resistance patterns are to the overall health of the animals and people colonized by these resistant organisms. Furthermore, the appropriate course of action to stem the development of bacterial resistance and maintain the production efficiency of our food systems continues to be a challenge. Demand for a safe, affordable and plentiful food supply encourages producers to do everything possible to seek economical advantages. Sometimes larger dairy farms, which are designed to maintain high levels of production efficiency and produce large amounts of inexpensive food, may require greater antibiotic administration and may create increased risk for food-borne disease outbreaks. As consumers become more concerned with the methods used to produce their food, their interests will likely shape the future of food production and the dairy industry.
In keeping with these consumer demands, dairy producers must find new and innovative ways to produce a plentiful supply of dairy products while improving the quality of animal welfare and decreasing their dependence on antimicrobials. Regardless of consumer opinion, more prudent use of antibiotics can likely be obtained through the implementation of simple practices that improve animal welfare and production. By studying the current prevalence of *E. coli* and *Salmonella* recovered from the fecal flora of dairy cattle we may gain more knowledge about the factors that influence shedding of these organisms and provide producers with additional opportunities to improve production standards. In addition, assessing changes to the minimum inhibitory concentrations to important antimicrobial drugs of *E. coli* and *Salmonella* isolated from dairy herds will help us gain a better understanding of antibiotic resistance trends allowing attention to be focused where there is the most critical need for change. Coupling the reduced susceptibility findings with rates of antimicrobial usage may establish a cause and effect relationship or perhaps lead us to a greater understanding of other factors that result in antimicrobial resistance.
Chapter 2: Changes in Antimicrobial Susceptibility of Fecal *Escherichia coli* Recovered from Dairy Cattle on 16 Farms in Ohio 2001-2011

INTRODUCTION

The development of antimicrobial drugs represents one of the most important advancements in medicine. Until the most recent decade, since their introduction, the number of antimicrobials available for use within the United States has been increasing steadily. Today more than 150 antimicrobial drugs comprising 20 different classes with 10 targets of action are utilized to treat and prevent both human and animal bacterial diseases of importance (Khachatourians, 1998). In its annually published summary report detailing approved antimicrobial use in food animal agriculture within the United States, the Food and Drug Administration’s Center for Veterinary Medicine estimated that between 15,000 to 16,000 tons of antibiotics were sold and distributed in 2009 and 2010 (FDA, 2009; FDA, 2010).

Antimicrobials are important to reduce the threat of disease in domesticated livestock. However, the use of veterinary antimicrobial agents to relieve pain and animal suffering and improve production efficiency does not come without criticism. The risks associated with antimicrobial usage within food animals, including the emergence of antimicrobial resistance, has created demand for further study on how antibiotics work and what their impact for human, animal and environmental health may be. As with other facets of the food production industry, antimicrobial usage and reduced susceptibility trends within the United States dairy industry continue to be a subject of
controversy and research. Some believe that the consumer and media concerns regarding antimicrobial use in animal agriculture are sensationalized and warrant no investigation, however, scientists have provided evidence that concerns surrounding the effects of selection pressures applied by veterinary antimicrobial use are valid. Once introduced into an environment, antimicrobials place selective pressure on the existing microbes which may foster the development of resistant phenotypes.

Additionally, of more recent concern, there is evidence to show that these resistant microbes may have capabilities of spreading resistance genes directly to other, previously susceptible microbes (McDermott, 2002; Tenover 2006).

Aside from the concerns regarding selection pressure, the risks associated with antimicrobial drug residues within the food supply are of equal importance. For example, consumption of dairy products containing antibiotics can lead to allergic reactions in susceptible consumers. The recognition of such risks prompted the Federal Food and Drug Administration in 1924 to establish the Standard Milk Ordinance now called the Pasteurized Milk Ordinance (USPHS, 2009). Under these established guidelines which were most recently updated in 1991 to include testing for beta-lactam antibiotics, chloramphenicol, and sulfonamides; all milk products are required to test negative for 6 of the most frequently used beta-lactam antibiotics including: Penicillin, Amoxicillin, Ampicillin, Cloxacillin, Cephapirin and Ceftiofur prior to processing for human consumption. Chloramphenicol administration to lactating dairy cattle is prohibited by law and sulfadimethoxine is the only approved sulfonamide for administration to lactating dairy cattle.
Reports of unprocessed milk products containing antibiotic residues are extremely rare, widely reported to be less than 0.5% (Schaik et al., 2002). When antibiotic residues are found the milk is destroyed and not sold for public consumption. However, after discovering increasing frequency of antibiotic residues in the meat of cull dairy cows, in order to ensure compliance of antibiotic free dairy products the FDA made an expanded milk testing proposal in 2010. Although this proposal is currently being contested, it would expand testing of milk products for residues from up to 27 different therapeutic agents, including non-antimicrobials. Understanding the types of antimicrobials used in the dairy industry, the purposes for their use, the primary risks associated with them, and resistance trends helps to better our understanding of how dairy populations can be managed to maximize animal welfare, production, and food safety. Monitoring reduced susceptibility trends and the changes of minimum inhibitory concentrations over time for common food pathogens such as E. coli is critical to determining the effects of using antimicrobial therapies in food producing animals.

The objectives of this study were to document the changes in antimicrobial susceptibility of fecal E. coli between 2001 and 2011 on 16 Ohio dairy farms. These results are expected to provide evidence regarding the impact of veterinary antimicrobial therapy on the susceptibility of enteric flora of food animals likely to enter the food supply.

2. MATERIALS AND METHODS

2.1 Study population

Fecal samples collected from Ohio dairy herds in two separate study periods were used to isolate E. coli over a 10 year period. The first study sample consisted of 9,253
fecal samples (approximately 50g each) collected via direct rectal palpation from dairy cattle located on 42 dairy farms in the state of Ohio in 2001. The second study sample consisted of 400 fecal samples (approximately 50-75g each) collected via direct rectal palpation from dairy cattle located on a subset of 16 dairy farms that had participated in the first study period. The subset of 16 farms from the first cohort contained 900 of the previous 9,253 fecal samples collected in the original study. Disposable plastic palpation sleeves were used to collect fecal samples and a new sleeve was used for each cow.

Herd for the first study period (2001-2002) were randomly selected from the existing ODA dairy division’s list of Grade A dairies in the state of Ohio. Three separate attempts were made to contact each herd owner listed before an alternate herd was randomly selected from the list. All herds that completed participation in the first study period were contacted to participate in the second study period (2011). Again, at least 3 separate attempts were made to contact each herd owner by phone using the previously recorded contact information from the first study period.

In the first study period a total of 4 individual fecal samples was obtained from every lactating cow on the farm over the course of 4 separate collection periods. One fecal sample was obtained from each cow during each visit. Only herds that completed at least three sample collections during the first study period have been included in this report and were recruited to participate in the second study period. The participating herds and methods of sample collection used were the same for both the first and second study periods. However, some differences between the first and second study period exist. In order to minimize producer inconvenience and increase study participation, only 25 cows were sampled from each herd that agreed to participate in the second study
period. In addition, the number of samplings was reduced from 4 in the first study to 1 from each of the 16 herds that participated in the second trial period.

To minimize selection bias, cows sampled in the second trial period were randomly selected from all lactating cows in the herd. While most participating farms contained only one group housing area, farms with multiple pens had a proportional sample randomly selected from each group housing area. Those cows randomly selected for sampling were restrained in headlocks or in the milking parlor for the sampling process. Fecal samples were collected from individual cows and placed into individually labeled 50 ml plastic centrifuge tubes for storage and transport.

2.2 Sample Storage and Processing

Following collection, sample tubes were stored for transport to the laboratory in a clean cooler which maintained samples at ambient temperature. Time of transport from farm to laboratory ranged from 2 to 6 hours. Samples were stored over night at 4°C for processing the next day. For the recovery of *E. coli*, 4-g fecal aliquots were homogenized into 36 ml nutrient broth. After overnight incubation, this broth was streaked onto MacConkey agar plates. *E. coli* confirmation was determined based on the isolation of lactose-positive and indole-positive isolates. We have previously described similar methods to successfully recover fecal *E. coli* isolates (Mollenkopf, et al, 2013).

A pure culture for each *E. coli* isolate from both the 2001 and 2011 study periods was grown using MacConkey agar. Fresh, unfrozen samples from the second study period and frozen samples from the first study period, recovered from frozen isolates prepared using standard glycerol freezing techniques were used as the source for the freshly produced pure cultures. Cultures were incubated overnight at 35°C and a bacterial
suspension was made using deionized water. The resulting suspension was standardized to 0.5 McFarland turbidity standard and confirmed using the BioMérieux Vitex Inc. Colorimeter. Following standardization, a 10µL volume was subsequently pipetted into a tube containing 10ml Mueller- Hinton Broth that was mixed for approximately 15s using a Vortex Mixer. Following the establishment of a homogenous mixture, 50µl was transferred into each well containing serial 2-fold antimicrobial dilutions of the NARMS panel using an 8 head repeat pipettor. After the inoculation of each well the plate was covered using an adhesive seal and incubated at 35°C overnight. Following incubation, growth in each well was determined manually and the same person recorded the automatic readings by using the Sensititre Sensitouch reader.

Changes to the NARMS panel over the previous 10 years prohibited all Minimum Inhibitory Concentration (MIC) data from being generated for each sample using the above described Sensititre protocol. To compare MIC data across all samples, agar dilutions following CLSI guidelines (CLSI, 2006) were performed for the antimicrobials not included on the revised NARMS panel available in 2011. Major changes that occurred to the standard NARMS panel between 2000 and 2011 that impacted this study include the removal of apramycin, cephalothin and sulfamethoxazole and the addition of sulfadoxazole and azithromycin. Minor changes include the removal of amikacin from the standard NARMS panel during the study period in 2011. Comparisons of the E. coli Reduced Susceptibility Proportion (RSP) and Reduced Susceptibility Index (RSI) for apramycin and azithromycin are not included in this study because E. coli phenotypes resistant to apramycin and azithromycin are extraordinarily rare; therefore, agar dilutions for these antimicrobials were not performed. The antimicrobial comparisons between the
first and second study period were made using antimicrobials contained in the 2001 NARMS panel (CMV1CNCD). To provide us with 13 of the 16 antimicrobials contained in the standard 2001 NARMS panel we used the revised NARMS panel (CMV1CNCD). However, because of the previously mentioned revisions resulting in the removal of cephalothin, sulfamethoxazole, and amikacin from the NARMS panel in 2011, comparisons of the *E. coli* RSP and RSI for cephalothin, sulfamethoxazole, and amikacin were determined via agar dilution. All isolates plated onto a NARMS panel that did not contain any one of these three antimicrobials removed after 2001 had RSP and RSI information obtained for the removed antimicrobial from MIC information that was obtained via agar dilution.

Agar dilutions were prepared using a standard Mueller Hinton Agar preparation following the Clinical and Laboratory Standards Institutes’ guidelines and protocols for microbroth dilution and agar dilution (CLSI, 2006). Differences in the approved MIC breakpoints between the 2001 and 2011 NARMS panels created additional comparison challenges. In order to maintain uniformity in the MIC values obtained for the antimicrobials recorded using the 2011 NARMS panel, we used the antimicrobial dilutions approved for the 2011 NARMS panel. In addition, we interpreted MIC values using the breakpoint values utilized by the CLSI and NARMS in 2011.

2.3 Statistical Analysis

MIC data were first used to produce a reduced susceptibility proportion and resistant proportion for each antimicrobial category within each herd. All samples below the intermediate breakpoint were classified as susceptible, those above were classified as
resistant and all within the intermediate breakpoint were excluded from the resistant
category but included in the reduced susceptibility proportion.

RSP values for each of the antimicrobials used to test the 1,262 E. coli isolates
comprising the two cohort study periods (900 in 2001 and 400 in 2011) were recorded
from established break points for each of the antimicrobial agents included within the
study. The RSP was calculated by totaling the number of isolates which were both
resistant or had reduced susceptibility and dividing by the total number of isolates tested
for each individual antimicrobial. Separate RSP values for each antimicrobial category
were determined for the 2001, as well as the 2011 study period using the 2011
breakpoints and used to create the reported RSP values for each of the antimicrobials
tested during both the 2001 and 2011 study periods.

In order to determine the statistical significance of the changes in the recorded
reduced susceptibility proportions during each study period, the mean RSP for each
antimicrobial category was calculated. The mean RSP value was calculated for each
study period by summing the individual herd RSP values and dividing by the total
number of herds included within each study. The resulting mean RSP for the herd tested
in the 2001 period was compared to the mean RSP for the same herd tested in the 2011
period and analyzed using a Wilcoxon signed-rank test. To further depict the magnitude
of change for each reduced susceptibility proportion, an odds ratio has also been reported.
The odds ratio was calculated by first subtracting the RSP value recorded for each
antimicrobial from 1 and then dividing this difference by the recorded RSP value. The
resulting dividend for the 2001 herd was then divided by the resulting dividend for the
2011 herd to produce the recorded odds ratio.
Additionally we have reported an overall reduced susceptibility index for each herd (RSI). The RSI for each herd was calculated by combining the number of isolates with reduced susceptibility to each antimicrobial and dividing by the total number isolates in each herd. The resulting RSI values for each of the herds in the 2001 and 2011 cohort studies were then averaged amongst the separate study cohorts to determine the overall mean RSI for both the 2001 and 2011 study period. This number helps to depict not just the number of resistant isolates within each antimicrobial segment but the overall reduced susceptibility of isolates within each study period.

3. RESULTS

As previously stated, the study encompasses two sampling periods from the same herds. The second study period includes data from 16 of the previously sampled 42 herds that were still in existence and willing to participate in this second study period. Herd owners from the previously sampled herds were only excluded if: they were unable to be successfully contacted by phone after at least 3 attempts (n=7), if they requested to not be included due to inconvenience (n=12), if the farm had been sold or cows had been dispersed (n=5) or if scheduling within the three month study period was prohibited (n=2). One herd willing to participate in the second study period was relocated and currently under new management however, no cattle had been purchased into the herd between the two studies and therefore, was enrolled in the second trial period.

The majority of fecal samples collected in this study were acquired from Jersey and Holstein cows. All animals were lactating at the time of collection in both studies. All currently lactating animals on the farms tested in 2001 were sampled. During the
2011 study period, cows were selected randomly to form a total sample of 25 cows per farm.

The mean RSP for each of the 16 antimicrobials tested shows an increase in 4 of them. The proportion of isolates with reduced susceptibility to ampicillin increased from 0 to 0.03 (P=0.01). In addition the mean RSP of cephalothin has increased from 0.11 in 2001 to 0.23 in 2011 (P=0.03). That of chloramphenicol increased from 0 to 0.09 (P=.0001). And finally, the mean RSP of sulfamethoxazole increased from 0.12 to 0.49 (P=0.01).

For the remaining 11 antimicrobials, there was no statistically significant increase in the proportion of isolates with reduced susceptibility. The mean RSP of ceftiofur increased from 0 to .01 (P=0.12). Additionally, the mean RSP of ceftriaxone increased from 0 to 0.01 (P=0.06) and ciprofloxacin increased from 0 to 0.01 (P=0.50). Furthermore, the mean RSP of streptomycin and tetracycline showed an increase over the ten year period, from 0.04 in 2001 to 0.07 in 2011 (P=0.37) and from 0.14 to 0.16 (P=0.7) respectively. Finally the mean RSP of trimethoprim/Sulfamethoxazole showed an increase from 0 to 0.01 (P=0.25).

There were a total of 4 of the 16 antimicrobials which showed no change over the 10 year period. Amikacin, gentamycin, and naladixic acid remained stagnant with an RSP value of 0 (P=1.0), (P=0.50), and (P=1.0) respectively. Finally, the fourth antimicrobial kanamycin had an RSP of 0.02 which remained unchanged during the 10 year period (P=1.0).

None of the antimicrobials monitored during the study period showed a significant decrease in RSP over the 10 year period, however, 2; amoxicillin/clavulonic
acid and cefoxitin showed a reduction from 0.03 to 0.02 (P=0.28) and from 0.02 in 2001 to 0.01 in 2011 (P=1.0) respectively.

The previous results indicate reduced antimicrobial susceptibility in *E. coli* is influenced by specific genetic variations. In the 2001 study period, the most common phenotype was pan-susceptible. The RSP results from the 2011 study period show the most common phenotype to be sulfa-resistant.

While the reduced susceptibility proportion (RSP) allows us to better our understanding of the changes over the 10 year study period occurring at the individual antimicrobial level, the overall reduced susceptibility index (RSI) between the two study periods can help us to understand the total number of isolates within each study period that have a reduced susceptibility to one or more antimicrobials. Using the previously described computational techniques, the RSI for the 2001 study period was 0.032 indicating that approximately 3.2 percent of the isolates within the 2001 cohort have a reduced susceptibility to one or more of the 16 antimicrobials tested. Likewise, we determined the RSI for the 2011 study period to be 0.062 indicated that approximately 6.2% of the isolates within the 2011 cohort have a reduced susceptibility to one or more of the 16 antimicrobials tested approximately twice that of the 2001 cohort.

Additionally, while not analyzed, no decrease in MIC value for the 16 antimicrobials tested has been observed.

4. DISCUSSION

Our results document the changes in susceptibility of enteric flora of dairy cows to 16 antimicrobials that have been included within the FDA’s NARMS panel within the 10 year period. Cephalosporins (cefoxitin, cephalothin, ceftriaxone, ceftiofur) and other
β-lactam antibiotics (amoxicillin, ampicillin) make up the two primary categories included on the panel. Both are of particular importance due to their common use to treat important veterinary and human infectious diseases. Food-borne pathogens such as *E. coli* and *Salmonella* are commonly treated with these antimicrobials in the human medical profession and formation of resistance to the products used to treat animals infected with these pathogens may put consumers at greater risk of acquiring a resistant food-borne infection.

The overall percentage of isolates that are pan-susceptible for the 2001 and 2011 studies are 67% and 42% respectively. During the course of the 10 year study period, the *E. coli* isolates tested went from a majority pan-susceptible phenotype to a majority sulfamathoxazole resistant phenotype. The 65% increase in the number of isolates with reduced susceptibility to sulfamathoxazole was far more significant than that of any of the other three antimicrobials. We observed a 3% increase in the number of isolates with reduced susceptibility to ampicillin over the study period; along with a 8% increase with reduced susceptibility to cephalothin and an additional 10% to that of chloramphenicol.

As the results of this study are interpreted, it is beneficial to consider the results of other retrospective studies tracking the changes in *E. coli* antimicrobial sensitivities. One such study tracked the *E. coli* susceptibility profiles to the antimicrobials contained in the NARMS panel from 1950 to 2002 (Tadesse et al., 2012) allows for some comparisons between the findings of this study. The most notable correlations are that in the aforementioned study, there were four significant antimicrobial agents with significant increases in reduced susceptibility: Ampicillin, cephalothin, sulfonamide and tetracycline. While our study findings did not correlate an increase in tetracyclines, this
may simply be the result of a decreased window of time. Additionally, our retrospective study assessed a more narrow population of animals managed under similar techniques throughout the duration of the study period. Because producers often adopt management protocols, including the selection of which antimicrobials they use to treat disease; this may decrease the sensitivity to the detection of change over time. Additionally, the Tadesse et al. study included data from chickens and pigs as well as dairy cattle which is likely to contribute further to the differences reported. Interestingly to note, while the Tadesse et al. study shows a substantial increase among animal *E. coli* isolates to cephalothin, a significant decrease in cephalothin resistance in the human population was observed over the same time period (Tadesse et al. 2012). This may support the hypothesis that greater use within a confined population increases selection pressure among the microbial population, creating a causal relationship between the use of antimicrobial agents and their decreased efficacy within the microbial population.

When interpreting the increased proportion of resistant isolates (ampicillin, cephalothin, chloramphenicol and sulfamethoxazole) it is important to recognize that while increases have been observed among the individual antimicrobial groups, it is not possible to directly confer a clinically significant resistance trend from the proportion of resistant isolates. Likewise, there is no statistical evidence to correlate the use of these antibiotics directly to decreased efficacy. In fact, in the case of chloramphenicol, its use in food animals has been prohibited since 1984 (Payne et al., 1999). One possible explanation lies in the fact that resistance plasmids coding for resistant phenotypes may provide cross-resistance to other, unrelated anti-microbial agents for which no selection pressure has been applied. To depict the point, many studies highlight the fact that in
spite of the United Kingdom’s reduction of the use of sulfonamides within the food animal population in 1995, the rates of sulfonamide resistance recovered from *E. coli* isolates continues to increase (Enne et al., 2001).

It is likely that co-selection for the resistance genotypes reported in this study has occurred; for instance, the common use of beta lactam antimicrobials to treat dairy cattle may have impacted the increased resistance to ampicillin and cephalothin on these farms. Additionally, it has been shown that resistance to chloramphenicol is maintained in enteric bacteria through generations as a result of co-selection (Akwar et al., 2008). Trimethoprim, which is allowed for use in food animals and commonly paired with sulfamethoxazole could offer one explanation for direct sulfamethoxazole resistance selection pressure while conferring co-selection pressure for enteric bacteria with reduced susceptibility to chloramphenicol (Harada et al., 2006).

Furthermore, the data reported within this study is from a 10 year retrospective analysis that has previously not been performed. While increases over this period have been noted, changes during this time period to the Animal Medicinal Drug Use Clarification Act and FDA regulations for approved use of antimicrobial therapies have reduced the continued increase in resistance. In the FDA’s 2011 Retail Meat Annual Report of the National Antimicrobial Resistance Monitoring System (NARMS) for instance, the resistance to many of the antimicrobials reported here is low. Furthermore, for those antimicrobials like florquinolones, the prevalence of isolates with reduced susceptibility has stopped increasing entirely following the prohibition of extra label use of this antimicrobial in food producing animals in 1997. Additionally, the FDA has restricted off-label veterinary use of extended spectrum cephalosporins to mitigate the
increases in resistance for *E. coli* and *Salmonella* isolates recovered from food animals and those regulations were not in place during this study period.

Finally, while we did observe increases in the proportion of *E. coli* recovered from the feces of dairy cattle with reduced susceptibility to ampicillin, cephalothin, chloramphenicol, and sulfamethoxazole our findings differ somewhat from those of the National Antimicrobial Research Monitoring System (NARMS). This discrepancy is likely a result of the different testing methods utilized. NARMS focuses on identifying resistant isolates from retail meat cuts, while our study was looking for isolates directly from fecal samples. These differences may also be due to geographical circumstances, differing sample size or other variables. NARMS data for the ten year study period seems to confirm our trend for increased resistance to chloramphenicol showing an increase from 1.0-1.4% between 2002 and 2011 respectively. However, NARMS reports a decrease for both sulfamethoxazole (9.8-7.9%) and ampicillin (6.1-3.7%). Information regarding the trends for cephalothin are unavailable after it was removed from the NARMS panel in 2004.

We did not observe any statistically significant increases in the proportion of isolates with reduced susceptibility to: ceftiofur, ceftriaxone, ciprofloxacin, streptomycin, tetracycline, trimethoprim/sulfamethoxazole amikacin, gentamycin, naladixic acid or kanamycin. From the available NARMS data, it appears our findings are consistent for gentamycin, ceftiofur, and ceftriaxone. There was however a decrease reported between the study periods for tetracycline (30.9-17.7%), kanamycin (2.4-1.4%), and streptomycin (9.5-6.5%). Additionally, NARMS data reports the number of isolates with reduced susceptibility to trimethoprim/sulfamethoxazole increased from (0.7-2.3%). Information
for naladixic acid and ciprofloxacin is not available from NARMS. Finally, we reported a
nonsignificant decrease in the number of isolates with reduced susceptibility to
amoxicillin/clavulonic acid and cefoxitin. The available NARMS data supports these
findings with a reported decrease from 2% to 0.5% and 1.4%-0.5% respectively.

In conclusion, the extended-spectrum cephalosporin ceftiofur is commonly used
in dairy cattle because of its broad spectrum of activity, perceived efficacy, management
benefits, and food safety benefits. While we could not detect an increase in reduced
susceptibility to extended-spectrum cephalosporins, their frequent use may be providing
selection pressure resulting in the increase in proportion resistant that we did observe.

Continued monitoring of resistance trends is essential to ensuring the safety of our
food supply as well as ensuring the continued efficacy of antimicrobials in the treatment
of both human and animal diseases. This and other studies depicting the susceptibility
trends of antimicrobials of importance will allow appropriate recommendations to be
made regarding the use of antimicrobials. All indications of increased resistance warrant
increasing attention toward the judicious use of antimicrobial therapies but caution
should be used when extrapolating these findings to a clinical paradigm.
Table 1: Minimum inhibitory concentrations to 16 antimicrobial drugs of fecal *E. coli* collected from dairy cows on 16 farms in Ohio in 2001.

<table>
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<tr>
<th>Drug</th>
<th>2001 MIC (μg/ml)</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
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<th>2.00</th>
<th>4.00</th>
<th>8.00</th>
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<th>32.00</th>
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<td></td>
<td>894</td>
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<td></td>
<td></td>
<td></td>
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<td>0.11</td>
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<td>0.55</td>
<td>0.22</td>
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<td>260</td>
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<td>531</td>
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Table 2: Minimum inhibitory concentrations to 16 antimicrobial drugs of fecal *E. coli* collected from dairy cows on 16 farms in Ohio in 2011.

<table>
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<tr>
<th>2011 MIC (μg/ml)</th>
<th>0.015</th>
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Table 3. Proportion of fecal *E. coli* recovered from dairy cows in 16 herds in Ohio in 2001 and 2011 with reduced susceptibility to 14 antimicrobial drugs.

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Figure 1. Culture Isolation and *E. coli* verification methodology schematic

*E. coli* isolation was performed via incubation within non-selective nutrient broth and MacConkey agar growth followed by selection of representative *E. coli* colonies. Only after successful confirmation of lactose fermentation and indole positivity were pure isolates confirmed to be those of *E. coli*. 
References


Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, MJ., et al. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals,


