

**Antimicrobial Resistance and Molecular Epidemiology of Extended-Spectrum
Beta-Lactamase-Producing *Escherichia coli* and *Klebsiella* Species in Dairy
Farms**

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DEDICATION

I dedicate this dissertation to those who have made tremendous sacrifices and contributions to the completion of this dissertation and who helped lay the groundwork for my academic career. Firstly, to my wonderful wife, Meseret Daba, who has selflessly put aside everything to help my academic pursuits, care for our precious kids, and provide unwavering support .during my study. The extent of your sacrifices is beyond measure, and I am deeply thankful for that. Secondly, to my children, Sindursen, Monera, Nega, and Ebba, who have endured the hardship of not having enough time with me for nearly four years. Your sacrifices are immeasurable in many aspects. I hope you realize and acknowledge the efforts I have put in for the past few years to secure a better future for you all.

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ABSTRACT

Extended-spectrum beta-lactamase (ESBL)-producing-*E. coli* and -*Klebsiella* species present major global health threats. However, there is limited information on the status and genomic epidemiology of these bacteria in the United States conventional dairy cattle farms that frequently use beta-lactam antibiotics. This dissertation is aimed to determine the prevalence, antimicrobial resistance, spread mechanisms, and public health implication of ESBL-*E. coli* and -*Klebsiella* spp. using microbiological and molecular tools. A total of 605 samples (rectal feces, bulk tank milk (BTM), manure, water, and feed) were tested. From these, 241 ESBL-*E. coli* and 59 ESBL-*Klebsiella* spp. were detected. The prevalence of fecal ESBL-*E. coli* and -*Klebsiella* spp. were 47.5% (n=508) and 7.2 % (n=508), respectively. Most *E. coli* isolates (94.4%, n=241) were multidrug-resistant (MDR), whereas only 19% (n=57) ESBL-*Klebsiella* spp. were MDR. Six families of beta-lactamase (*bla*) genes, along with several other resistance genes (ARGs), were detected in both *E. coli* and *Klebsiella* spp. The *bla*_{CTX-M} and *bla*_{TEM} were the most frequent in *E. coli*, whereas *bla*_{SHV} and *bla*_{CTX-M} were dominant in ESBL-*Klebsiella* spp. A 47 distinct sequence types (STs), 13 unknown, and 4 novel STs of ESBL-*E. coli* and 15 distinct STs, and 10 unknown STs of ESBL-*K. pneumoniae* were identified. High-risk *E. coli* clones accounted for 28% (n=67) of the identified STs. Both ESBL-*E. coli* and -*K. pneumoniae* STs were clustered within farms, suggesting animal-to-animal transmission. Evidence of horizontal spread of *bla* genes via plasmid was also observed. ESBL-*E. coli* and -*Klebsiella* spp. were detected in 24.2% and 6.1 % of BTM, respectively. About 41% of dairy farms had ESBL-*E. coli* in their BTM. All ESBL-*E. coli* isolates from BTM were MDR and co-carried the *bla*_{CTX-M}, other ARGs, and virulence genes. Isolates with matching STs and resistance patterns were detected in BTM and fecal samples collected from the same farm. Four ESBL-*E. coli* STs obtained from BTM were high-risk pandemic clones. MDR ESBL-*E. coli*, including high risk-clones, and ESBL-*Klebsiella* spp. were widespread in dairy farms and BTM. These findings indicate that dairy cattle can serve as a reservoir for zoonotic ESBL-*E. coli* and -*K. pneumoniae*.

Keywords: antimicrobial resistance, *Escherichia coli*, *Klebsiella species*, extended-spectrum-beta-lactamase, sequence types, dairy farm, dairy cattle, bulk tank milk, resistance genes, mobile genetic elements, whole genome sequencing

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Chapter 1. General Introduction

Antimicrobial resistance (AMR), the ability of microorganisms to overcome the antimicrobial drugs created to destroy them, is a significant global health concern (1, 2). The rapid and continuous increases in AMR jeopardize progress in human and animal health, food production, and, ultimately, life expectancy worldwide (1, 3). A report indicated that 4.95 million deaths were associated with AMR bacteria in 2019 alone, with 1.27 million deaths attributable to bacterial AMR across the globe (2). Over 2.8 million illnesses and over 35,000 fatalities in the United States (U. S.) occur annually due to AMR (1). Scientists are warning that the world may be approaching a post-antibiotic era due to the alarming rise in infections and deaths caused by antibiotic-resistant infections and the lack of development of new antimicrobials (4, 5).

Among AMR bacteria, extended-spectrum beta-lactamases (ESBLs)-producing *Enterobacteriaceae* emerged as one of the significant threats to human and animal health globally (6, 7). The ESBL-producing *Enterobacteriaceae* (ESBL-*Enterobacteriaceae*) mainly exhibit multidrug-resistant (MDR) traits and rapidly increase in frequency and distribution in humans and food-producing animals such as dairy cattle (1, 7). Extended-spectrum beta-lactamases (ESBLs) are mainly detected in *Escherichia coli* and *Klebsiella* species which are normal residents of the gastrointestinal tracts of animals and humans (8-10). The ESBLs confer resistance to the beta-lactam antibiotics such as penicillin, monobactams, and cephalosporins, including the third-generation cephalosporins (3GCs) (11), and are inhibited by beta-lactamase inhibitors. The ESBLs are of great clinical importance and are often encoded on plasmids, which also frequently contain genes for resistance to other classes of antibiotics (11-13). As a result, the World Health Organization (WHO) identified ESBL-producing *E. coli* and *-Klebsiella* spp. as members of the priority pathogens (14, 15) and a 3GC as the highest priority critically important class of antibiotics (16).

The overuse and/or misuse of antimicrobial drugs in multiple sectors, including humans, animals, and agriculture, has been identified as a key driver for the rapid increase of resistance among bacteria (17-19). Many studies have shown a direct association between the use of antimicrobial drugs in food animals such as dairy cattle and the occurrence and rise of ESBL-*Enterobacteriaceae* carriage among food-producing animals (20-24). As a result, there have been

suggestions that the extensive use of penicillin and cephalosporins in food-producing animals has played a role in the worldwide spread of human infection caused by ESBL-*E. coli* and -*Klebsiella* spp. (25-27).

Beta-lactam antibiotics, such as ceftiofur (a veterinary 3GC), first-generation cephalosporins, and penicillin, are the top three frequently used antibiotics in the U. S. dairy farms (28-30). According to a recent report, ceftiofur is a primary antibiotic used to treat 50.5 % of mastitis cases, 77.6 % of respiratory diseases, 58.7 % of lameness, 57.4 % of gastrointestinal disorders, and 45.6 % of reproductive diseases (31). Similarly, ceftriaxone and cefotaxime, human 3GCs, are the drug of choice to treat invasive infections caused by Gram-negative bacteria such as *Salmonella* and *E. coli* in humans in the U. S. (15, 32, 33). Both veterinary and human 3GCs have similar chemical structure, mode, and spectrum of action and are thus susceptible to ESBL enzymes (34, 35). As a result, ESBL-*Enterobacteriaceae* occurring in dairy cattle may pose an indirect threat to the human health sector via cross-resistance (36).

The widespread use of beta-lactam antibiotics in dairy farms can exert selection pressure that may lead to the emergence of ESBL-*E. coli* and -*Klebsiella* spp., commonly known to colonize the recto-anal junction of dairy cattle (9, 37). A growing body of research indicated a rise in ESBL-*E. coli* in U.S. dairy cattle (36, 38-42). Information on the status of ESBL-*Klebsiella* spp. in dairy farms in the U.S. is virtually unavailable. Although most ESBL-*Enterobacteriaceae* in dairy cattle's gastrointestinal tracts (GIT) are considered harmless, the detection and carriage of ESBL-*E. coli* in the U.S. dairy cattle may still pose a potential threat to public health (9). Also, ESBL-*Enterobacteriaceae* from dairy cattle can spread to humans through contaminated food products such as raw milk, undercooked meat, direct contact with dairy cattle, and the environment (26, 36). Once these bacteria enter the human GIT, they can transfer the ESBL gene to other harmful bacteria, such as *Salmonella*, via mobile genetic elements such as plasmids (43). In rare cases, these bacteria may be one of the high-risk clones that cause human intestinal or extra-intestinal infections (26, 27, 44-46). A high-risk clone of *E. coli* strains is characterized by their worldwide spread, carriage of multiple antimicrobial resistance genes, capacity to inhabit

and persist within hosts, ability to transmit between hosts effectively, increased pathogenicity and adaptability, and the potential to induce severe and/or recurring infections (47).

In the past decades, the U. S. Centers for Disease Control and Prevention (CDC) reported a rapid and continuous increase in infections and fatalities attributed to the *Enterobacteriaceae* resistant to 3GC mainly due to plasmid-borne ESBL production (48). Consequently, CDC categorized the ESBL-*Enterobacteriaceae* as a serious threat (48). Previously, most infections caused by ESBL-*Enterobacteriaceae*, mainly *E. coli* and *K. pneumoniae*, were associated with health care (49, 50). Recent reports showed a change in the epidemiology of these infections, where nearly half of the cases were community-associated (48). The primary sources of community-associated ESBL-*Enterobacteriaceae* are still unknown and have become a topic of debate. It should be noted that this study was not intended to address this question. The CDC's report did not directly indicate dairy cattle or other animals as a source of this rapidly increasing ESBL-*Enterobacteriaceae*.

However, the widespread use of 3GCs and other beta-lactam antibiotics in the U.S. dairy cattle could contribute to the rise of ESBL-*Enterobacteriaceae*. Despite the clear indication that ESBL-*Enterobacteriaceae*, primarily *E. coli*, are on the rise in the U. S. dairy farms (41, 51-55), the prevalence of ESBL-*E. coli*, a main resistance indicator bacterium, and -*Klebsiella* spp. in dairy cattle remains unknown. Furthermore, lack of comprehensive and high-resolution studies on ESBL-*E. coli* and -*Klebsiella* spp. in dairy cattle limits our understanding of the genetic diversity of these bacteria, the full range of genes that encode ESBL enzymes and other co-harbored resistance genes, and their mechanism of spread within dairy farms and beyond. Understanding the prevalence and molecular epidemiology of ESBL-*E. coli* and -*Klebsiella* spp. isolated from dairy cattle, farm environment, and raw milk could help inform evidence-based intervention and potential public health risks arising from dairy farms.

Thus, it is hypothesized that the common presence of commensal *E. coli* and *Klebsiella* spp. in the gut of dairy cattle, along with the frequent exposure of dairy cattle to third-generation cephalosporins, may lead to the widespread occurrence of ESBL-*E. coli* and -*Klebsiella* spp. and

the ESBL-genes that can spread both vertically and horizontally within the dairy cattle production making dairy cattle a reservoir of these bacteria.

The Structure of The Dissertation

The dissertation comprises seven chapters. A general introduction, a literature review that has been published, a published research article, three research chapters in preparation for publication, and a general conclusion. Chapter 1 presents a general overview of the Ph.D. research topic and its significance, and part of this chapter was published as a book chapter (56). Chapter 2 is a literature review that critically analyzes the existing research on the status of ESBL-*Enterobacteriaceae* in the U. S. dairy farms, identifies research gaps, and was published in the journal of *Antibiotics* (36). Chapter 3 presents a preliminary study on antimicrobial-resistant *E. coli* conducted in a limited number of dairy farms and published in the journal of *Foodborne Pathogens and Disease* (57). Chapter 4 explicitly examines the prevalence, antimicrobial resistance, and molecular epidemiology of ESBL-producing *E. coli*. Chapter 5 covers the prevalence, antimicrobial resistance, and molecular epidemiology of ESBL-producing *Klebsiella* spp. Chapter 6 presents molecular epidemiology and pathogenomics of ESBL- *E. coli* and -*K. pneumoniae* isolates from bulk tank milk. The final chapter of the dissertation presents a summary of the research chapters and offers concluding remarks. Tables and figures are labeled according to their respective chapters and placed in appendix at the end of each chapter.

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Chapter 2. Literature Review

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Abstract

Antimicrobial resistance (AMR) is one of the top global health threats of the 21 century. Recent studies increasingly report the rise in extended-spectrum beta-lactamases producing *Enterobacteriaceae* (ESBL-Ent) in dairy cattle and humans in the U.S. The causes of the increased prevalence of ESBL-Ent infections in humans and commensal ESBL-Ent in dairy cattle farms are mostly unknown. However, the extensive use of beta-lactam antibiotics, especially third-generation cephalosporins (3GCs) in dairy farms and human health, can be implicated as a major driver for the rise in ESBL-Ent. The increase in ESBL-Ent, particularly ESBL-*Escherichia coli* and ESBL-*Klebsiella* species in U.S. dairy cattle, is not only an animal health issue but also a serious public health concern. The ESBL-*E. coli* and -*Klebsiella* spp. can be transmitted to humans through direct contact with carrier animals or indirectly through the food chain or via the environment. The U.S. Centers for Disease Control and Prevention reports also showed a continuous increase in community-associated human infections caused by ESBL-Ent. Some studies attributed the elevated prevalence of ESBL-Ent infections in humans to the frequent use of 3GCs in dairy farms. However, the status of ESBL-Ent in dairy cattle and their contribution to human infections caused by ESBL-producing enteric bacteria in the U.S. is the subject of further study. This review aims to give in-depth insights into the status of ESBL-Ent in the U.S. dairy farms and its implication for public health and highlight some critical research gaps that need to be addressed.

Keywords: extended-spectrum beta-lactamase, *Enterobacteriaceae*, beta-lactam antibiotic, dairy cattle, public health, antimicrobial resistance, antibiotic resistance gene

2.1 Introduction

Antimicrobial resistance (AMR) is one of the most critical global health challenges (1, 2). Globally, about 700,000 deaths were attributed to diseases caused by antibiotic resistant organisms. The major concern is that if proper intervention measures are not implemented, this figure will rise to 10 million deaths annually in 2050 (2). Every year, in the U.S. alone, 2.8 million people suffer from infections caused by antibiotic resistant bacteria (ARB) and a significant number of deaths (3). The drivers of AMR emergence are complex and multifactorial. But the widespread use and misuse of antibiotics in livestock and human medicine are recognized as the leading driver of AMR (4).

Antimicrobials are widely used in food-producing animals throughout the globe. Intensive food animal production systems such as dairy, beef, poultry, and swine productions frequently use medically important antimicrobials (MIAs) for therapeutic, prophylactic, and metaphylactic purposes (5, 6). For instance, antibiotics, regarded as the highest priority and critically important (e.g., third-generation cephalosporins-3GCs) for treating human infections that are refractory to other antibiotics, are widely used in dairy farms for the prevention and treatment of various diseases in dairy cattle (6-8). Cattle carry many bacteria in the group of *Enterobacteriaceae* in their gastrointestinal tract, which are frequently exposed to these critically important classes of antibiotics (CIAs) and MIAs (9). Critically important antibiotics (CIAs) are antimicrobial drugs used to treat enteric pathogens that cause foodborne disease and a last-resort therapy or one of few alternatives to treat serious human disease where first-line antibiotics have not worked. Medically important antibiotics (MIAs) are antibiotics that are important for treating human diseases, including critically important, highly important, and important antibiotics (10).

The continuous exposure of *Enterobacteriaceae* to CIAs such as 3GCs can lead to the selection and spread of ARB and their antimicrobial resistance genes (ARGs). The ARB and ARGs could spread to humans through direct contact or indirect routes (11-14). Extended-spectrum beta-lactamases (ESBL) encoding genes mediate resistance to third- and sometimes to fourth-generation cephalosporins, the “highest priority and CIAs” (7, 15). So the rise in the incidence of ESBL-Ent, such as *E. coli* and *Klebsiella* spp., in dairy farms is of significant public health

concern since these antibiotics are the highest priority and critically important ones for the treatment of human infections caused by Gram-negative bacterial pathogens (16).

Ceftiofur, a 3GC, is one of the top three most frequently used antibiotics to treat and prevent mastitis and other diseases in dairy cattle (5, 17). Recent studies indicated that resistance to beta-lactam antibiotics, specifically resistance to 3GCs, is rising among commensal *Enterobacteriaceae* isolates from U.S. dairy cattle (18). Resistance to 3GCs is mainly mediated by the production of ESBL, a group of enzymes that break down a beta-lactam ring of the extended-spectrum cephalosporins such as 3GCs (19). Among *Enterobacteriaceae*, *E. coli* and *Klebsiella* spp. are among the most frequently identified bacteria carrying ESBL-encoding genes, such as *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} (20-24). Ceftriaxone and cefotaxime are similar 3GCs antibiotics used to treat severe infections caused by pathogenic strains of *Enterobacteriaceae* in humans (25, 26). The use of the same generation of cephalosporins with the same chemical structure, active ingredients, and spectrum of activity in dairy cattle farms and human health settings may lead to cross-resistance that can be transferred to humans or vice versa via direct and indirect routes (27-29). This review aims to give a detailed account of the current status of ESBL-Ent in the U.S. dairy cattle farms and its implication for human health and highlight research gaps that need to be addressed.

2.2 Mechanisms of Resistance to Beta-Lactam Antibiotics

Beta-lactam antibiotics, including 3GCs such as ceftiofur, ceftriaxone, and cefotaxime, are widely used against Gram-negative pathogens (30). These antibiotics act by covalently binding to penicillin-binding proteins (PBP), an enzyme that catalyzes the polymerization and transpeptidation of peptidoglycan (31). The binding of antibiotics to PBP will lead to their inactivation, thereby inhibiting cell wall synthesis and the death of susceptible bacteria (31, 32).

Enterobacteriaceae resistance to 3GCs has become an alarming and growing public health challenge (3, 21). Bacteria employ three resistance mechanisms against beta-lactam antibiotics. These include (1) mutations that change the structure of penicillin-binding proteins, (2) changes in cell permeability (disruptions of porin proteins in the outer membrane or increase in efflux

pumps), and (3) production of beta-lactamase enzymes, which hydrolyzes the beta-lactam ring in beta-lactam antibiotics (33-35).

In *Enterobacteriaceae*, resistance to 3GCs is primarily mediated by the production of beta-lactamases (36). In addition to ESBL, resistance to extended-spectrum beta-lactam antibiotics could be mediated by carbapenemase (encoded by *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-48}, etc.), plasmidic AmpC (pAmpC; commonly encoded by the *bla*_{CMY} genes), and mutations in AmpC promoter regions in the chromosome (20, 37). However, this review focuses on ESBL, as ESBL production is one of *Enterobacteriaceae*'s most important and common resistance mechanisms against extended-spectrum beta-lactam antibiotics (21, 38). The definition of ESBL is ambiguous, and in this review, we adopt a more comprehensive ESBL definition as beta-lactamases that hydrolyze or confer resistance to penicillins, cephalosporins (First- to third-generations), monobactams (e. g., aztreonam) but not the cephamycins (e.g., cefotetan and cefoxitin) or carbapenems (e.g., meropenem and imipenem) and are inhibited by beta-lactamase inhibitors (e.g., clavulanate) (39, 40).

Although ESBL shares common biochemical properties, they all break down extended-spectrum beta-lactam antibiotics and are inhibited by clavulanate; the genes encoding these enzymes are diverse (41, 42). The most frequent variants of ESBL include the CTX-M (cefotaxime-hydrolyzing beta-lactamase), SHV (sulfhydryl reagent variable), and TEM enzymes. The TEM was determined initially in a single strain of *E. coli* isolated from the blood of a human patient (43, 44). The parent type of SHV (SHV-1) and TEM (TEM-1, 2) are narrow-spectrum beta-lactamases that give rise to their respective ESBL variants through mutations (45). Amino acid substitutions or mutations in the genes encoding these enzymes give rise to expanded substrate specificity or enhanced hydrolytic activity (41, 46). As a result, the number of identified variants of the TEM and SHV families is continuously rising, most of which have emerged and are also currently emerging via stepwise mutations (41).

TEM-1, the first TEM-type beta-lactamase, was reported almost six decades ago (in 1965) in Greece from a patient named Temoneira, which was designated as TEM (40, 43). This enzyme

variant resists narrow-spectrum beta-lactam antibiotics (penicillin and the first-generation cephalosporins before it expanded its spectrum via mutations (40).

The SHV enzymes were first reported nearly five decades ago (in the 1970s), and the first reported variant (SHV-1) exhibited activity against the penicillins and first-generation cephalosporins (45, 47). In the 1980s, the SHV- and TEM-ESBL variants mutants of the parent enzymes were the prominent cause of resistance to 3GCs among *Enterobacteriaceae* (48). As of July 5, 2022, 229 *bla*_{SHV} and 246 *bla*_{TEM} variants have been reported (49).

As opposed to the TEM- and SHV-ESBL variants, CTX-M (cefotaximase) type enzymes did not evolve from mutations of existing enzymes; the gene encoding this enzyme was acquired from *Kluyvera* spp. through horizontal gene transfer (50). After mobilization of this gene from the chromosome into a plasmid, mutations lead to further diversification and allow hydrolytic activity to other extended-spectrum cephalosporins such as ceftazidime (51). In animals, the CTX-M enzyme was first identified in 1988 from *E. coli* isolated from dogs' feces in Japan (52). Since the 2000s, the CTX-M-ESBL variant has become the most prevalent and widespread cause of resistance to extended-spectrum cephalosporins among the *Enterobacteriaceae* across the globe, both in humans and food animals such as dairy cattle (21, 24, 38, 53-57). Based on the order of identification of the group's founder and amino acid sequence identity, the CTX-M family of ESBL is phylogenetically categorized into five distinct groups designed as 1, 2, 8, 9, and 25 (53, 56). Each of the five groups differs by at least 10% in amino acid sequence identity (58). There are several minor variants within each group, and currently, at least two groups of CTX-M (groups 1 and 9) are described in U.S. dairy farms (24, 59). As of July 5, 2022, 252 *bla*_{CTX-M} ESBL variants have been described (60).

2.3 Use of Beta-Lactam Antibiotics in the U.S. Dairy Cattle Farms

Beta-lactam antibiotics are the most frequently used class of antibiotics characterized by the beta-lactam ring, a similar biochemical structure across the class (32). These include penicillin, cephalosporins, monobactams, cephamycins, carbapenems, and beta-lactamase inhibitors (34). Beta-lactam antibiotics are the most frequently prescribed class of antibiotics in human health (32) and on dairy farms (5, 17). In the U.S., cephalosporins and penicillin are the most

commonly used beta-lactam antibiotics to treat or prevent mastitis and other common diseases of dairy cattle (17, 61). According to the US Food and Drug Administration (FDA) 2019 report, from a total of 29,830 kg of cephalosporins sold and approved for use in food-producing animals, the vast majority (81%) were distributed to cattle production (6). Similarly, Nora et al. (62) also reported that the largest amount of cephalosporins (10.5 grams per cow year) and penicillin (4.49 grams per cow year) are used in dairy cattle farms compared to other classes of antibiotics whose use is less than 1 gram per cow year.

Ceftiofur and cephalixin are the only cephalosporins licensed for use in the US food animals, including dairy cows (63, 64). Cephalixin is only approved as an intramammary infusion for the treatment of mastitis caused by *Streptococcus* and *Staphylococcus* species (65). Cephalixin is the active ingredient in cephalixin sodium (Brand name: toDAY) is used for the treatment of mastitis in lactating cows, and cephalixin benzathine (Brand name: ToMORROW) is used for the treatment of mastitis in dry cows. Ceftiofur is approved in two forms, injectable and intramammary infusion. In the U.S., three parenteral and two intramammary formulations of ceftiofur are approved for use in dairy cattle. The two intramammary formulations of ceftiofur are (1) Ceftiofur hydrochloride suspension for the treatment of lactating cows (Brand name: SPECTRAMAST LC) and (2) Ceftiofur hydrochloride suspension for the treatment of dry cows (Brand name: SPECTRAMAST DC). The parenteral formulations include 1) ceftiofur sodium (Brand name: Naxcel), (2) ceftiofur hydrochloride (Brand name: Excenel), and (3) ceftiofur crystalline-free acid (Brand name: Excede). All these formulations are approved for treating bovine respiratory disease (BRD) and footrot but with different treatment regimens. In addition, ceftiofur hydrochloride (Brand name: EXCENEL RTU- Excenel ready to use) and ceftiofur crystalline-free acid are used to treat acute metritis at different dosage regimens. Ceftiofur hydrochloride suspensions for lactating (Brand name: SPECTRAMAST LC) and dry (Brand name: SPECTRAMAST DC) cows are used for treating clinical mastitis caused by non-aureus staphylococci also known as coagulase-negative *Staphylococcus* species (CNS), *Streptococcus dysgalactiae* (*S. dysgalactiae*), and *E. coli* during lactation and subclinical mastitis caused by these bacteria at the time of drying off (65-68), respectively.

The most common infectious diseases of dairy cattle treated with antibiotics include mastitis, lameness, and respiratory and digestive diseases (5, 69). In dairy cattle, ceftiofur is indicated to treat mastitis, bovine interdigital necrobacillosis, bovine respiratory disease, acute postpartum metritis, and mastitis caused by coliform bacteria. Ceftiofur is the most widely used 3GCs for preventing and treating mastitis in dairy cattle [45-47, 65]. The recent U.S. National Animal Health Monitoring System (NAHMS) survey report showed that the highest proportion (27.6%) of pre-weaned heifers and about 7.2% of weaned heifer calves were treated for diarrhea with ceftiofur as a primary antibiotic. Similarly, 10.3 % of pre-weaned heifers and 13.4% of weaned heifers were given ceftiofur as a primary antibiotic for treating BRD (17). A relatively recent survey on several dairy herds in the U.S. also reported frequent use of ceftiofur to prevent and treat respiratory and digestive diseases in dairy calves (5, 70).

Almost all studies found that mastitis is the primary reason for using antibiotics in dairy cattle (5, 71-74). According to NAHMS, ceftiofur is a primary antibiotic used to treat 50.5 % of mastitis cases, 45.6 % of reproductive disorders, 58.7 % of lameness, 77.6 % of respiratory infections, and 57.4 % of digestive tract diseases (17). The broad spectrum of activity and the shorter milk withdrawal period make ceftiofur the most popular and ideal antimicrobial drug for dairy cows (75). Cephalosporins use in US food-producing animals (cattle, swine, turkey, and chickens) has shown a general increasing trend in the last decade(76) (Figure 2.1A, placed under the appendix)

Accurate data on cephalosporins used in U.S. dairy cattle are not available. The U.S. Food and Drug Administration Center for Veterinary Medicine report showed the total quantity of cephalosporins sold and distributed in the U.S. for cattle use (Figure 2.1B, placed under the appendix). However, the amount of cephalosporins used in dairy and beef cattle farms is not explicitly described and thus is unknown (6). The absence of the actual quantity of cephalosporins administered or given to dairy cattle is one of the biggest challenges in assessing the impact of its use on the emergence of resistance to this class of antibiotics.

A recent study on *Klebsiella* isolates from cases of mastitis indicated that the prevalence of ceftiofur-resistant *Klebsiella* spp. was low from 2008 to 2016 and abruptly increased between 2016 and 2017, and then decreased in 2019 (Figure 2.1B). The increase in prevalence could be

related to the rise in the use of ceftiofur (Figure 2.1B) for the prevention and treatment of mastitis and other diseases of dairy cattle during the specific year, which might have increased in response to the increased selection pressure on *Klebsiella* spp. (77).

Both experimental and observational studies indicated that antibiotic use and, thus, the resultant selection pressure is an important factor driving the emergence and persistence of ARB and their resistance genes (78-85). Mastitis is the most frequent disease of dairy cattle; ceftiofur is the most commonly used antimicrobial drug to manage it (5, 69). This implies mastitis is an important indirect driver of the emergence of ESBL-producing bacteria in U.S. dairy farms [6,9,20]. However, despite increased usage of ceftiofur in dairy farms, several studies examining the AMR status of mastitis pathogens in dairy cattle did not find a rise in the prevalence of resistance to ceftiofur (86-90). Nevertheless, it should be noted that AMR among commensals and foodborne pathogens from dairy cows and dairy manure is increasing (91). Our published and ongoing studies also showed an increased prevalence of ESBL-Ent (e.g., *E. coli*, *Klebsiella* spp.) from dairy manure and bulk tank milk (18).

Thus, heavy reliance on ceftiofur for the prevention and treatment of mastitis should be reassessed, and prudent and antimicrobial stewardship-focused utilization of ceftiofur is important to reduce the development of resistance against this CIA. The use of other alternative dairy cattle disease control measures such as good hygienic and biosafety practices, use of teat sealants at drying off, vaccines, and full implementation of mastitis control plan with a good plan of nutrition and management should be considered to reduce the incidence of mastitis and other diseases of dairy cattle (92-95). A study showed that blanket application of teat sealants to all cows at drying off had a protective effect against the emergence of ESBL at the herd level (93, 96). Thus, the widespread use of this method may help reduce cephalosporin use and, thereby, the emergence of ESBL-Ent.

2.4 Molecular Epidemiology of ESBL-*Enterobacteriaceae* in the U.S. Dairy Farms

The epidemiology of ESBL-Ent is complex and rapidly changing (4, 97). The epidemiology of ESBL genes is influenced by the horizontal transfer of resistance genes, presence of additional resistance gene/s, rapid mutation of existing resistance genes to generate new variants, expansion of bacterial host range carrying the gene, and sometimes convergence of ESBL genes and virulence genes in the host bacteria (38, 98).

In *Enterobacteriaceae*, ESBL genes are primarily associated with mobile genetic elements (MGEs) such as plasmids, insertion sequences (IS), transposons, integron cassettes, and prophages-mediated intracellular and intercellular movement (99). The MGEs play a vital role in the spread of ESBL genes; for example, IS, transposons, and integron cassettes may mediate the movement of ESBL genes within the same bacterial genome, whereas plasmids can transfer genes between different bacteria cells (99, 100).

ISEcp1, an insertion element situated upstream of the CTX-M-encoding gene, causes the movement of this ESBL gene from the chromosome of *Kluyvera* spp. onto plasmid (101, 102). Then, the plasmid can spread to other populations of bacteria, such as *E. coli* and *Klebsiella* spp. (97, 100). Some ESBL-harboring plasmids have a broader host range, whereas others have a limited or restricted host range. Broad-host range plasmids are characterized by their ability to replicate and easily transfer between different species of bacteria. Thus, enhancing interspecies transmission and spread of ESBL genes and other ARGs (100, 103). These groups of plasmids include those belonging to the family of IncA/C, IncI, IncN, IncHI2, IncL/M, IncK, and IncN (97, 100).

In contrast, narrow-host range plasmids tend to be restricted to certain species or strains within a given species. Thus, their role is also limited to intraspecies dissemination of ESBL genes (103, 104). Narrow-host range plasmids play a crucial role in disseminating ESBL genes, particularly the CTX-M-variants (103). For instance, IncF plasmids, also known as “epidemic resistance plasmids,” have a strong tendency to acquire and disseminate ESBL genes and other ARGs among members of *Enterobacteriaceae* (105).

Some studies reported the worldwide distribution of *bla*_{CTX-M-15} gene variants of ESBL is due to its frequent association with IncF plasmids (24, 38, 97, 106). Molecular epidemiological studies have shown that these plasmids frequently harbor additional genes involved in fitness, virulence, and other ARGs (e.g., *qnr*, *qepA*, *tetA*, *floR*, *sul2*, and *cmlA*) that may help the host bacteria to survive and thrive in human and animal hosts (18, 98, 107-109). *E. coli* ST131 is the classic example of the most successful ESBL-producing pathogenic strains associated with IncF plasmids. The high-risk clone of ESBL-*E. coli* has been reported in humans and animals, including dairy cattle in the U.S., Canada, Spain, Korea, and India (24, 103, 105, 106, 110). It has been suggested that the successful spread of this virulent strain of *E. coli* globally is related to its association with the IncF plasmids, particularly the FIA and FII replicon types (105, 110, 111).

Molecular studies have consistently shown that ESBL genes in *Enterobacteriaceae* isolates are physically associated with MGEs that carry other resistance genes mediating resistance to an unrelated class of antibiotics (112, 113). Co-resistance to multiple classes of antibiotics such as fluoroquinolones, tetracyclines, chloramphenicol, aminoglycosides, sulfonamides, and /or trimethoprim has been frequently reported in *E. coli* and *Salmonella* species isolated from dairy cattle and other food-producing animals (e.g., in Pigs and poultry) in the US and elsewhere (e.g., India, China, and Egypt (18, 24, 59, 113-116).

Among the ESBL families, CTX-M-type is rapidly evolving and spreading in the US and worldwide (15, 21). Currently, CTX-M- genes appear to be the dominant type of ESBL genes in *Enterobacteriaceae*. The cause of the dominance of the CTX-M variant of ESBL over the TEM and SHV variants is not clearly understood. Some authors speculated that the success of *bla*_{CTX-M} might be related to the less fitness cost of expressing CTX-M enzymes in the host bacteria, more effective mobilization of the gene by MGEs, and selection pressure from antibiotics (24, 58).

This ESBL-variant is mainly associated with multidrug resistance (MDR) encoding plasmids (117). MDR CTX-M-producing *E. coli* in US dairy cattle and dairy cows' manure is frequently related to transferable resistance genes to other critically important and highest-priority classes of antibiotics. For instance, plasmid-mediated fluoroquinolone resistance genes (e.g., *qnr*) and

macrolides resistance genes (e.g., *mph(A)*) have been reported together with CTX-M genes in *E. coli* isolated from the US adult dairy cattle, dairy calves, and dairy cows' manure (8, 18, 59). As a result, the detection of ESBL genes in *Enterobacteriaceae* is considered a hallmark of MDR. This MDR phenotype poses a serious threat to public health if the commensal *E. coli* passes the resistance gene cassettes to other enteric human pathogens such as *salmonella* spp. or pathogenic strain of *E. coli* or *Klebsiella* spp. (59).

2.5 Emergence and Status of ESBL-*Enterobacteriaceae* in the U.S. Dairy Farms

In the U.S., ESBL-Ent has long been reported in humans and animals, including dairy cattle. In humans, the TEM family ESBL was first described in 1987 (118), and the SHV-type ESBL in late 1980 from Boston, Massachusetts (119). The CTX-M type ESBL was first described in 2003 from *E. coli* isolates obtained during hospital surveillance in five States (Virginia, Idaho, Ohio, Washington, and Texas) (120).

In the U.S. dairy cattle, ESBL-Ent was first reported in Ohio more than two decades after it was reported in humans (27). Since then, studies have shown that the number of bacterial isolates resistant to 3GCs is rapidly rising in dairy cattle. Widespread use of ceftiofur has been linked to increased isolation of 3GCs-resistant fecal bacteria from dairy cattle. In the U.S., ESBL-*E. coli* and -*K. pneumoniae* have been isolated from milk of cows with mastitis, bulk tank milk (18, 77), rectal fecal samples, lagoon, and soil amended by cow manure (18, 24). This highlights that ESBL-*E. coli* and -*K. pneumoniae* may enter the food supply through contaminated milk and beef products of dairy cows' origin.

In the U.S., few studies have investigated the status of ESBL-Ent in dairy cattle, dairy cattle-derived food products, and their environment (18, 24, 27, 57, 121-124). A recent global review of the status of ESBL-Ent in cattle clearly showed the availability of very limited information on the prevalence of ESBL-Ent, including *E. coli* and *Klebsiella* spp. in US dairy farms (125). Furthermore, comparing findings from these studies is difficult because these studies vary widely in terms of design, sampling methods, sample types, and ESBL detection methods (Table 2.1, placed under the appendix). As a result, data from these studies may not be generalizable, and

thus, not possible to determine the status of ESBL-Ent in the dairy cattle population. This impedes proper estimation of the public health risk arising from ESBL-Ent in the dairy farm, affecting the design and implantation of appropriate intervention measures.

Previous studies often focused on phenotypic detection of resistance to 3GCs in *Enterobacteriaceae*, mainly in *E. coli* and *Salmonella* spp. in dairy farms. Only recently, a few studies attempted to identify the genetic determinants of the observed phenotypes. Most earlier reports indicated *bla_{CMY-2}* genes as dominant genes mediating resistance to extended-spectrum cephalosporins in *Enterobacteriaceae* in the US dairy farms, while ESBL genes were reported relatively recently (126). Recent reports suggested that *bla_{CTX-M}* genes are the most prevalent ESBL genes responsible for resistance to extended-spectrum cephalosporins among *Enterobacteriaceae* in the US dairy farms (Table 2.2, placed under appendix).

Among *bla_{CTX-M}* variants, *bla_{CTX-M-15}*, which belongs to CTX-M group 1, was the most prevalent allele reported in US dairy farms. The gene is frequently associated with the IncI1 plasmid replicon type, an easily transmissible narrow-host-range plasmid that might have contributed to its widespread. In addition to ncI1, ESBL genes reported from US dairy farms were associated with other plasmid replicon types such as IncFIB, IncF, IncFIA/FIB, IncN, IncFIA, IncFIA/11, and IncB/O, IncA/C, IncN (24)

In addition to studies from various U.S. Universities and other research institutes, the U.S. National Antimicrobial Resistance Monitoring System (NARMS) survey also reported different kinds of beta-lactamase encoding genes in *E. coli* and *Salmonella* species isolated from the US dairy operations (127). The NARMS report (Table 2.3, placed under the appendix) showed that most beta-lactamase genes detected in *E. coli* were *bla_{TEM-1}* (69%). In contrast, the most frequent and widespread beta-lactam antibiotics resistance gene detected in *Salmonella* isolates of dairy origin was *bla_{CMY-2}* (72.6%).

In addition to *E. coli*, *Klebsiella*, and *Salmonella* spp., other ESBL-Ent members such as *Enterobacter* spp. could be important both as a mastitis-causing pathogen and as a possible vector of ESBL genes. To the authors' knowledge, there is no at least published report of ESBL-

producing *Enterobacter* spp. from US dairy farms. However, detailed studies are required to determine the contribution of *Enterobacter* species to the ESBL-Ent.

2.6 Public Health Implications of ESBL-*Enterobacteriaceae* in Dairy Farms

The association between levels of ESBL-Ent in cattle and its occurrence in humans is complex. It could be influenced by bacterial strains, MGEs, and the frequency of direct and indirect interaction among cattle, humans, and environments (23, 128). Previous studies have shown that farm workers are more likely to be colonized by multidrug-resistant *E.coli* than people without direct contact with animals (129). Thus, direct exposure to dairy cattle and farms may provide an important mechanism for spreading ESBL-Ent from dairy farms to the community. In dairy cattle, *Klebsiella* spp. (*K. pneumoniae*, *K. oxytoca*), and *E. coli* are the most common coliform bacteria that cause bovine mastitis (77, 130, 131). However, the strains that cause bovine mastitis and those that cause human infections may not be the same. But some strains of ESBL-*E. coli* and -*K. pneumoniae* of dairy origin can pass to humans or transfer their resistance genes to human pathogenic strains. In humans, pathogenic strains of ESBL-*E. coli* and -*K. pneumoniae* are associated with hospital-acquired and community-acquired urinary tract and bloodstream infections (3, 132). As previously mentioned, non-pathogenic strains of these bacteria may pass the ESBL genes to human pathogenic strains causing cross-resistance to other 3GCs, such as ceftriaxone and cefotaxime, which are considered critically important for the treatment of human infections (7).

Currently, ESBL-Ent infections are rising in humans (133, 134). The CDC in the U.S. reported a steady increase in community-associated infections caused by ESBL-producing *Enterobacteriaceae*. The data showed a 9% average annual increase in the number of hospital patients with ESBL infections over five years, with an estimated 197,400 cases of ESBL infections and 9,100 deaths among hospitalized patients in the US in 2017 alone (Figure 2.2, placed under the appendix). The estimated healthcare cost for these infections was USD 1.2 billion(3).

The sources of community-acquired ESBL-Ent infections are unknown and can be from multiple sources; a system-based study is required to understand the contribution of different sources. Some researchers believe that extensive use of ceftiofur in production animals, such as in dairy farms, is a risk factor for rising ESBL-Ent infections in humans in the US (117, 135). This argument is acceptable to a certain extent, as previous studies have found that the therapeutic use of antibiotics in animals could increase the prevalence of antibiotic resistant *Enterobacteriaceae* such as *E. coli* in animals and its risk of transmission to humans (136, 137). However, only a few epidemiological studies have demonstrated strong evidence of transmission of 3GCs-resistant *Enterobacteriaceae* from cattle to humans in the US [75,127,156,157].

Nevertheless, it should be noted that the lack of strong evidence of ESBL-Ent transmission occurring between dairy cattle and humans may not necessarily suggest the absence of transmission. It might be related to a lack of utilization of high-resolution molecular techniques such as WGS, lack of sensitivity of sampling (inability to detect ESBL-Ent from diverse populations in a sample), transient colonization of ESBL-Ent, which may escape detection at the time of sampling, and possible recombination events or increased mutation rate (138, 139). Robust data showing transmission of ESBL-Ent, or ESBL genes from cattle to humans, is important to develop appropriate intervention measures and policies on beta-lactam antibiotics use and stewardship and infection control in dairy cattle and other farm animals (140). The use of WGS technologies along with an appropriate study design (that shows temporal and spatial connection) are critical tools to generate valid inferences on the extent of ESBL-Ent and ESBL genes transmission at dairy cattle-human interfaces as well as the spread among dairy farms, humans, and the environments [141,160,161].

Two mechanisms of spread of ESBL genes are expected among *Enterobacteriaceae* in dairy farms and humans. These are (1) clonal and (2) horizontal spread of ESBL genes (128). The ESBL-Ent and ESBL genes can transmit from cattle to humans through direct or indirect routes (Figure 2. 3, placed under the appendix). Direct transmission involves close contact between animals and humans (hand to mouth). Indirect transmission can occur via the food chain, such as food of animal origin (consumption of unpasteurized raw milk, undercooked meat, and

unpasteurized fresh fruits and vegetables contaminated with ESBL-Ent) or via contaminated environmental sources such as soil, crops, and surface water (Figure 2.3, placed under the appendix) (140).

The gastrointestinal tract of dairy cattle contains many enteric bacteria, such as *E. coli*, *Klebsiella*, and *Salmonella species*. Ceftiofur use in dairy cattle selects for ceftiofur-resistant *Enterobacteriaceae*, which might contaminate food of dairy cattle origin (milk and ground Beef) and eventually pass to humans. ESBL-Ent can also enter the food supply as contaminants of fresh leafy vegetables from manure or wastewater used to fertilize fruits and vegetables. Dairy farm workers and individuals working in agricultural farmlands have a higher risk of exposure to dairy cattle harboring ESBL-Ent or their manure containing ESBL-Ent. Thus, they may become carriers and transmit the resistant bacteria to their close contacts (household members) and the broader community (Figure 2. 3). ESBL- Ent may be maintained in the dairy farm through oral-fecal transmission via contaminated feed and water (141).

Ingestion of ESBL-bacteria such as *E. coli* and *Klebsiella* spp. via direct or indirect routes may result in colonizing the human gastrointestinal tract. Upon colonization, based on its pathogenic potential, the growth of ESBL-producing bacteria could lead to an infection or persistence as a commensal. Both conditions will allow ESBL-producing bacteria to spread to other humans clonally (142) or horizontally transfer ESBL genes to other bacteria via MGEs (143).

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2.6.1 Unpasteurized Milk and Undercooked Beef as Sources of ESBL-*Enterobacteriaceae*

It is well-recognized that unpasteurized milk is a source and vehicle for transmitting several bacteria of animal origin (144-146). More recently, a study estimated that raw milk is consumed by only 3.2% of the population, and cheese is consumed by only 1.6%, but responsible for 96% of diseases caused by contaminated dairy products in the U.S. (147). The same group of researchers estimated that the odds of unpasteurized milk and milk products causing illness are 840 times that of pasteurized products. Similarly, a study conducted over 14 years indicated that three-fourths of outbreaks linked to dairy products occur in states that allow the sale of raw milk (148).

Commensal bacteria that acquire ESBL genes in dairy farms may spill over to humans indirectly through the consumption of unpasteurized milk and milk products (149). Consumption of these products may favor mixing these bacteria with the human enteric microbiota or other more pathogenic strains of bacteria (150). Mixing ESBL-producing bacteria with pathogenic strains enhances the chance of ESBL genes being shared through horizontal gene transfer, creating a threat to human health (18, 151, 152). In our recent study, we detected cefotaxime resistant *E. coli* in bulk tank milk (18). All cefotaxime resistant *E. coli* isolates identified in this study carried *bla*_{CTX-M} type ESBL gene together with other multiple resistance genes conferring resistance to several classes of antibiotics (18). Similarly, a previous study reported that 3GCs resistant *E. coli* and *Klebsiella* species isolates from bulk tank milk had MDR phenotypes (150).

In addition to the risk of transferring ESBL genes to other clinically important strains, some members of *Enterobacteriaceae*, such as *E. coli* and *Salmonella* species, are reported to be

among the most important milkborne human pathogens in the U.S. (153). For instance, a study reported an outbreak of *E. coli* O157:H7 infections caused by consuming unpasteurized milk from a specific dairy farm in Portland, Oregon. The study reported homologous strains (pulsotypes) of *E. coli* O157:H7 isolates from human cases and the dairy herd where milk had originated (154). *E. coli* causes a wide range of severe infections in humans (155) and is also among the frequent causes of environmental mastitis in dairy cattle (156), along with colibacillosis in calves (157).

Some reports indicated that more than 30 states in the US allow the legal sale of raw milk (158). Previous data also showed that most foodborne outbreaks are reported from states allowing the sale of raw milk (153). The rise in MDR ESBL-producing bacteria such as *E. coli* and the increase in legalization and consumption of raw milk will create a high-risk synergy that jeopardizes public health safety. Previous studies have not shown strong evidence of transmission of EBLs-Ent from milk to humans. However, studies have shown that milk is a source of an outbreak caused by *E. coli* O157:H7 but AMR pattern of strain that caused the outbreak was undetermined (154). Cody et al. (159) reported identical pulsotypes of MDR *Salmonella* Typhimurium in the outbreak, which was later linked to the consumption of raw milk cheese in California. Fuenzalida et al. recently reported 32 isolates of *Klebsiella* species resistant to 3GCs from 483 isolates collected over 12 years from milk samples in Wisconsin State (77).

More than 80% of ground beef is obtained from beef cattle in the U.S.; however, 18% is produced from dairy cows sent to slaughterhouses due to their old age or drop in milk yield (160). Dairy cows constitute about 9.4 % of the cattle slaughtered for meat in the U.S. in 2021 (161). Due to the extensive use of 3GCs in dairy cattle, ground beef produced from dairy cattle has a high risk of contamination with ESBL-enteric pathogens (162-164). Thus, undercooked ground beef from culled dairy cows could be a possible source of ESBL-Ent for humans (164, 165). A study by Iwamoto and his collaborators showed a strong correlation between 3GCs resistant *Salmonella* serotype Newport from humans and ground beef, suggesting possible transmission of 3GCs resistant *Salmonella* to humans (165). The study showed evidence that

dairy cattle are important reservoirs of 3GCs resistant *Enterobacteriaceae*, such as *Salmonella* spp., which caused 36 human illnesses in the U.S. (165).

Similarly, another study linked the 1987 *Salmonella* Newport outbreak to contaminated ground beef from slaughtered dairy cows in California (166). Again, the source of the recent outbreak of *Salmonella* newport that caused 106 illnesses and several hospitalizations was traced back to ground beef produced from slaughtered dairy cows (163). The 2019 outbreak of Shiga toxin-producing *E. coli* O103 that affected 209 people in 10 States was linked to ground beef through the AMR pattern of the outbreak strain of *E. coli*, and the source of the ground beef was not reported (167). Several recent and past multistate outbreaks caused by pathogenic strains of *E. coli* reported by the CDC have been linked to ground beef (167-172).

Given the widespread use of ceftiofur in dairy cattle, the increased consumption of raw milk, and undercooked meat, the risk of ESBL-Ent transmission to humans is high. The resulting infections could be difficult to treat with extended-spectrum cephalosporins or fluoroquinolones. Because both classes of antibiotics are used in dairy cattle and human medicine, co-resistance is also a common phenomenon (8, 172-175). Though many studies have investigated the types of bacteria in raw dairy and beef products (144, 148, 154, 168, 176-179), only a few looked at the bacterial response to antimicrobial agents (150, 180). Thus, continuous testing of raw milk and beef for AMR bacteria such as ESBL-Ent and identifying the source of contamination is important to reduce the public health risk that may arise from the consumption of these products. In addition, more robust restrictions on selling unpasteurized milk and milk products can help reduce safety hazards arising from their consumption.

2.6.2 Direct Contact (hand to Mouth) with Dairy Cattle or their Excretions

Dairy cattle and dairy products have long been reported as potential reservoirs for 3GCs resistant *Enterobacteriaceae*. Recently, CDC reported a *Salmonella* serotype Heidelberg outbreak affecting 56 people in fifteen States. The outbreak's source was identified as direct contact with dairy calves. The *Salmonella* isolates involved in the outbreak were MDR, including resistance to 3GCs (181). Similarly, another study showed strong evidence of transmission of 3GCs resistant *Salmonella enterica* serotype Typhimurium from cattle to humans in the U.S. (75). The

study employed pulsed field gel-electrophoresis-based analysis of plasmids and beta-lactamases to confirm a link between a domestically acquired ceftriaxone resistant *Salmonella* infection in a child and clonally related isolates from cattle, suggesting cattle could be the source of 3GCs resistant pathogenic and commensal bacteria (75). Similarly, Gupta et al. (182) also reported 3GCs resistant *Salmonella enterica* serotype Newport isolates with the same pulsotypes and antibiogram patterns from sick dairy cattle, farm workers, and cattle on the farms in Massachusetts and Vermont, suggesting possible transmission through direct contact.

2.6.3 Fresh Vegetables and Fruits as a Source of ESBL-*Enterobacteriaceae*

Several fresh vegetables, fruits, and herbs, frequently eaten raw, could be contaminated and serve as a source of ESBL-Ent infection in humans (141, 183-187). This could happen through multiple routes, including when these products are contaminated with manure containing ESBL-Ent in the field, when untreated manure is used as fertilizer, or when these leafy greens are irrigated with water contaminated with feces (Figure 2. 3) [206,208,209]. Similarly, antimicrobial agents used for crop diseases may increase pressure on microbes to become resistant, or the crop could be contaminated by manure used as fertilizer (Figure 2. 3). In the U.S., fresh green leafy vegetables have been associated with several outbreaks of enteric pathogens (188-192). Similarly, fresh vegetables may carry AMR bacteria such as ESBL-Ent that can enter the human gut when consumed uncooked (193-195).

Recently, Liao et al. isolated several ESBL-*E. coli* strains from ready-to-eat lettuce collected from Northern California (186). A significant number of the ESBL-*E.coli* strains isolated from lettuce in this study (186) were previously reported from dairy cattle elsewhere (196), suggesting cow manure's possible role in contaminating vegetables. The *bla*_{CTX-M} variant was the most frequent ESBL gene detected in *E. coli* isolates obtained from ready-to-eat lettuce. This suggests an increased public health threat linked to vegetable consumption as *bla*_{CTX-M} is increasingly prevalent and expressed by pathogenic strains of *E. coli* in the U.S. and globally (125, 132, 186, 197). Similarly, several previous studies also identified ESBL-Ent, such as *E. coli* and *Klebsiella* spp., from U.S. green leafy vegetables (e.g., lettuce, spinach, and romaine) ready for human consumption (183, 193, 195, 198). However, further studies are needed to identify other indirect

transmission routes and possible wild animals and birds that may serve as vectors carrying these pathogens among farms and environments, as well as sources of contamination of leafy vegetables and fruits.

2.7 Priority Research Gaps that Need to be Addressed

Based on the current literature, we pinpointed the following research gaps that need to be addressed to enhance our understanding and the control of ESBL-Ent in the U.S. dairy farms. A major weakness of current studies is the lack of reliable data on the amount of beta-lactam antibiotics, especially 3GCs used in dairy farms. For example, cephalosporins sales data is often used as an indicator for cephalosporins use, which is unreliable. Furthermore, the sale data does not separately show the amount sold for use in dairy and beef cattle productions. In the absence of these data, it is difficult to assess the impact of their use, develop appropriate interventions, and evaluate the impact of interventions (e.g., the effect of reducing the use of cephalosporins on the prevalence of resistance to cephalosporins). Thus, improving surveillance data on preexisting (baseline) resistance to 3GCs and their use and resistance dynamics after use is crucial to understanding how antibiotic use may influence antibiotic resistance.

Currently, the prevalence of ESBL-Ent in the U.S. dairy farms is mostly unknown. For instance, despite the veterinary and public health importance of *Klebsiella* spp., information on its prevalence and the variants of ESBL genes carried by *Klebsiella* spp. isolates from dairy farms are mostly unknown. Further research should address the status of ESBL-Ent in dairy farms and their potential risk to human health.

Among the significant ESBL genes, *bla*_{CTX-M} encoding lineages are establishing themselves as dominant ESBL in *Enterobacteriaceae*, particularly among *E. coli* in the U.S. dairy farms and across the globe. However, the driver of the successful dissemination of this gene variant is not understood beyond speculation. Understanding the mechanisms for its rapid dissemination in *E. coli* and other members of *Enterobacteriaceae* may help to reduce the emergence and spread of antibiotic resistant commensals and pathogenic strains. Thus, further study is critically important

to unravel the mechanisms for widespread dissemination of *bla*_{CTX-M} encoding genes and the bacteria hosting these genes

Recently, cases of community-acquired ESBL-Ent infection have been rising in the U.S. Despite the widespread speculation, there is a lack of adequate scientific data on the level of ESBL-Ent transmission from dairy cattle and their farm environments to humans. A further detailed investigation is needed to address the potential transmission of ESBL-Ent from dairy farms to humans using high-resolution genome sequencing technologies such as WGS in epidemiologically linked settings in a system-based one-health approach. This will help to develop a prudent usage plan and antimicrobial stewardship and infection control policies through one health approach consisting of animals, humans, and environments.

Factors such as antimicrobial usage and farm management practices that may drive the increased prevalence, spread, persistence, and diversity of ESBL-Ent in dairy farms are not adequately investigated in the U.S. dairy farms. Such studies are needed to enhance our understanding of factors that influence the occurrence and spread of ESBL-Ent so that evidenced-based control measures can be devised.

Archived and contemporary isolates of the members of *Enterobacteriaceae* should be tested to track any temporal changes in the trends (changes) of phenotypic and genotypic resistance to 3GCs over time in the U.S. dairy farms.

Conclusion

The widespread use of extended-spectrum cephalosporins in dairy cattle production exposes many healthy cows to antibiotics, resulting in increased selective pressure favoring the propagation of ESBL-producing bacteria. The growing body of evidence suggests that ESBL-producing commensal *E. coli*, *Klebsiella*, and *Salmonella* spp. are on the rise in dairy cattle. The surge in ESBL-organisms in dairy farms can be a significant public health risk, as some of these bacteria are zoonotic and can transmit to humans via various routes. In addition, the resistance genes from commensal bacteria can be transferred horizontally through MGEs to human pathogens, which leads to cross-resistance to antibiotics as the same genetic determinants are

responsible for resistance against 3GCs used for treating severe infection in humans. However, the prevalence of ESBL-Ent in dairy cattle and dairy cattle's contribution to the burden of ESBL-Ent infections in humans in the US is unknown.

Further studies involving temporally and spatially matched samples from dairy cattle, humans, and environments in a system-based one-health approach are needed to generate more robust evidence of direct and indirect transmission of ESBL-producing *o* Ent between humans and dairy cattle using high-resolution techniques such as WGS. Currently, the level of resistance to 3GCs among mastitis-causing pathogens seems low. However, the increase in resistance to 3GCs among commensal *Enterobacteriaceae* can affect dairy cattle's health by raising the prevalence of MDR bacteria and the horizontal exchange of these ARGs. Thus, better knowledge of the major species and their transmission and spread and driving factors of ESBL-producing organisms and ESBL genes in the U.S. dairy farms is needed to develop appropriate mitigation strategies.

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Appendix

Table 2.1. Prevalence of extended-spectrum beta-lactamases producing *Enterobacteriaceae* in the U.S. dairy farms.

Sample from	Method	Study design/Population	Pathogens/Prevalence	State/ Region	Reference
Conventional Farm					
Manure, bulk tank milk, manure fertilized soil	CAM, CSM, PCR of ESBL genes	A cross-sectional study on four dairy farms	Prevalence of CTX ^r <i>E. coli</i> was 20.5%, about 36% of BTM isolates were CTX ^r . Over 83% of CTX ^r isolates carried ESBL genes	TN	(18)
Feces, swabs (pre-evisceration and carcass)	CSM, PCR of ESBL genes	Prospective study on veal calves from four cohorts (farms)	CTX ^r <i>E. coli</i> were 91%, 34% & 19% in feces, pre-evisceration and final carcass swabs, respectively. ESBL genes were detected in 89% of CTX ^r <i>E. coli</i>	OH	(199)
Feces	CSM, WGS	Matched-pair longitudinal study in CEF-treated and non-treated cows	More than 19 CEF ^r <i>E. coli</i> isolates and multiple ESBL genes found	TX, NM	(8)
Feces	Culture	A longitudinal study on cattle with clinical signs of salmonellosis and asymptomatic ones	The proportion of CEF ^r and CTR ^r <i>Salmonella</i> were 16.5% and 16%, respectively	CA	(200)
Feces	CSM, PCR of ESBL genes	A cross-sectional study on 747 dairy cattle from 25 conveniently selected dairy farms	More than 9% of <i>E. coli</i> isolates were CEF ^r , CTX ^r , and CPD ^r . All the 70 <i>E. coli</i> isolates carried the ESBL genes	OH	(201)
Feces	CSM, PCR of ESBL genes	On-farm from healthy dairy cattle and dairy cattle submitted for diagnostic purposes	Prevalence of CEF ^r <i>Salmonella</i> isolates were 35.8 % and 1.8% among diagnostic and on-farm isolates, respectively		(202)
Feces, lagoons, and milk filters	CSM, PCR of ESBL genes	A retrospective study on <i>E. coli</i> isolates banked from a previous survey of 30 dairy farms	The proportions of <i>E. coli</i> with ESBL genes were 53.5%, 57.1%, and 50.0% in feces, lagoon, and milk filters from 28 farms, respectively	WA	(57)
Feces	CSM, PCR of ESBL genes	A longitudinal study on 20 dairy heifer calves monthly for five months	About 93% of heifers harbored CEF ^r <i>E. coli</i> . The proportion of CEF ^r <i>E. coli</i> was 100%. ESBL and cephamycinase genes detected	PA	(113)
Milk	Culture, WGS	A cross-sectional study on milk from cows with mastitis from four farms	The prevalence of CEF ^r <i>K. pneumoniae</i> was 2.8%. ESBL genes detected	NY	(124)

Table 2.1. Continued.

CAM: culture on antibiotic supplemented media; CSM: culture on selective media; CEF^r: ceftiofur resistant; CTX^r: cefotaxime resistant; CTR^r: ceftriaxone resistant; CPD^r: cefpodoxime resistant; WGS: Whole genome sequencing; AST: antibiotic susceptibility testing; TN: Tennessee; OH: Ohio; TX: Texas; NM: New Mexico; CA: California; WA: Washington, PA: Pennsylvania; NY: New York; WI: Wisconsin; VT: Vermont; MA: Massachusetts; CT: Connecticut; SW: Southwestern Region of US; MI: Michigan; MN: Minnesota.

Table 2.1. Continued.

Sample from Conventional Farm	Method	Study design/Population	Pathogens/Prevalence	State/ Region	Reference
Milk	Culture, AST	A retrospective study on 483 <i>Klebsiella</i> isolates from milk submitted for testing mastitis	The prevalence of CEF ^r <i>Klebsiella</i> spp. was 6.6%	WI	(77)
Feces	Culture, AST	A cross-sectional study on healthy and sick dairy cattle under different management systems	About 95% and 93 % of <i>E. coli</i> isolates were CEF ^r and CTR ^r , respectively	CA	(203)
Composite manure	Culture, AST, and PCR of ESBL genes	A cross-sectional study on 80 dairy farms	CEF ^r and CTR ^r <i>E. coli</i> were identified in 31.2% and 36.4% of calves, respectively. Similarly, 6.2% and 5% of cows had CTR ^r and CEF ^r <i>E. coli</i> isolates, respectively. <i>E. coli</i> carrying <i>bla</i> _{CTX-M} was identified in about 5% of the farms	PA	(204)
Feces	Culture and AST	A prospective study on <i>Salmonella</i> suspected cases over eight months from 2,565 dairy cattle in 412 farms	The prevalence of CEF ^r <i>Salmonella</i> spp. was 60.4%	NY, PA, VT, MA, CT	(205)
Feces from pen floors	CSM and AST	A cross-sectional study on healthy and sick cows from four large-sized dairy farms	More than 51% of <i>Salmonella</i> isolates were CEF ^r , and all were susceptible to CTR	SW	(206)
Feces	CSM and AST	A longitudinal study on 110 dairy herds with five times sampling at a two-month interval	Prevalences of CEF ^r <i>Salmonella</i> isolates were 2.4%, 10%, and 10.8% in healthy cows, sick cows, and calves, respectively	NY, MI, MN, WI	(207)

CAM: culture on antibiotic supplemented media; CSM: culture on selective media; CEF^r: ceftiofur resistant; CTX^r: cefotaxime resistant; CTR^r: ceftriaxone resistant; CPD^r: cefpodoxime resistant; WGS: Whole genome sequencing; AST: antibiotic susceptibility testing; TN: Tennessee; OH: Ohio; TX: Texas; NM: New Mexico; CA: California; PA: Pennsylvania; NY: New York; WI: Wisconsin; VT: Vermont; MA: Massachusetts; CT: Connecticut; SW: Southwestern Region of US; MI: Michigan; MN: Minnesota.

Table 2.2. Extended-spectrum beta-lactamases genes detected in *Enterobacteriaceae* in the U.S. dairy cattle

ESBL Gene Type	Bacteria	State/Region	Sample	Reference
CTX-M-1	<i>E. coli</i>	OH, WA, SW, TX	Fecal	(8, 59, 205, 208)
	<i>K. pneumoniae</i>	NY, IA	Mastitic milk	(124, 209)
CTX-M-12	<i>E. coli</i>	WA	Fecal	(126)
CTX-14	<i>E. coli</i>	OH, WA	Fecal	(24, 201)
CTX-15	<i>E. coli</i>	OH, WA, SW, TX	Fecal	[9, 113,132]
CTX-M-24	<i>E. coli</i>	WA	Fecal	(24)
CTX-M-27	<i>E. coli</i>	WA, SW, TX	Fecal	(8, 24, 59)
CTX-M-32	<i>E. coli</i>	SW, TX	Fecal	(8, 59)
CTX-M-55	<i>E. coli</i>	WA, SW	Fecal	(8, 24)
CTX-M-65	<i>E. coli</i>	WA, SW, TX	Fecal	(8, 24, 59)
CTX-M-79	<i>E. coli</i>	OH	Fecal	(27)
CTX-M-102	<i>E. coli</i>	TX	Fecal	(59)
CTX-M-174	<i>E. coli</i>	TX	Fecal	(59)
CTX-M	<i>Salmonella</i> spp.	Not available	Feces from clinical case	(202)
	<i>E. coli</i>	WA	Fecal	(210)
	<i>E. coli</i>	TN	Fecal & BTM	(18)
	<i>E. coli</i>	OH	Fecal & carcass swabs	(199)
	<i>E. coli</i>	PA	Fecal	(204)
SHV	<i>Salmonella</i> spp.	Not available	Feces from clinical case	(202)
	<i>E. coli</i>	WA	Fecal	(210)
	<i>K. pneumoniae</i>	NY	Mastitic milk	(124)
TEM	<i>E. coli</i>	OH, WA	Fecal	(126, 201)
	<i>E. coli</i>	PA	Fecal	(113)
	<i>Salmonella</i> spp.	Not available	Fecal	(202)
OXA-27	<i>E. coli</i>	WA	Fecal	(126)

TN: Tennessee; WA: Washington; NY: New York; OH: Ohio; PA: Pennsylvania; SW: Southwestern Region of U.S.

Table 2.3. Beta-lactam antibiotics resistance genes of dairy origin reported by NARMS from 2014 To July 2022.

Beta-Lactam Antibiotic Resistance Gene	Host Bacterium	State	The Proportion of Total Beta-Lactam ARGs of Dairy Source	Time (year)
<i>bla</i> _{CTXM-27}	<i>E. coli</i>	WA, TX, OH and SD	7.2% (7/97)	2017, 2019 and 2021
<i>bla</i> _{CTXM-55}	<i>E. coli</i>	TX, ND	2.1% (2/97)	2019 and 2020
<i>bla</i> _{CXM-14}	<i>E. coli</i>	PA	1% (1/97)	2019
<i>bla</i> _{CTXM-15}	<i>E. coli</i>	TX	1% (1/97)	2018
<i>bla</i> _{CTXM-65}	<i>E. coli</i>	FL	1% (1/97)	2020
<i>bla</i> _{TEM-1}	<i>E. coli</i>	TX, UT, WI, TN, WA, NY, OH, KS, MI, SD, CA, AZ, ID, PA and NE	69.1% (67/97)	2014–2020
<i>bla</i> _{CMY-2}	<i>E. coli</i>	SD, MD, CA, PA, MI, ID, WA and WI	14.4% (14/97)	2018
<i>bla</i> _{AmpC}	<i>E. coli</i>	WI	3.2% (1/31)	2018
<i>bla</i> _{OXA-2}	<i>E. coli</i>	MI	1/97	2019
<i>bla</i> _{CARB-2}	<i>E. coli</i>	CA	1/97	2018
<i>bla</i> _{SHV-12}	<i>Salmonella</i>	CA	1/95	2018
<i>bla</i> _{CMY-2}	<i>Salmonella</i>	UT, WA, WI, CA, TX, ID, UT, CO, AZ, TN and SC	72.6% (69/95)	2015–2020
<i>bla</i> _{TEM-1}	<i>Salmonella</i>	WI, ID, CA, SD, WA, TX, UT, RI, IA and GA	22.11% (21/95)	2015–2019
<i>bla</i> _{CARB-2}	<i>Salmonella</i>	WA, CA	4.21% (4/95)	2014 and 2016

TX: Texas; UT: Utah; WI: Wisconsin; TN: Tennessee; WA: Washington; NY: New York; OH: Ohio; KS: Kansas; MI: Michigan; CA: California; AZ: Arizona; ID: Idaho; CO: Colorado; IA: Iowa; GA: Georgia; RI: Rhode Island; SC: South Carolina; SD: South Dakota; MD: Maryland; MI: Michigan; PA: Pennsylvania; and NE: Nebraska.

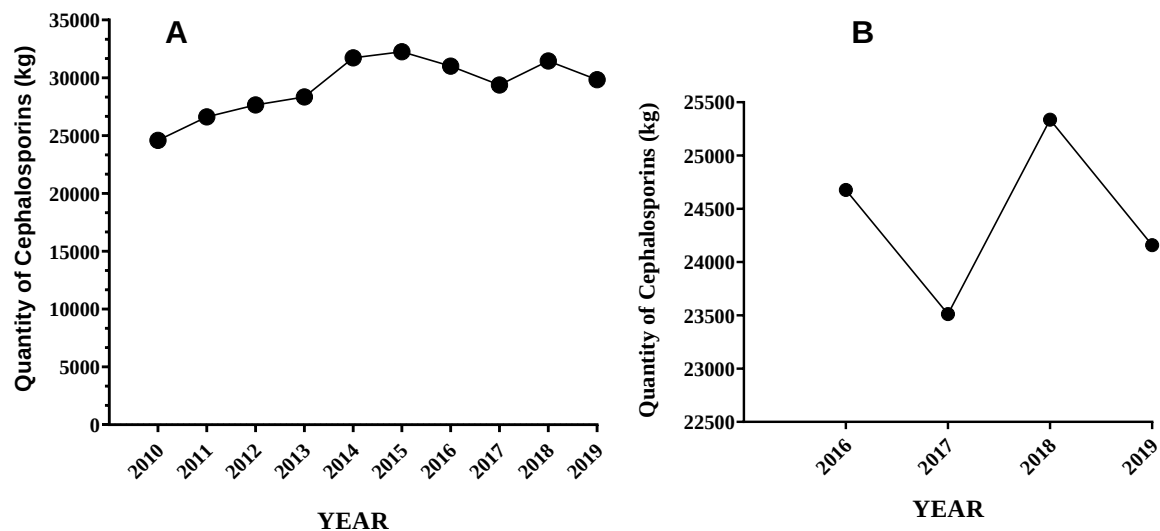


Figure 2. 1. Cephalosporins sold for use in food-producing animals over 10 years (2.1A) and for cattle over 4 years (B) in the U.S.

The cephalosporins sold for cattle use were declined from 2016 to 2017, followed by an increase from 2017 to 2018 and a declining trend from 2018 to 2019 (Figure 2.1 B) (76).

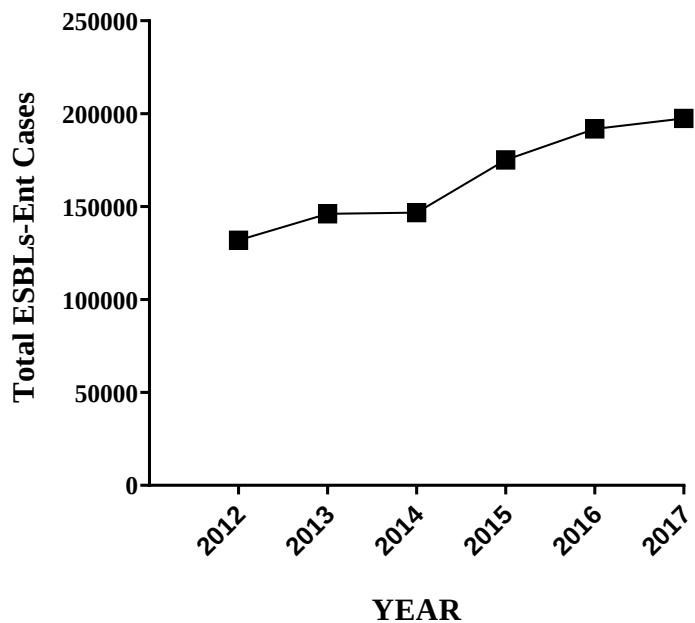


Figure 2. 2. Trends of extended-spectrum beta-lactamases-producing *Enterobacteriaceae* infections in humans in the U.S.

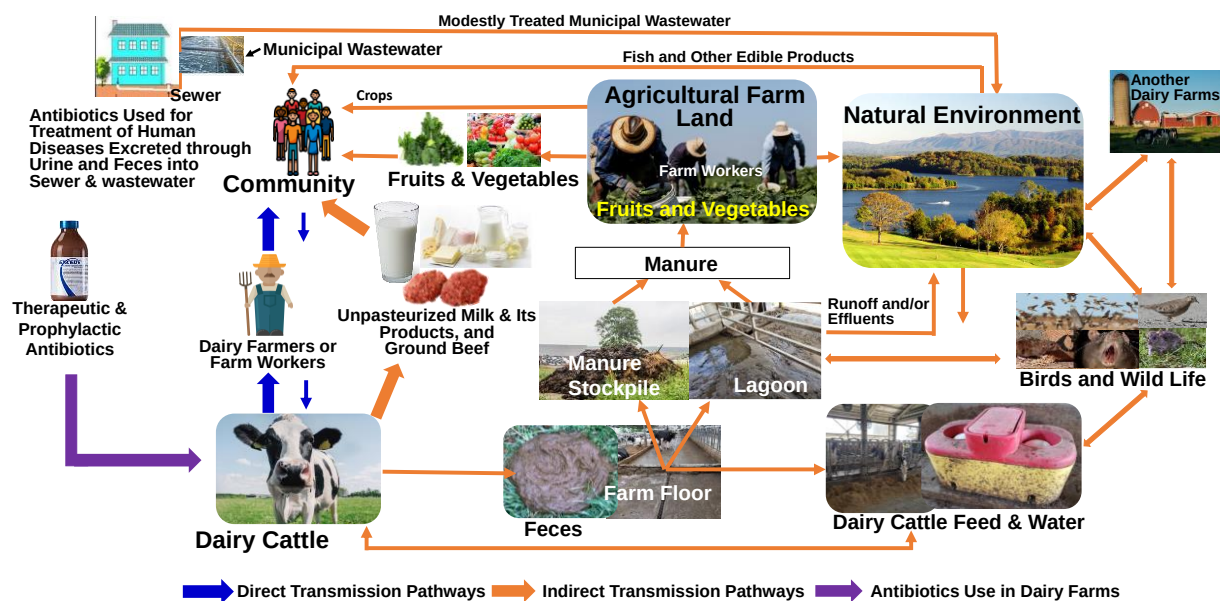


Figure 2. 3. Extended-spectrum beta-lactamases-producing *Enterobacteriaceae* transmission, and maintenance within one health settings

Arrows in the figure indicate potential transmission routes, and the thickness of the arrows shows the more likely transmission routes from dairy cattle to humans or vice versa through direct or indirect routes.

Chapter 3. Prevalence of Antimicrobial Resistant and ESBL-Producing *E. coli* in Dairy Cattle Farms in East Tennessee

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Abstract

Antimicrobials have been widely used in dairy farms to prevent and control dairy cattle diseases since the 1960s. This led to the emergence of antimicrobial-resistant bacteria (ARB) that, along with their antimicrobial resistance genes (ARGs), can spread from dairy farms to humans. Therefore, regular antimicrobial resistance (AMR) monitoring is important to implement proper mitigation measures. The objective of this study was to determine the prevalence of AMR and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in dairy cattle. A cross-sectional study was conducted in four dairy cattle farms (A - D) in East Tennessee. A total of eighty samples consisting of twenty samples each of bulk tank milk, feces, dairy cattle manure amended soil, and prairie soil adjacent to the farms were collected and cultured for the isolation of *E. coli*. Tetracycline (TET^r)-, third-generation cephalosporin (TGC^r)- and nalidixic acid (NAL^r)-resistant *E. coli* (n=88) were isolated and identified on agar media supplemented with tetracycline, cefotaxime, and nalidixic acid, respectively. TGC^r *E. coli* were tested for ESBL and other co-selected ARGs. TET^r (74%, n=88) was the most common, followed by TGC^r (20%) and NAL^r (8%). Farms had significant ($P<0.001$) differences: the highest prevalence of TGC^r (55%) and TET^r (100%) were observed in farm D, while all NAL^r isolates were from farm C. Over 83% of TGC^r isolates (n=18) harbored ESBL gene *bla*_{CTX-M}. The majority (78%) of the *E. coli* isolates were multidrug-resistant (MDR), being positive for beta-lactams (*bla*_{CTX-M}), tetracyclines *tet*(A), *tet*(B), *tet*(M)), sulphonamides (*sul2*), aminoglycosides (*strA*), and phenicols (*floR*). This study indicates the widespread occurrence of MDR ESBL-*E. coli* in dairy cattle farms in East Tennessee. AMR surveillance of more dairy farms and identification of farm-level risk factors are important to mitigate the occurrence and spread of ARB of significant public health importance, such as ESBL *E. coli*.

Keywords: Dairy cattle; Antimicrobial resistance; *E. coli*; Extended-spectrum beta-lactamase; Antimicrobial resistance genes; PCR, Dairy cattle manure

3.1 Introduction

Dairy farming is one of the most significant users of antibiotics (1), including medically important antibiotics such as third-generation cephalosporins (TGC), tetracycline, fluoroquinolones, and macrolides (1-3). These antimicrobial drugs are administered to dairy cattle parenterally, orally, or through intramammary infusion (IMMI) (4, 5). Orally administered antimicrobials directly affect the gut microbiota by indiscriminately killing the susceptible population while favoring the propagation of the resistant population (6, 7). Antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) are excreted into the environment through feces, urine, and milk (8, 9), which may spread to humans indirectly through the food chain (milk and meat) or manure land application as a soil amendment for vegetables and crops for human consumption (10-13). Similarly, ARB can spread from the environment to animals (10-13). Parenterally administered antibiotics are bio-transformed in the body and excreted through milk, urine, or feces with some active ingredients in environments (14). Similarly, IMMI may leak into the environment with milk. At the same time, some are absorbed across the mammary gland membrane/blood barrier into the body and bio-transformed and excreted into the environment with feces or urine (9).

Antimicrobials are extensively used on dairy farms to prevent and treat mastitis (15, 16) and other diseases such as metritis, digital dermatitis or lameness, pneumonia, diarrhea, and others (17, 18). The extensive use of these antibiotics can create selection pressure on the normal intestinal microbiota of dairy cattle that could affect humans (19, 20).

Escherichia coli is among the most important Gram-negative bacteria in the GIT of cattle as normal microbiota (3). Bacteria in the GIT are frequently exposed to antimicrobials directly, orally, or indirectly through the parenteral route (6), leading to intense pressure and the emergence of resistant strains (7). Resistant bacteria in the animal body are continuously shed through feces, urine, or milk onto the dairy farm floor and grazing land in the farm environments (8, 9). ARB and ARGs in the animal body may spread to humans through direct contact with carrier animals by farm workers or indirectly through consumption of raw milk or undercooked ground meat from culled dairy cows, consumption of vegetables contaminated with manure used

as fertilizer, or indirectly through exposure to environments contaminated by run-off water from dairy farms (14).

Continuous monitoring of individual dairy cattle, bulk tank milk, and manure is vital to determining the status of ARB and ARGs and implementing appropriate mitigation measures at the farm level and along dairy value chains, preventing their spread to humans. We hypothesized that dairy cattle farms are potential reservoirs for ARB and ARGs, particularly *E. coli*, the dominant Gram-negative foodborne pathogen that also causes mastitis in dairy cattle. Therefore, the objective of this study was to determine the prevalence of antimicrobial resistant and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* on four dairy farms in East Tennessee. This information on harborage sites and prevalence helps implement proper mitigation, sanitation, and control practices to prevent the spread of ARB and ARG in the environment.

3.2 Materials and Methods

3.2.1 Sample Collection

A cross-sectional study was conducted on four conventional dairy farms designated A, B, C, and D that regularly use antibiotics as part of their management practices in East Tennessee. The four farms were conventional commercial Holstein dairy farms with average milking cows of 225. All four farms regularly use antibiotics as dry cow therapy (DCT) and for the treatment of cases of mastitis and other diseases of dairy cows. The housing system is a free stall divided into different pens, and cows were fed a total ration with supplemental feeding and had pasture lands for grazing. All farms were on year-round calving and enrolled in Dairy Herd Improvement Association (DHIA). A total of 80 samples consisting of four types of samples, which include bulk tank milk (BTM, n=20), pooled feces (n=20) from different spots on the farm floor, dairy cattle manure fertilized soil (Agsoil, n=20), and prairie soil from land adjacent to the farm with no access to dairy cattle (Psoil, n=20) were collected in five replicates. The sample types from dairy cattle were selected based on possible exit routes of antimicrobial resistant bacteria (ARB) and antibiotic resistance genes (ARGs). During each sampling, 50 mL of BTM and 200 grams each of feces and soil were collected into screw-capped sterile 50 mL falcon tubes (Thermo

Fisher Scientific, Waltham, MA, USA). Samples were transported on ice to the laboratory and processed within 4 -12 h of collection.

3.2.2 Identification and Determination of Antimicrobial Resistant *E. coli*

For the isolation of *E. coli*, 10 g of feces or soil samples were suspended in 90 mL of tryptic soy broth (TSB) with phosphate buffer (TSB-PO₄) (Becton, Dickinson and Company, Sparks, MD, USA) in a filter barrier bag (Thomas Scientific, Houston, TX, USA) and homogenized by hand massage for 15 s. For the milk samples, 20 mL was suspended in 80 mL TSB-PO₄. Pre-enrichments were incubated at 25°C for 2 h, then at 42°C for 6 h, and held at 4°C until the secondary enrichments were carried out the following day. For secondary enrichments, 0.5 mL of the broths were transferred to 2.5 mL MacConkey broth (BD and Company) and incubated at 42°C for 18 - 24 h. After incubation, secondary enrichments were swabbed onto CHROMagar™ *E. coli* (CEC) agar (DRG International Inc., Springfield, NJ, USA) and incubated at 37°C for 18 h. Up to two suspect colonies were picked from each sample, inoculated into 96 deep wells containing TSB, and incubated overnight at 37°C. Sterile glycerol (final concentration of 80%) was added to the TSB culture in 96 well blocks (sterile serum blocks) and stored at -80°C until further analysis.

To determine AMR *E. coli*, frozen isolates were thawed and cultured on CEC. Isolates were confirmed by PCR targeting the *uidA* gene that encodes beta-glucuronidase enzyme as described (21). Tetracycline (TET^r)-, TGC^r- and nalidixic acid (NAL^r)- resistant *E. coli* isolates were determined on CEC media supplemented with tetracycline (16 mg/L), cefotaxime (4 mg/L), and nalidixic acid (32 mg/L), respectively. Cultures were incubated at 37°C overnight. All antibiotics were obtained from Sigma-Aldrich, Inc., St. Louis, MO, USA, and their concentration was based on Clinical Laboratories Standards Institute's breakpoints (22).

3.2.3 Characterization of TGC^r Isolates for Antimicrobial Resistance Genes

Genomic DNA was extracted from an overnight culture of *E. coli* isolates for PCR detection of ESBL and other co-selected resistance genes. DNA was extracted using Promega Wizard® Genomic DNA Purification Kit (Madison, WI, USA) following the manufacturer's instructions.

Phenotypically TGC^r *E. coli* isolates were tested for ESBL genes (*bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM}) using PCR as previously described (Table 3.1, placed under the appendix). The TGC^r isolates were also tested by PCR for the presence of ARGs in six other antimicrobial classes using published primers and protocols (Table 3.1) as well as newly designed primers by Clone Manager version 9.3 for Windows (Sci Ed Software LLC, Cary, NC, USA). Other co-selected ARGs tested include tetracycline-(*tet*(A), *tet*(B), and *tet*(M)), sulfonamide-(*sul*1, *sul*2), quinolone-(*qnr*A, *qnr*B, *qnr*S, and *qep*A), phenicol-(*flo*R), fosfomycin-(*fos*A3) and streptomycin-(*str*A). The PCR reaction was conducted in a total volume of 20 µL consisting of 10 µL of 2X PCR master mix (Thermo Fisher Scientific), 7 µL of nuclease-free water, 1 µL of 100 ng DNA, 1 µL of 10 µM each primer (forward and reverse). The PCR reaction was run on a Thermal cycler (Bio-Rad, Hercules, CA, USA) using the following conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 53.7°C for 35 sec and extension at 72°C for 1 min. The final extension was at 72°C for 7 min. A 5 µL of PCR product was mixed with 1 µL of 5X DNA loading dye (Thermo Fisher Scientific) and loaded onto a 1.5% (w/v) agarose gel (Lonza, Walkersville, MD, USA) with GelRed nucleic acid stain (Phenix Research Products, Candler, NC, USA) and electrophoresed at 85 V for two hours in Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) buffer. A 1 kb plus DNA molecular weight marker (Thermo Fischer Scientific) was used to estimate the sizes of the PCR products. Images were taken using a ChemiDoc Touch Imaging System (Bio-Rad) and exported to Image Lab 5.2.1 Software (Bio-Rad).

3.2.4 Data Analyses

Raw data were entered into Microsoft Excel for Windows (2010, Microsoft Corp., Redmond, WA, USA) and imported to SPSS for Windows Version 23.0 (IBM Corp, Armonk, NY, USA) for statistical analysis. Initial descriptive statistics were done to summarize the sample and farm-level prevalence. The association between these factors and the prevalence of ARGs was performed using the Chi-Square test or fisher exact test. A P-value of 0.05 was used to determine the significance level. Multilevel effects logistic regression was conducted in STTA 16 (StataCorp, College Station, TX, USA). In these models, sample type was included as a fixed categorical variable with the *E. coli* isolates from the prairie soil serving as a reference category,

and the farm was included as a random variable to account for clustering of observations within the farm (five samples were collected per farm for each sample type).

3.3 Results

3.3.1 Prevalence of Antimicrobial Resistant *E. coli*

A total of 88 PCR confirmed *E. coli* isolates were obtained from the four sample types and further characterized for AMR. Across all samples, the highest resistance prevalence (73.9%) was to tetracycline, followed by TGC^r (20.5%) and NAL^r (8%). The proportion of AMR *E. coli* isolates varied significantly ($P < 0.001$) by farms for the three antimicrobials tested. The prevalence of AMR *E. coli* to a given antibiotic within and between farms ranged from 0 for NAL to 100% for TET. All NAL^r *E. coli* isolates were from farm B, whereas all isolates from the other three farms were susceptible (Table 3.2, placed under the appendix).

The highest proportion of resistant isolates for all three antimicrobial drugs was obtained from fecal samples, followed by dairy cow manure-fertilized soil isolates. The proportion of resistant isolates was the least in the prairie soil compared to the other sample types (Table 3. 3, placed under the appendix). Over one-third (36%) of *E. coli* isolates ($n=14$) from raw BTM samples were TGC^r. The prevalence of TET^r ($P=0.116$) and TGC^r ($P=0.078$) in the *E. coli* isolates did not significantly differ by sample type. NAL^r prevalence was only marginally significant (Fisher's exact $P=0.049$). NAL^r *E. coli* isolates were not detected from BTM and Prairie soil samples.

When adjusted for the farm effect, the prevalence of TET^r ($P=0.024$) and TGC^r ($P=0.034$) isolates from fecal samples was significantly higher than that of the Prairie soil samples. However, the prevalence of TET^r isolates from the Agsoil and prairie soil ($P=0.269$) and from BTM and Prairie soil ($P=0.456$) did not differ. Similarly, the prevalence of TGC^r isolates from Agsoil and Prairie soil ($P=0.748$) and from BTM and Prairie soil ($P=0.140$) did not vary. Since NAL^r *E. coli* was not detected from Prairie and BTM, similar modeling could not be done.

A quarter (26%) of the *E. coli* isolates ($n=88$) were susceptible to the tested drugs representing three antimicrobial classes. The remaining three-fourths (73.9%) were TET^r only (50%), or TET^r

co-resistant with TGC (15.9%) and nalidixic acid (3.4%). The remaining 4.6% of the isolates were phenotypically multidrug-resistant (MDR) with resistance to three antimicrobial classes (Table 3.4, placed under the appendix).

3.3.2 Detection of Antimicrobial Resistance Genes among TGC^r *E. coli*

ARGs detected among the TGC^r *E. coli* isolates by antimicrobial classes are presented in Table 3.5 (placed under the appendix). While all TGC^r isolates (n=18) carried *tet* genes, most (83%) were also positive for *bla*_{CTX-M}, an ESBL gene. Genotypically determined MDR *E. coli*, defined as carriage of ARGs of ≥ 3 antimicrobial classes, was isolated from all sample types, including BTM. Over three-fourths (78%) of the TGC^r isolates were genotypically resistant to five antimicrobial classes.

Among the 18 phenotypically TGC^r *E. coli* isolates, 15 were *bla*_{CTX-M} positive, making them ESBL strains (Figure 3.1, placed under the appendix). None of the isolates were positive for other beta-lactamase genes (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CMY-2}). Three TGC^r isolates, one each from BTM, feces, and Agsoil, were negative for any of the targeted beta-lactam genes.

All 18 TGC^r *E. coli* isolates carried genes for tetracycline resistance (Figure 3.2, placed under the appendix). All isolates had *tet*(A). Two isolates carried *tet*(B), and one isolate carried *tet*(M) in addition to *tet*(A). The sulfonamide resistance gene *sul1* was not detected, but *sul2* was detected in 89% of the TGC^r *E. coli* isolates (n=18) (Figure 3.3, placed under the appendix). Two fecal isolates were negative for the *sul2* gene. Of 18 TGC^r *E. coli* isolates, 15 were positive for streptomycin resistance gene *strA*. An additional florfenicol resistance gene (*floR*) was detected in 14 isolates (Figure 3. 4, placed under the appendix). Fosfomycin (*fosA3*), quinolone (*qnrA*, *qnrB*, and *qnrS*) resistance, and other beta-lactamase genes (*bla*_{CMY-2}, *bla*_{TEM}, and *bla*_{SHV}) were not detected.

3.4 Discussion

The prevalence (20.5%) of TGC^r *E. coli* in cattle from dairy farms in the present study is similar to that of beef cows (20). The occurrence of TGC^r could be due to selection pressure as most US

dairy farms use β -lactam antibiotics such as cephalosporins, including ceftiofur, a 3rd generation cephalosporin, for the treatment and prevention of various diseases (1, 23). While Both cefotaxime and ceftiofur are TGC, ceftiofur is commonly used in dairy farms only, whereas cefotaxime is widely used to treat gram-negative bacterial infections in humans. Thus, the use of ceftiofur in dairy farms may contribute to the cross-resistance of *E. coli* to cefotaxime (24). A recent study (1) reported that cephalosporins such as ceftiofur are the most frequently used antibiotics to treat and prevent mastitis in some US dairy farms. This extensive use may cause selection pressure and subsequently lead to the rise of resistance to cefotaxime, an antibiotic with a similar chemical structure (25).

The high prevalence of TGC^r *E. coli* is significant from a public health point of view. Some of these *E. coli* strains could be zoonotic (26). Resistant strains or their genes can be transmitted to humans directly through contact with animals or indirectly through foods of animal origin, posing a threat to public health (27). Besides, resistance genes such as the ESBL gene (*bla*_{CTX-M}) detected in *E. coli* isolates from dairy cattle in this study can transmit horizontally to pathogenic bacteria (28). As reported frequently in previous studies, ESBL genes on mobile genetic elements such as plasmids can spread horizontally to different bacteria (29).

TET^r *E. coli* were the most prevalent ($\approx 80\%$) isolates observed in this study. The widespread occurrence of TET^r concurs with previous reports from dairy farms in Washington State (26), Pennsylvania (93%), the USA (30, 31), and Europe (32). The high prevalence of TET^r *E. coli* isolates detected in all farm samples may reflect the frequent use of tetracycline across these farms (1). Another important reason is that TET^r can be selected indirectly due to the use of other antibiotics, such as TGCs (20, 33). In this study, all TGC^r *E. coli* strains were also TET^r, both phenotypically and genotypically. Previous studies showed that TET^r genes are often found on transmissible genetic elements such as plasmids and conjugative transposons (34), contributing to its widespread occurrence (23).

In this study, *bla*_{CTX-M} was the only ESBL and β -lactamase gene detected among the TGC^r *E. coli*, consistent with other studies in the USA (35). The widespread occurrence of *bla*_{CTX-M}

harboring TGC^r *E. coli* may be related to its effective mobilization and dissemination through mobile genetic elements, low fitness cost, and antibiotic selection pressure (36). Based on the frequent usage of ceftiofur in dairy cattle production and previous observations in which *bla*_{CTX-M} was significantly higher among ceftiofur treated than non-treated cows (Agga et al., 2016), ceftiofur may select for *bla*_{CTX-M}. Similarly, recent observation in farmed mink (37) showed that ceftiofur was selected mainly for ESBL-producing bacteria.

All TGC^r and ESBL- *E. coli* exclusively carried *tet*(A), as similarly reported (Agga et al., 2016). Detection of *tet* genes in all ESBL-*E. coli* isolates and the other genes (*sul*, *strA*, and *floR*) in most isolates suggest their co-selection with the ESBL genes (38). Our finding indicates that TGC^r and especially the ESBL-producing population are MDR, as also reported by Agga et al. (33, 37). Similarly, others (31, 39) reported the presence of *tet*(A), *sul2*, and *strA* in cattle in the USA. Other studies also indicated that *bla*_{CTX-M} could select for other resistance genes to different classes of antibiotics (40, 41), as most ESBL-producing isolates showed resistance to several classes of antibiotics (42). In addition, *bla*_{CTX-M} is mainly located on mobile genetic elements such as plasmids so that it may coexist with multiple resistance genes on the same plasmid (26, 43-45).

MDR *E. coli* was detected in all sample types, including BTM samples. Detection of MDR ESBL-*E. coli* in raw milk has a significant public health risk, especially if raw milk is consumed, which is still practiced in some parts of the USA (46, 47). In addition to its zoonotic potential, MDR *E. coli* in raw milk may pose an additional public health threat due to its resistance to available antibiotics. They were reported to carry mobile resistance determinants that can be transmitted to other more pathogenic or commensal bacteria in the human gut (47, 48).

Detection of statistically significant variation in the prevalence of *E. coli* isolates resistant to the three tested antibiotics between the study farms suggests differences in antibiotic use patterns. This implies that the prevalence of AMR bacteria depends on farm-level factors, including antibiotic use and herd health management (49). Variation by farm and not by sample type indicates the need to sample more farms for future studies rather than collecting more samples within farms. The World Organization for Animal Health (OIE) ranked TGC and TET as critically important classes of antibiotics for veterinary medicine. The high prevalence of TGC- and TET-resistance has

significant implications on animal health as these antibiotics are commonly used to treat severe animal diseases (50). Similarly, WHO also lists TGCs, such as cefotaxime, as a critically important class of antibiotics used to treat severe infections caused by *Salmonella* spp. and *E. coli* in humans (51). Thus, using structurally and chemically similar antibiotics in dairy cattle production and human medicine can lead to cross-resistance and further speed up the emergence and spread of ESBL-producing bacteria.

Conclusions

In this study, we reported the widespread occurrence of TET^r *E. coli* followed by TGC^r *E. coli* in dairy cattle, environment, and milk, which varied between farms. Almost all TGC^r *E. coli* were ESBL-producing strains with MDR genotypes. The widespread occurrence of MDR *E. coli*, and more importantly ESBL producing *E. coli* indicates that the dairy cattle production system and its environment are important reservoirs for resistant bacteria of public health importance. Detection of ESBL *E. coli* poses a direct and immediate food safety risk if unpasteurized milk or milk products are consumed. This study indicates the need for continued surveillance of AMR bacteria by sampling more dairy farms to identify risk factors responsible for their occurrence and persistence in dairy production systems. This will lead to the implementation of appropriate mitigation strategies once harborage areas and modes of transmission, persistence/prevalence, are better understood.

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Appendix

Table 3. 1. Primer sequences, and expected product sizes of the targeted resistance genes

Primer	Sequence (5'-3')	Product size (bp)	Reference
<i>bla</i> _{CTX-M-F}	5'-TTTGCATGTGCAGTACCAGTAA- 3'	544	(1)
<i>bla</i> _{CTX-M-R}	5'-CGATATCGTTGGTGGTGCCATA - 3'		
<i>bla</i> _{TEM-F}	5'-CATTTCCGTGTCGCCCTTATTC- 3'	846	(2, 3)
<i>bla</i> _{TEM-R}	5'-CCAATGCTTAATCAGTGAGGC- 3'		
<i>bla</i> _{CMY2-F}	5'-ATGATGAAAAAATCGTTATGCTGC- 3'	1117	(4)
<i>bla</i> _{CMY2-R}	5'-GCTTTTCAAGAATGCGCCAGG- 3'		
<i>bla</i> _{SHV-F}	5'-GCCGGGTTATTCTTATTTGTCGC- 3'	1016	(3, 5)
<i>bla</i> _{SHV-R}	5'-ATGCCGCCGCCAGTCA- 3'		
<i>tetA-F</i>	5'-GCT ACATCCTGC TTGCCTTC-3'	210	(6)
<i>tetA-R</i>	5-'CAT AGATCG CCG TGA AGA GG-3'		
<i>tetB-F</i>	5'-TTG GTT AGG GGC AAG TTT TG-3'	659	(7)
<i>tetB-R</i>	5'-GTA ATG GGC CAA TAA CAC CG-3'		
<i>tetM-F</i>	5'-GTG GAC AAA GGT ACA ACG AG-3'	406	(8)
<i>tetM-R</i>	5-CGG TAA AGT TCG TCA CAC AC-3'		
<i>floR-F</i>	5' TGCTGCTGATGGCTCCTTTC 3'	1168	This study
<i>floR-R</i>	5' TAGACGACTGGCGACTTCTC 3'		
<i>strA-F</i>	5' GGTGAATCGCATTCTGACTG 3'	774	This study
<i>strA-R</i>	5' CCAAGTCAGAGGGTCCAATC 3'		
<i>sul1-F</i>	5' GGTGGCGTTACGCTGGTTTG 3'	1801	This study
<i>sul1-R</i>	5' GCTCGGATCTCAGGACGAAG 3'		
<i>sul2-F</i>	5' TCGGCATCGTCAACATAACC 3'	775	This study
<i>sul2-R</i>	5' TCTTTCAGCGCCGCCAATAC 3'		
<i>fosA3-F</i>	5' GTCAGCGATCTGGCGTCAAG 3'	306	This study
<i>fosA3-R</i>	5' CTCCAGCTTATGGCCGTCAG 3'		
<i>qnrA-F</i>	5'-ATTCTCACGCCAGGATTTG-3'	516	(9, 10)
<i>qnrA-R</i>	5'-GATCGGCAAAGGTTAGGTCA-3'		
<i>qnrB-F</i>	5'-GATCGTGAAAGCCAGAAAGG-3'	469	(9, 10)
<i>qnrB-R</i>	5'-ACGATGCCTGGTAGTTGTCC-3'		
<i>qnrS-F</i>	F: 5'-ACGACATTCGTCAACTGCAA-3'	417	(9, 10)
<i>qnrS-R</i>	5'-TAAATTGGCACCCCTGTAGGC-3'		

Table 3. 2. Overall and farm-level prevalence of antimicrobial resistant *E. coli*

Farm code	Number of isolates	Number (proportion) of isolates resistant to specific antibiotics		
		Tetracycline	Cefotaxime	Nalidixic Acid
A	25	10 (40%)	0	0
B	21	18 (85.7%)	2 (9.5%)	0
C	22	17 (77.3%)	5 (22.7%)	7 (31.8%)
D	20	20 (100%)	11 (55%)	0
Total	88	65 (73.9%)	18 (20.5%)	7 (7.95%)

Table 3. 3. Antimicrobial resistance of *E. coli* isolates by sample type

Sample	Number of isolates	Tetracycline resistance (%)	Cefotaxime resistance (%)	Nalidixic Acid resistance (%)
Feces	26	23 (88.5)	8 (30.8)	5 (19.2)
Agronomic soil	24	18 (75)	3 (12.5)	2 (8.3)
Prairie soil	24	14 (58.3)	2 (8.3)	0
Bulk tank milk	14	10 (71.4)	5 (35.7)	0
Total	88	65 (73.9)	18 (20.5)	7 (8)

Table 3. 4. Resistance profiles of *E. coli* isolates (n=88) obtained across four dairy farms and sample types

Resistance pattern	Frequency	Percentage
Pan susceptible	23	26.1
TET	44	50.0
TET-NAL	3	3.4
TET-TGC	14	15.9
TET-TGC-NAL	4	4.6

TET= Tetracycline; TGC=3rd generation cephalosporin; NAL=nalidixic acid

Table 3. 5. Antibiotic resistance genes detected in 18 third-generation cephalosporin resistant *E. coli* isolates by antimicrobial classes

Antibiotic class	Resistance gene	Number of <i>E. coli</i> isolates carrying the gene
Beta-lactam	<i>bla</i> _{CTX-M}	15
Tetracycline	<i>tet(A)</i> , <i>tet(B)</i> and <i>tet(M)</i>	<i>tet(A)</i> (18), <i>tet(B)</i> (2) and <i>tet(M)</i> (1)
Sulphonamide	<i>sul2</i>	16
Phenicol	<i>floR</i>	14
Streptomycin	<i>strA</i>	14

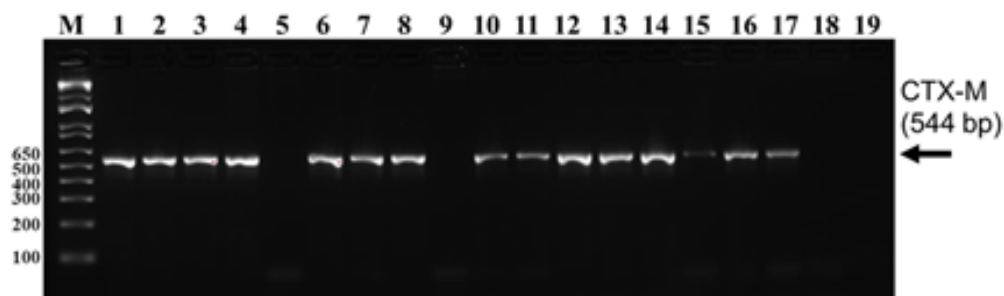


Figure 3. 1. Gel image showing *bla*_{CTX-M} PCR positive third-generation cephalosporin resistant *E. coli*.

Isolates were obtained from dairy manure fertilized soil (lanes 1, 2, 11, 17, 18); bulk tank milk (lanes 3, 5-7, 14); feces (lanes 4, 9-10, 13, 15-16); prairie soil (lanes 8, 12). M: 1 kb DNA molecular weight marker; Lane 19: negative control; Three TGC^r isolates (lanes 5, 9, 18) were negative for *bla*_{CTX-M}.

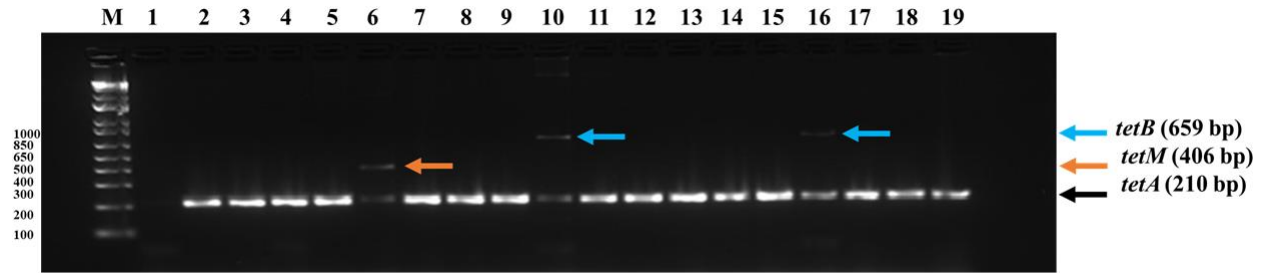


Figure 3. 2. Tetracycline resistance gene positive third-generation cephalosporin resistant *E. coli*.

M: 1 kb DNA molecular weight marker; Lane 1: negative control; Isolates were from dairy manure fertilized soil (lanes 2, 3, 12, 18, 19); bulk tank milk (lanes 4, 6–8, 15); feces (lanes 5, 10, 11, 14, 16, 17); prairie soil (lanes 9, 11).

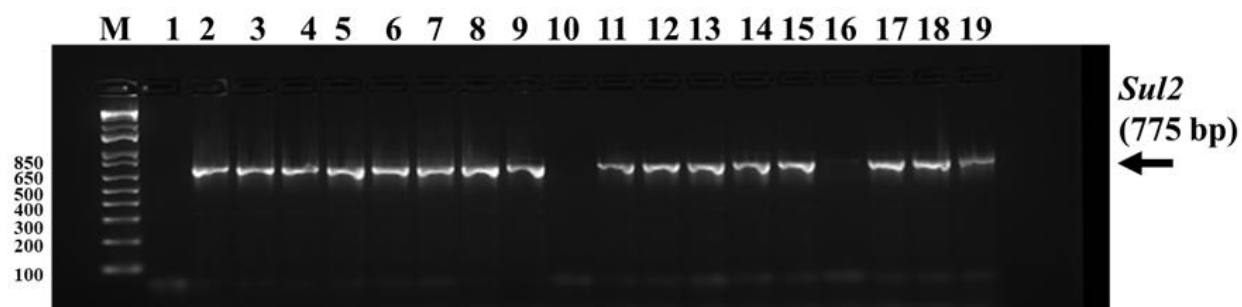


Figure 3. 3. Sulfonamide resistance gene (*sul2*) positive TGC^r *E. coli* isolates.

M: 1 kb DNA molecular weight marker; Lanes 1: negative control; TGC^r *E. coli* isolates were obtained from dairy manure fertilized soil (lanes 2, 3, 12, 18, 19); bulk tank milk (lanes 4, 6 - 8, 15); feces (lanes 5, 10, 11, 14, 16, 17); and prairie soil (lanes 9, 11). Two fecal isolates (lanes 10, 16) were negative for *sul2* gene.

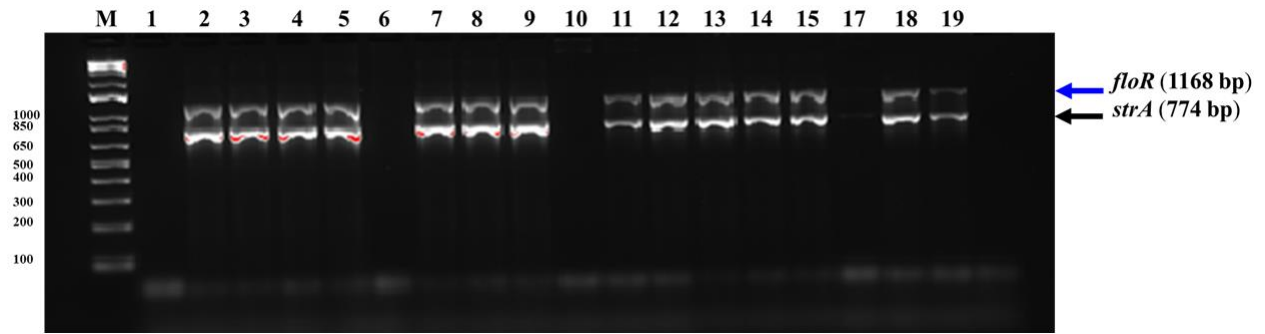


Figure 3. 4. Florfenicol and streptomycin resistance among TGC^r *E. coli* isolates.

M: 1 kb DNA molecular weight marker; Lanes 1: negative control; Isolates were from dairy manure fertilized soil (lanes 2, 3, 12, 18, 19); bulk tank milk (lanes 4, 6–8, 15); feces (lanes 5, 10, 11, 14, 16, 17); and prairie soil (lanes 9, 11).

Chapter 4. Antimicrobial Resistance and Molecular Epidemiology of ESBL-Producing *E. coli* in East Tennessee Dairy Farms

Abstract

The extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, such as *E. coli*, are emerging as a serious threat to global health due to their rapid spread and their multidrug-resistant (MDR) phenotypes. ESBL-*E. coli* is evidently on the rise in U.S. dairy cattle. However, limited information is available regarding the prevalence and molecular epidemiology of ESBL-*E. coli* in dairy farms. This study aimed to determine the prevalence, antimicrobial resistance, and spread mechanism of ESBL-*E. coli* and other ESBL-co-harbored resistance genes (ARGs) in East Tennessee dairy cattle farms. Rectal fecal samples from dairy cattle (n= 508) and manure, water, and feed samples (n=64) were collected from 14 farms in East Tennessee. The samples were processed, and the bacteria were isolated using CHROMagar™ ESBL. The presumptive *E. coli* isolates were confirmed by MALDI-TOF MS. Antimicrobial susceptibility testing (AST), whole genome sequencing (WGS), and in silico typing were performed on the ESBL-*E. coli* isolates. A total of 233 ESBL-*E. coli* were identified (41%, n=572) from the tested samples. The prevalence of fecal ESBL-*E. coli* was 47.5% (95% CI: 46.2 - 49.2). The most common resistance phenotypes to beta-lactam antibiotics were against ampicillin (99.1%; n=231 isolates) and ceftriaxone (98.7%; n=231). Most ESBL-*E. coli* isolates (94.4%) were MDR (resistance to ≥ 3 antimicrobial classes). Beta-lactamase genes (*bla*) were detected in 98.7% (n=233) of *E. coli* isolates. Six families of the *bla* genes were detected, including blaCTX-M, blaTEM, blaCMY-2, blaSED-1, blaLAP-2, and blaOXA-10. About 39% of ESBL-*E. coli* isolates had mutations in the *ampC* gene's promotor region. Most (97%, n=233) of the ESBL-*E. coli* isolates were genotypically MDR carrying resistance genes against nine classes of antibiotics. A47 distinct sequence types (STs), 13 unknown, and 4 novel STs of ESBL-*E. coli* were detected. Most ESBL-*E. coli* STs exhibited clustering within farms, indicating transmission between animals. About 28% (n=233) of the detected STs belonged to high-risk *E. coli* clones, including ST10 and ST69. The detection of MDR high-risk clones of ESBL-*E. coli* suggests that dairy cattle can be a reservoir for these bacteria, highlighting the importance of implementing effective control measures to reduce the associated public health risk.

Keywords: ESBL-*E. coli*, Prevalence, Dairy farm, sequence types, Multidrug resistance, resistance genes, high-risk clones

4.1 Introduction

Antimicrobial resistance (AMR) is recognized as one of the top five global public health threats of this century (11). Of great concern are extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae*, including *E. coli*, due to their rising prevalence in food-producing animals (12). Some studies identified the use of β -lactam antibiotics, especially the third-generation cephalosporins (3GC) in dairy cattle farms, as a major risk factor for the rise of ESBL-producing *Enterobacteriaceae* (13, 14).

In the United States (U.S.), β -lactam antibiotics such as 3GC (e.g., ceftiofur), first-generation cephalosporins (e.g., cephapirin), and penicillin are the top three most frequently used antibiotics in dairy cattle. These antibiotics are mainly used to treat or prevent mastitis, metritis, endometritis, lameness, pneumonia or bovine respiratory disease complex, and neonatal calf diarrhea (15-17). According to FDA's 2019 report, from 29,830 kg of cephalosporins sold and approved for use in food-producing animals, the vast majority (81%) was distributed to cattle production (18). Similarly, Nora *et al.* (19) also reported that dairy cattle use the largest amount of cephalosporins and penicillin, both of which are β -lactam antibiotics.

According to WHO's risk-based classification systems, third-generation cephalosporins are categorized as a 'critically important class of antibiotics' (20). They are used as antimicrobial drugs of choice for the treatment of severe infections caused by *E. coli* and *Salmonella* in humans (21, 22). The use of the same generation of cephalosporins in dairy cattle farms and human health settings may lead to cross-resistance to similar cephalosporins used for the treatment of human infections or vice versa if resistant bacteria transfer from carrier animals to humans through direct contact or indirectly through the food chain or environmental sources (23, 24).

In *Enterobacteriaceae*, resistance to 3GC is mainly mediated by the production of ESBLs, a group of enzymes that hydrolyze the β -lactam ring of the 3GCs (25). Among *Enterobacteriaceae*, *E. coli* is the most frequently identified bacteria carrying ESBL genes, such as *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} families (25, 26). ESBL genes are usually carried on mobile

plasmids and other unrelated genes conferring resistance to tetracycline, aminoglycosides, fluoroquinolones, sulfonamides, macrolide, and phenicols (27-32). *E. coli*, the most frequent colonizer of the gastrointestinal tracts of cattle, are frequently exposed to β -lactam antibiotics that exert selection pressure, and certain strains of *E. coli* can cause severe infections in humans (24, 33-36). Recent studies in the U.S. (23, 37-41) have increasingly reported the emergence of ESBL-producing *E. coli*, in dairy cattle since its first report in Ohio in 2010 (42), posing a significant threat to both animal and human health.

Recent reports from the U.S. Centers for Disease Control and Prevention (CDC) indicated a continuous rise in human infections caused by community-associated ESBL-producing *Enterobacteriaceae* (43). The CDC report revealed a yearly rise of approximately 9% in hospitalizations and an increase in deaths attributed to ESBL bacteria. There is speculation that dairy farms may serve as reservoirs of ESBL-producing human pathogens because of the frequent use of 3GC (44-48). The CDC has not directly implicated dairy farms as a source of ESBL-*Enterobacteriaceae* infection. However, the frequent use of 3GCs and other β -lactam antibiotics in dairy farms may play a role in the overall increase of ESBL-*Enterobacteriaceae*.

Previous studies on the U.S. dairy cattle farms showed an increasing trend in the occurrence of ESBL-*E. coli* (40, 49-53). A recent review of available literature on U.S. dairy cattle farms and recent global reviews on the status of ESBL- *Enterobacteriaceae* in cattle indicated that there is limited information on the prevalence and molecular epidemiology of ESBL-*Enterobacteriaceae*, including ESBL-*E. coli* in the U.S. dairy farms (23, 54). The prevalence of ESBL-*E. coli* and factors affecting their occurrence in dairy farms are poorly understood. Furthermore, mechanisms of transmission and spread of ESBL-*E. coli* within dairy farms, a full range of ESBL gene types, and other co-harbored resistance genes were not identified using high-resolution molecular tools. Understanding the prevalence and spread of ESBL-*E. coli* in dairy cattle farms using advanced molecular tools is crucial to informing the associated public health risks and devising effective control measures. The overall objective of this study was to determine the prevalence of ESBL-*E. coli*, identify their genetic diversity and possible spread mechanism among dairy cattle and farm environments.

4.2 Materials and Methods

4.2.1 Study Design and Sample Size

This study was approved by the University of Tennessee's Institutional Animal Care and Use Committee (IACUC) Registration Number:2782-0720. The study farms were conventional (non-organic) dairy farms in East Tennessee. A cross-sectional study was conducted in 14 dairy farms across eight counties in East Tennessee.

East Tennessee was selected due to its proximity to the University of Tennessee, and most of the dairy farms (59%, n=183) in Tennessee are found in this part of the State.

Dairy farms were randomly selected (using a random number generator) from a sampling frame of 108 dairy farms found at the beginning of this study in East Tennessee. Since some farmers may not be interested in the study, the first 60 dairy farms were randomly selected. Dairy farmers on the list were then contacted one at a time by phone and asked whether they were willing to participate in the study. When the first selected farm refused to participate or could not be contacted, the next farms on the list were contacted and asked for their willingness to participate in the study. In the end, of the contacted dairy farms (n=50), only 14 agreed to participate in the study.

To estimate the sample size, 50% expected prevalence of ESBL-*E. coli* at an animal level, 0.05 desired precision at a 95% confidence interval was used. Accordingly, the minimum required sample size was 384. To account for the possible clustering effect (design effect), the minimum sample size (n=384) was multiplied by 1.34 (design effect) (51, 55). Accordingly, 507 dairy cattle were sampled (9 to 74 animals per farm). The herd size of the farms ranges from 14 to 1700 dairy cattle. Convenience sampling was used to select an individual animal in the herd. Although an attempt was made to select the number of animals to be sampled from each farm proportional to the herd size of the respective farm, it was practically impossible because the farmers had limited time to provide animal-related information and restrain cows for sample collection. On the day of the farm visits, a brief questionnaire survey was conducted with a dairy farmer (producer) before sample collection. The questions included farm-related information such as herd size, predominant breed of cows, major disease problems in the farm, common

antibiotics used (for therapeutic and prophylactic purposes), use of a blanket dry cow therapy (BDCT) versus selective dry cow therapy (SDCT), management of waste milk (milk from antibiotic-treated cows), use of medicated milk replacer, management of manure, and type of farm (open or closed), if open the recent introduction of animals from other herds. Individual sampled animal-related information such as age, breed, parity, physiological status (dry/lactating), recent treatment history, and use of ceftiofur within the last six months before sampling were recorded.

4.2.2 Sample Collection

Following the recruitment of the farms, one-time visits were made to each farm between August 2020 and July 2021 for survey data and sample collections. Fresh rectal fecal samples were collected from individual cows and calves using sterile disposable rectal long-arm gloves. Farm environmental samples such as manure (pooled from different pen surfaces and slurry), feed (pooled feed), and water (from troughs) were collected from the study farms. Approximately 100 g of individual cow and 20 g of individual calf rectal feces were collected into 50 mL sterile falcon tubes (Thermo Fischer Scientific, Waltham, MA, USA) and labeled with individual animal IDs. Similarly, about 200 g of manure and feed samples and 50 mL of water from water troughs were collected into 50 mL sterile tubes. The samples were immediately placed on ice in an icebox, transported to the Lab, and processed in less than 24 h of collection.

The study recruited 14 dairy farms (designated A-N) across eight counties in East Tennessee (Table 4.1, placed under appendix). A total of 424 dairy cows (average parity: 2.3 lactations, range: 1 to 8 lactations; average age: 3.9 years, range: 2 to 13 years) and 84 calves (average: 2.7 months old, range: 0.4 to 8 months). A pooled manure (n=30), pooled water samples (n=19) from water troughs, and pooled feed samples (n=15) were collected from the farms (Table 4.1). Two samples (from farm N), one collected from a lagoon and the other collected from a runoff from the farm to the environment, were included as manure. A feed sample was not collected from one of the farms (Farm M) as the farmer was less comfortable. One of the two collected feed samples in farm G was a liquid whey fed to cattle.

4.2.3 Laboratory Analysis of Collected Samples

In the laboratory, 10 g of individual animal rectal feces, feed, and manure samples were combined with 90 mL of Tryptic soy broth (TSB-PO₄, MG Scientific, Pleasant Prairie, WI, USA) in a Whirl-Pak bag (Whirl-Pak, Pleasant Prairie, WI, USA) and massaged by hand for 15-20 sec to mix the samples with the TSB-PO₄. For water samples, 20 mL of sample was mixed with 80 mL TSB-PO₄. The homogenized sample was placed at room temperature for about 2 h. Then, 50 µL of the homogenized sample was spirally plated on CHROMagar ESBL plates (DRG International Inc., Springfield, NJ, USA) and incubated at 37°C for 24 h to isolate ESBL-*E. coli* and -*Klebsiella* spp. Presumptive ESBL-*E. coli* (dark pink to reddish) colonies were subcultured onto a new CHROMagar ESBL plate and incubated for another 24 h at 37°C. Well-isolated pure colonies were transferred to 1 mL Luria-Bertani broth (LBB) (Thermo Fischer Scientific) into a 2 mL 96 well-serum block and incubated at 37°C for 24 h. A 0.5 mL of the culture was transferred to a new 96-well serum block, mixed with an equal volume of sterile 80% glycerol, and stored at -80°C for further analysis.

4.2.4 Sample Preparation and MALDI-TOF MS Based *E. coli* Identification

Presumptive *E. coli* isolates stored at -80°C were thawed and plated on CHROMagar ESBL plates and incubated at 37°C for about 18 h. A single colony of *E. coli* was picked and subcultured on blood agar (Thermo Fischer Scientific) at 37°C for about 18 h. Subsequently, *E. coli* was identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described by the manufacturer (Bruker Daltonics, Billerica, MA, USA) at the University of Tennessee, College of Veterinary Medicine, Diagnostic Bacteriology and Mycology Lab. The samples were prepared using a formic acid (FA) extraction method (56). Briefly, a small amount of the bacteria from a single fresh colony was picked using the sterile wire loop or toothpick and directly smeared onto a duplicate target spot or well on the MALDI-TOF MS steel anchor plate (Bruker Daltonics). The smear was allowed to air dry for about 5 min. A 1 µL of 70% FA was added to the smeared samples in each well (Sigma-Aldrich, St. Louis, MO, USA) and allowed to dry for about 5 min. After drying, the sample was overlaid with 1 µL of the matrix (α -cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics) to form

crystals. Following matrix addition, samples were allowed to air dry for about 5 min before placing them inside the Biotyper (Bruker Daltonics) for MALDI-TOF MS analysis.

With each run, 1µL of the Bacterial Test Standard (BTS; Bruker Daltonics) overlaid with matrix was added on the plates' first two spots; negative controls (formic acid and matrix) were added at the end of the sample wells. The barcode on a plate containing the dried sample was scanned, and the plate was placed inside the MALDI Biotyper (Bruker Daltonics). The data relating to the isolate was entered at appropriate slots of MBT (MALDI Biotyper) Compass software (Bruker Daltonics). Then, the Biotyper was allowed to run once the vacuum status was adjusted and ready. The MALDI Biotyper library version 11.0 and MALDI Biotyper software version 4.1.100 (Bruker Daltonics) were used for this analysis. For identifying *E. coli*, the manufacturer-recommended cutoff score values of 2.0 to 3.00, which is considered high-confidence identification, were used.

4.2.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed on MALDI-TOF MS confirmed *E. coli* isolates against 14 antimicrobials representing ten classes of antimicrobials using the broth microdilution method. Commercially available 96-well microtiter plates containing the 14 antimicrobial panels (Sensitre™ CMV4AGNF) (Thermo Fisher Scientific) were used. The 14 antimicrobials in the 96-well plates include β -lactams (ampicillin, ceftriaxone, cefoxitin, amoxicillin-clavulanic acid, meropenem), aminoglycosides (gentamicin and streptomycin), folate pathway inhibitors (sulfisoxazole, trimethoprim/sulfamethoxazole), macrolides (azithromycin), phenicols (chloramphenicol), quinolones (ciprofloxacin and nalidixic acid), and tetracyclines. The minimum inhibitory concentrations (MIC) of the 14 antimicrobials were determined following the manufacturer's recommended protocol and following CLSI M100 Clinical and Laboratory Standards Institute guidelines (CLSI M100: *Enterobacteriaceae*) (57) and summarized below (Table 4.2, placed under appendix). Briefly, 3 - 5 colonies of pure overnight bacterial culture were picked with a sterile swab and emulsified in 5 mL of demineralized water. The bacterial suspension was placed in DenseCHECK Plus (bioMerieux Inc., Durham, NC, USA) to confirm 0.5 McFarland. A 10 µL of the bacterial suspension was transferred to 11 mL

of Sensitire™ Cation Adjusted Muller-Hinton Broth (MHB) (Thermo Fisher Scientific, Lenexa, KS, USA). The tube was vortexed multiple times to homogenize the bacterial suspension. The bacterial suspension in MHB was transferred into a designated reservoir. From the reservoir, a multichannel pipette was used to inoculate 50 µL of the inoculum suspension into the 96-well plates containing the control and various concentration of the antimicrobials. The plates were covered with an adhesive seal and incubated at 37 °C for 16 - 20 h.

For quality control, immediately following the inoculation of the plates, using a 1µL loop, the inoculum suspension in the reservoir was streaked onto blood agar (Thermo Fischer Scientific) to check for purity and growth. Test results were valid only if a pure growth was detected on the blood agar. In addition, the Sensitire™ CMV4AGNF plates (Thermo Fisher Scientific) have positive and negative control wells. Test results were valid only when distinct growth was observed in the positive control and no growth in the negative control well at the end of the incubation period. *Escherichia coli* ATCC 25922 was used as a daily control.

After 16 - 20 h, the plates were removed from the incubators and placed inside the V3 image analysis (Giles Scientific, Santa Barbara, CA, USA). The results were automatically read and interpreted using the BIOMIC V3 Microbiologic system (Giles Scientific). The results were reported as susceptible (S), Intermediate (I), and Resistant (R) along with the MIC values, the lowest concentration (µg/mL) of an antibiotic that completely inhibits visible growth of the tested isolates using CLSI M100 guidelines for interpretation. A Sensititre Manual reader (Thermo Fischer scientific, Oakwood Village, OH, USA), was used when the image produced by V3 image analysis was not clear enough for interpretation.

An MIC breakpoint was not available on CLSI M100 guidelines for streptomycin and, thus, National Antimicrobial Resistance Monitoring System (NARMS) interpretive criteria (breakpoints) for *E. coli* antimicrobial susceptibility testing (<https://www.cdc.gov/narms/antibiotics-tested.html>) was used. Accordingly, an MIC ≥ 32 µg/mL was defined as resistant to streptomycin. For sulfisoxazole, CLSI defines an MIC ≤ 256 µg/mL as susceptible and an MIC ≥ 512 mL as resistant, and there are no breakpoints for intermediate resistance. Thus, *E. coli* isolates that have an MIC ≥ 256 µg/mL were reported as

resistant to sulfisoxazole in this study. In addition, for analyses, a few *E. coli* isolates that displayed intermediate resistance, mostly to ciprofloxacin, were recategorized as resistant. Multidrug resistance (MDR) was defined as acquired resistance to at least one antimicrobial agent in three or more antimicrobial classes (58).

4.2.6 DNA Extraction

The MALDI-TOF MS confirmed, and AST tested *E. coli* isolates stored at -80°C were thawed and plated on CHROMagar ESBL plates. After overnight incubation, 2 - 3 colonies were picked with a sterile wire loop, subcultured into 5 mL LBB and incubated at 37°C with shaking at 200 rpm for 18 - 24 h. Bacterial DNA was extracted using the Qiagen MagAttract HMW DNA kit (Qiagen, Germantown, MD, USA), a magnetic based-protocol that involves four main steps following the manufacturer procedure. These are lysing, binding, washing, and eluting. The detailed procedure is slightly modified as described below. Briefly, 2 - 3 bacterial colonies were taken from a pure overnight culture using a sterile wire loop and dissolved into 5 mL LBB (Thermo Fisher Scientific). The broth was incubated overnight at 37°C, and 2 mL was transferred into the 2 mL microcentrifuge tube. The tube was placed in a centrifuge (Thermo Fisher Scientific) and centrifuged at 5000 x g for 10 min. Then, the supernatant was discarded, and the pellet was suspended in a lysis solution, placed in shaker-incubator series (VWR International, Philadelphia, PA, USA), and shaken at 500 rpm for 1.25 h at 57°C. After adding 4 µL RNase and incubating for 2 min at room temperature, 15 µL magnetic beads and 280 µL binding buffer (Buffer MB) were added to the lysate solution and pulse vortexed and placed in a MagAttract magnetic rack (Qiagen) and shaken in a shaker-incubator series (VWR International) at 500 rpm for 15 min. The Magnetic Rack was placed on the magnetic base so that the magnetic beads, along with the DNA, would get separated by sticking to the side of the tube towards the magnetic base, and the supernatant was removed. The magnetic beads (together with the DNA samples attached) were washed twice each by Buffer MW1 and Buffer PE (Qiagen). The magnetic beads were rinsed twice with distilled water while the tube holder was on the magnetic base, and the beads were fixed to the walls of the microcentrifuge tube. Then, the tube holder of the Magnetic Rack was removed from its magnetic base, and 150 µL Buffer AE (elution buffer) (Qiagen) was added. The tube holder of the Magnetic Rack was placed in

incubator-shaker series (VWR International) and incubated at room temperature for 15 min at 500 rpm. The tube holder of the Magnetic Rack was placed on its magnetic base to separate the beads from the supernatant. Finally, the supernatant (elute) that was expected to contain high-molecular-weight DNA was transferred into a new sample tube. DNA concentration and purity were measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

4.2.7 Library Preparation and Whole Genome Sequencing

Genomic libraries were prepared using the Nextera XT DNA Library prep kit (Illumina Corporation, San Diego, CA, USA) following the manufacturer's instructions. Final concentrations were confirmed on a Qubit fluorometer (Thermo Fischer Scientific). Some libraries were checked for size and concentration on a high-sensitivity Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were normalized and pooled, and a final quality confirmation was performed on a Bioanalyzer (Agilent Technologies). The pooled samples were diluted to a final loading concentration of 0.5 nM (nanomole) and combined with 5% PhiX control (Illumina Corporation). The pooled sample and PhiX were then loaded on one lane of an SP flow cell reading 250 nucleotides paired-end (2 X 250) on the Illumina NovaSeq (Illumina Corporation). The DNA library was sequenced using Illumina NovaSeq PE250 at the University of Tennessee Genomics Core (Knoxville, TN, USA).

4.2.8 Whole Genome Sequence Data Analyses

To analyze the entire genome sequence data, first, the sequence data was demultiplexed. Then, FastQC v0.11.9 (59) and multiQC v1.14 (60) were used to assess the quality. Trimmomatic v0.39 was applied to remove adapters, filter out, and trim raw reads with low quality (61). After preprocessing, the raw reads were de novo assembled using the default settings of the SPAdes genome assembler v3.15.5 (62). The coverage, N50, number of scaffolds, maximum scaffold length, and percentage of predicted genome size were calculated for each assembled genome. Accordingly, the coverage (the mean depth) for all isolates varied from 25 to 227, the mean total scaffold count was 653, and the mean scaffold sequence total (bp) was 4547436.

To identify acquired antimicrobial resistance genes (ARGs) or mutations leading to resistance, we uploaded the assembled genomes of the isolates to the online bioinformatics tools found at the Center for Genomic Epidemiology (CGE) at the Technical University of Denmark, <https://www.genomicepidemiology.org/>). We used ResFinder version 4.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>) (63, 64) and *E. coli* specific panels to detect ESBL and other ARGs for in silico antibiograms [6]. ESBL-encoding genes and other resistance determinants were considered present when at least 60% of the length of the best-matching gene in the ResFinder database was covered with a sequence identity greater than or equal to 95% (63). In some cases, Comprehensive Antimicrobial Resistance Database (CARD) (<https://card.mcmaster.ca/analyze/rgi>) was used in addition to CGE (65).

The multilocus sequence types (MLST) of ESBL-*E. coli* were identified from the draft genome of the bacteria according to the Achtman seven-locus MLST approach, which is based on the sequences of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (66, 67). STs were identified from the assembled genome using a web-based bioinformatic pipeline, MLST 2.0.9 (<https://cge.food.dtu.dk/services/MLST/>) [(67, 68). The best matching MLST allele with 100% coverage and identity was chosen, and the ST of each ESBL-*E. coli* was determined based on the combination of MLST alleles.

Phylogenetic analyses of the ESBL-*E. coli* isolates were automated using GToTree (version 1.7.08) (69). A set of 172 Gammaproteobacterial single-copy genes was searched against the genome assemblies. Reconstructed gene lengths that varied from the median gene length by 20% or more were filtered from the analysis on a per-sample basis. IQ-TREE (version 2.2.0.3) was used to create a consensus tree by automated mixed-model selection and 1000 bootstrap replicates (70). Tree visualizations were produced using ggtree (71).

4.2.9 Statistical Data Analyses

Raw data were entered in Microsoft Excel for Windows (2010, Microsoft Corp., Redmond, WA). Data were imported to SPSS for Windows and analyzed using IBM SPSS Statistics for Windows Version 27.0. (IBM Corp, Armonk, NY, USA). For all statistical analyses, the unit of

analysis was the sample obtained from a given source (cow, calf, manure, water, and feed), the farms, and the bacterial isolates. Descriptive statistics were done to summarize the animal and farm-level prevalence of ESBL-*E. coli* and the prevalence of the bacteria from different sources (fecal, water, feed, and manure samples) of the farms. The individual animal was classified as ESBL-positive when ESBL-*E. coli* isolates were identified phenotypically on the Chromagar ESBL plate and confirmed by MALDI-TOF MS (50, 60) in the feces of the dairy cow or calf and the other samples.

A farm (herd) was classified as ESBL-positive when ESBL-*E. coli* is detected at least from one animal in the herd. Descriptive statistics were used to calculate the prevalence of ESBL-*E. coli* as the number of samples tested positive for ESBL-*E. coli* divided by the total number of samples tested for each sample category. Sampling weight was used to calculate the within-herd and individual animal level prevalence to account for the unequal probability of sampling employed during the sample collection. The weighted sample for a specific farm was determined by dividing the actual number of animals sampled on the farm by the herd size of that farm. The reciprocal of this quotient was then taken as the weighted sample for the farm. To calculate the overall weighted prevalence, the unweighted prevalence of ESBL-*E. coli* in each of the 14 herds (farms) was multiplied by the corresponding herd proportion. These products were then summed up, and the result was divided by 100. A univariate and multivariable mixed-effects logistic regression model, which accounts for the clustering effect of cattle or isolates within farms, was used in the analysis. A mixed effects logistic regression analysis (weighted) was performed to evaluate the association between selected predictors (farm or animal level data) and the odds of ESBL-*E. coli* fecal carriage (dependent variable) in cattle.

In addition, the multidrug resistance (MDR) status of each bacterial isolate obtained from the study was treated as a dichotomous outcome variable (MDR vs. non-MDR), and their possible variation between sample sources was evaluated. The MDR isolates were further dichotomized (whether they were resistant to at least six classes of antibiotics or not) and assessed if there was a variation between the sample sources. A Pearson χ^2 or Fisher exact test (as appropriate) was used to test the association between different categorical variables (ignoring clustering) when the

mixed effect model fails to converge. A p-value of 0.05 was used for all statistical analyses to determine the significance level.

4.3 Results

4.3.1 Questionnaire Survey Results

The questionnaire survey showed that half of the farms practice closed production systems where replacement heifers originated from the same farm, whereas the other half buy replacement heifers/cows from outside sources in addition to raising their own. Thirteen of the fourteen participating dairy farms use cow manure as fertilizer, mainly on pasture, and one farm (Farm G) uses manure for biogas production. Most of the farms (71%) discard waste milk from cows on antibiotic treatment (dump down the drain or dump on the pasture field), whereas the remaining four farms (Farm C, F, G, and I) feed it to calves. A blanket dry cow treatment program was routinely practiced in all the farms except two, which used selective dry cow therapy and teat sealant. The most frequently used antibiotics for dry cow therapy were Spectromast[®] DC (ceftiofur hydrochloride), toMORROW (cephapirin benzathine), and Quartermaster (procaine penicillin G and dihydrostreptomycin). Eleven farms predominantly have Holstein-breed cows, whereas Jersey cows dominate the remaining three. The herd size of the 14 farms ranged from 14 to 1700 cattle, with a median herd size of 750 animals.

As expected, all farmers mentioned mastitis as the most frequent herd health problem, followed by hoof problems. Beta-lactam antibiotics (penicillin and cephalosporins) were the most common antibiotics used in all farms. All study farms frequently use different generations and formulations of cephalosporins. Major disease problems and frequently used antibiotics in each farm are listed in Table 4.3(placed under the appendix)

4.3.2 Microbiological Result

According to CHROMagar[™] ESBL result, a total of 306 presumptive ESBL-*E. coli* was isolated from rectal feces, manure, water, and feed samples. Out of 306 presumptive ESBL-*E. coli* isolates, 233 (76%) were confirmed to be *E. coli* by MALDI TOF MS. The remaining bacteria include *Citrobacter sedlakii* (n=69 isolates); *Citrobacter freundii* (n=2) and *Acinetobacter*

baumanii and *Enterococcus faecalis* one each. Except for AST, which was conducted on 231 isolates, all statistical analyses and inferences were based on the 233 *E. coli* isolates. Most of the *E. coli* isolates (90 %, n=233) were isolated and identified from rectal fecal samples, whereas the remaining (10%) were from farm environmental samples (manure, water, and feed samples).

4.3.3 Prevalence of ESBL-Producing *E. coli* Isolates

The weighted prevalence of fecal ESBL-*E. coli* in dairy cattle was 47.5% (95% CI: 46.2 - 49.2). ESBL-*E. coli* was detected from all farms. The prevalence varies widely between farms, with the highest within-herd weighted prevalence of 100% from farm A, whereas the least prevalence of 8% was from two farms (farms E and N). ESBL-*E. coli* was isolated from fecal samples of all fourteen farms, resulting in a herd-level prevalence of 100%. The herd size of the farms, the number of animals tested from each farm, the number of animals that tested positive and within-herd weighted prevalence, and the 95 % confidence interval (95% CI) of the estimate for each farm are presented in Table 4.4 (placed under appendix).

The Chi-Square (χ^2) test of independence was performed (without accounting for within farm clustering effect of the isolates) to test if there was a statistically significant difference in the weighted prevalence of ESBL-*E. coli* between farms. The test showed an overall statistically significant variation ($\chi^2=21$; $p<0.001$) between the within-herd prevalence of ESBL-*E. coli*. Similarly, a mixed-effect logistic regression (controlling the random effect of the farm) showed an overall significant variation in the fecal ESBL-*E. coli* prevalence between farms ($p<0.001$). A comparison of the within-herd prevalence of fecal ESBL- *E. coli* between individual farms is shown in Figure 4.1(placed under the appendix).

The weighted prevalence of fecal ESBL-*E. coli* was 44% and 61.7% in cows and calves, respectively. Calves were twice [OR = 2.12 (95% CI: 1.78 - 2.53), $p<0.001$] more likely to carry ESBL-*E. coli* in their feces compared to cows using mixed effect logistic regression analysis with farms as a random variable and herd structure (calf vs. cow) as the sole fixed variable (Table 4. 5, placed under appendix).

ESBL-*E. coli* was recovered from over a quarter of all sample types (26%). The highest proportion of ESBL-*E. coli* was obtained from pooled manure samples (46.7%), whereas the least (26.3%) was from water samples (Table 4. 6, placed under appendix).

4.3.4 Factors Affecting Fecal ESBL- *E. coli* Prevalence in Dairy Cattle

After matching the farm-level survey data with the findings of the MALDI-TOF results from individual animals, a mixed-effect logistic regression analysis was carried out to determine the association between the variables collected during the survey and the probability of isolating ESBL-*E. coli* in samples. The farm-level collected data include herd size, farm type (closed vs. open), predominant breed in the farm, use of blanket dry cow therapy (BDCT) vs. selective dry cow therapy (SDCT), types of antibiotics used for DCT, presence of treatment ward in the farm, management of waste milk from treated cows (discard vs. feed to calves). None of these farm-level variables were associated with the detection of ESBL-*E. coli* in fecal samples, in univariate and multivariable analysis ($P > 0.05$), and, thus, were not included in this report.

The relationship between individual animal-related surveys such as the recent (up to six months prior to sampling collection) use of beta-lactam antibiotics such as ceftiofur, parity, lactation status of the cows (lactating vs. dry or non-lactating), age (of cows), and the breed was assessed for possible association with fecal ESBL- *E. coli* carriage. First, a univariate analysis was conducted to test the association between independent variables and the likelihood of isolating ESBL-*E. coli* in fecal samples. Then, only those variables with a P-value ≤ 0.2 in univariate analyses were used in a multivariable analysis controlling the random effect of the farm. Overall, recent treatment with ceftiofur, cow parity, and the lactation status of the cows was independently associated ($p < 0.001$) with the prevalence of fecal ESBL-*E. coli*. The odds of ESBL-*E. coli* fecal carriage in cows recently treated with ceftiofur was higher than [$OR_{adj} = 1.35$ (95% CI: 1.1-1.65), $p = 0.004$] those which did not receive the treatment. Lactating cows were more than three times [$OR_{adj} = 3.42$ (95% CI: 2.79 - 4.10), $p < 0.001$] more likely to carry ESBL-*E. coli* in their feces compared to dry cows.

Age and parity of the cows were highly correlated ($p < 0.001$; $R=0.73$) since parity increases with age. Thus, the age of the cows was not included in the analysis, and parity was retained in the model. Odds of retrieving ESBL-*E. coli* was 33% higher ([OR_{adj} = 1.33 (95% CI: 1.13 -1.63), $p=0.01$] in cows with parity greater than two compared to those cows with parity less than or equal to two (Table 4. 7, placed under appendix).

4.3.5 Antibiotic Resistance Profile of ESBL-*E. coli* Isolates from All Sample Type

Overall, 230 (99.6%) of 231 *E. coli* tested for phenotypic resistance were resistant to at least one of the 14 antimicrobial agents tested (Table 4. 8, placed under appendix). One isolate was susceptible to all tested antimicrobials. As expected, the most common resistance phenotypes were against beta-lactam antibiotics, ampicillin (99.1%; $n=231$), and ceftriaxone (98.7%, $n=231$). Only three isolates showed susceptibility to ceftriaxone; two of them were recovered from cows. After ceftriaxone and ampicillin, the isolates showed the highest resistance to tetracycline (80.1%), sulfisoxazole (60.2%), streptomycin (55%), and chloramphenicol (46.8 %).

Among the critically important classes of antibiotics, the higher level of resistance after ceftriaxone was to streptomycin (55 %), followed by ciprofloxacin (18.6%) and azithromycin (13.4%). The prevalence of resistance to gentamycin and nalidixic acid, the other critically important antimicrobials tested in this study, were 10% and 9%, respectively.

The resistance level to other β -lactam antibiotics was 10 for ceftiofur and 9.2% for amoxicillin-clavulanic acid. All ESBL-*E. coli* isolates in this study were susceptible to meropenem.

4.3.6 Multidrug Resistance Profile of ESBL-Producing *E. coli* Isolates

As expected, most of the ESBL-*E. coli* isolates (94.4%; 218/231) displayed multidrug resistance (MDR) phenotypes (acquired resistance to at least one agent in three or more antimicrobial classes) (58). All isolates recovered from manure ($n=5$) and water samples ($n=5$) showed MDR phenotypes. Most of the isolates from calves (96.3%; $n=27$), cows (93.9%; $n=180$), and feed (80%; $n=4$) were MDR. Among the 14 farms, all isolates obtained from 10 of the farms were MDR. From 218 MDR isolates, 42.6% showed concurrent resistance to at least one antibiotic in

six classes of antibiotics. ESBL-*E. coli* isolates that showed concurrent resistance to ≥ 6 antimicrobial classes were obtained from all sample sources (Table 4. 9, placed under appendix).

4.3.7 Distribution of ESBL-*E. coli* Resistant to Multiple Antimicrobial Agents

Overall, resistance to multiple classes of antibiotics is widespread across the study farms (Table 4.10, placed under the appendix). The most common (52.8%, 122/231) and widespread multidrug resistance pattern was concurrent resistance to ceftriaxone, ampicillin, streptomycin, and tetracycline, detected in all farms and sample types. Similarly, simultaneous resistance to ceftriaxone, ampicillin, sulfisoxazole, and tetracycline was frequent (48.5%, 112/231) and detected in all farms and sample types. Concurrent resistance to at least one antimicrobial agent in all critically important classes of antibiotics (ceftriaxone, streptomycin, azithromycin, and ciprofloxacin) was relatively less frequent (6.5%, 15/231) and limited in the scope of spread among farms (detected in only three farms). Fourteen of fifteen *E. coli* isolates with this resistance pattern were retrieved from cow fecal samples. Seventeen (7.4%) of *E. coli* isolates were co-resistant to all beta-lactam antibiotics tested in this study (ceftriaxone, ampicillin, cefoxitin, and amoxicillin-clavulanic acid). *E. coli* isolates with this co-resistance phenotype were detected in six farms, and all of them were isolated from fecal samples except one recovered from a feed sample.

4.3.8 Whole Genome Sequence Result of *E. coli* Speciation

All 233 ESBL-*E. coli* isolates identified by MALDI-TOF MS were subjected to Whole Genome Sequencing (WGS). Analysis of WGS confirmed that all 233 *E. coli* were indeed *E. coli* species using KmerFinder(<https://cge.food.dtu.dk/services/KmerFinder/>). Thus, further genomic analysis was carried out on all 233 *E. coli* isolates.

4.3.9 Genetic Determinants of Resistance to Beta-Lactam Antibiotics

All except four isolates (98.3%, 229/233) had at least one beta-lactamase encoding gene. Six families of beta-lactamase genes were detected at different frequencies in the *E. coli* genomes. These include *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{CMY-2}, *bla*_{SED}, *bla*_{LAP-2}, and *bla*_{OXA}. The *bla*_{CTX-M} gene was most prevalent and detected in 222 isolates (95.4%), followed by *bla*_{TEM} and *bla*_{CMY-2}, which

were detected in 122 (52.4%) and 18 (7.7%) isolates, respectively. Most isolates (72%) carrying the *bla*_{CMY-2} gene (n=18) were obtained from the same farm. Nearly half of the isolates carrying the *bla*_{CMY-2} gene did not harbor ESBL gene variants, and these eight isolates had mutations in the promotor regions of the *ampC* gene. The *bla*_{SED-1} was detected in four isolates, whereas *bla*_{LAP-2} and *bla*_{OXA} were detected in one isolate each. The *bla*_{SHV} was not detected in any *E. coli* isolates.

Among 29 *bla*_{CTX-M} variants detected, *bla*_{CTX-M-65}, *bla*_{CTX-M-32}, and *bla*_{CTX-M-15} were most frequently seen in 67, 61, and 49 ESBL-*E. coli* isolates. Other common *bla*_{CTX-M} variants include *bla*_{CTX-M-1} and *bla*_{CTX-M-27}, detected in 27 and 25 isolates, respectively. Nearly half, 48% (112/233) of the ESBL-*E. coli* isolates co-harbored *bla*_{CTX-M} and *bla*_{TEM} genes (the *bla*_{CTX-M} - *bla*_{TEM-1A} / *bla*_{TEM-1B} gene combinations were common). Among *bla*_{TEM}, the most frequent variants were *bla*_{TEM-1A} and *bla*_{TEM-1B}, detected in 60 and 55 isolates, respectively. The *bla*_{TEM} gene was the most diverse, and 37 variants of these genes were detected in the *E. coli* genomes in this study. Beta-lactamase inhibitor-resistant (BIR) *bla*_{TEM} variants such as *bla*_{TEM} (-33, -34, -40, -82, -97, -99, -122, and -163) were also detected in the ESBL-*E. coli* genomes. Other less frequently detected *bla*_{CTX-M} and *bla*_{TEM} variants are shown in Table 4.11 (placed under the appendix). In addition to detecting these beta-lactamase genes, a mutation in promotor regions of the *ampC* gene was detected in the genome of about 39% (90/233) of the ESBL-*E. coli* isolates.

4.3.10 Detection of Other Acquired Resistance Genes in ESBL- *E. coli*

In addition to beta-lactamase genes, ESBL-*E. coli* were found to harbor resistance genes against nine distinct classes of antibiotics in their genomes (Table 4.12, placed under the appendix). Most (97%) of ESBL-*E. coli* isolates were genotypically multidrug resistant (MDR), containing resistance genes for as many as eight categories of antibiotics. These included resistance genes against three critically important classes of antibiotics, namely (fluoro)quinolones, macrolides, and aminoglycosides, as well as other important classes of antibiotics such as folate pathway inhibitors, tetracycline, chloramphenicol, lincosamides, rifamycins, and fosfomycin.

Most of the isolates carried ARGs against critically important antibiotics (CIAs). Multiple types and subtypes of resistance genes against specific classes of antibiotics were detected. For instance, two types (*qnrB* and *qnrS*) and twelve subtypes of plasmid-mediated quinolone resistance genes (*qnr*) were detected in ESBL-*E. coli*. Among the *qnr* genes, three *qnrB* subtypes (*qnrB19*, *qnrB5*, and *qnrB81*) and nine *qnrS* subtypes (*qnrS7*, *qnrS1*, *qnrS3*, *qnrS12*, *qnrS13*, *qnrS8*, *qnrS11*, *qnrS10*, and *qnrS4*) were identified.

A large proportion of ESBL-*E. coli* (77.3%, 180/233) contained at least one *qnr* gene, one of the most common genes co-harbored with ESBL genes. Of the *qnr* genes, *qnrB19* and *qnrS1* were the most frequently detected in 168 (72%) and 54 (23%) isolates, respectively. Both *qnrB19* and one or more variants of *qnrS* genes co-occurred in 44 (19.8%) isolates. Additionally, in most ESBL-*E. coli* isolates, one or more-point mutations in the "quinolone resistance-determining regions" (QRDRs), such as *parC*, *gyrA*, *gyrB*, and *parE*, were detected. Among these mutations, *parC* and *gyrA* were the most frequently detected in 229 (98.3%) and 109 (47%) isolates, respectively.

A high proportion (55.8%, 130/233) of ESBL-*E. coli* isolates were found to possess one or more aminoglycoside resistance genes. Four different types (*aadA*, *aph(6)*-Id, *aac(3)*-VIa, and *rmtE*) and ten subtypes of aminoglycosides resistance genes were detected. The Aminoglycoside-phosphotransferase genes, *aph(6)*-Id and *aph(3)*"-Ib), were most frequently detected in 95 and 91 isolates, respectively. Both resistance genes were found to co-occur in 90 isolates. More than 85% (110/130) of the isolates bearing aminoglycoside resistance genes had two or more types of aminoglycoside phosphotransferase (*aph*) genes. Additionally, 30% (70/233) of ESBL-*E. coli* isolates were found to have the macrolide resistance gene, *mph(A)*.

The tetracycline resistance genes were the most frequently detected among the ESBL genes and were found to co-exist with ESBL genes in 80% (186/233) of the isolates. Three tetracycline resistance genes, *tet(A)*, *tet(B)*, and *tet(M)*, were detected, with *tet(A)* being the most frequently detected gene in 76% of the isolates. Over half (51.5%, 120/233) of the ESBL-*E. coli* isolates had chloramphenicol resistance genes, particularly *floR*. Additionally, 75% (175/233) of the isolates had resistance genes to folate pathway inhibitors. Two types of genes, including *sul* and

dfrA, as well as three subtypes of *sul* genes (*sul1*, *sul2*, and *sul3*), and seven subtypes of *dfrA* genes (*dfrA1*, *dfrA8*, *dfrA12*, *dfrA14*, *dfrA17*, *dfrA23*, and *dfrA24*) were detected. In 48% (84/175) of ESBL-*E. coli* isolates that harbored these genes, at least two *sul* genes and/or *dfrA* genes co-occurred. The most common resistance genes against folate pathway inhibitors were *sul2* and *dfrA*, found in 61% (142/233) and 34% (79/233) of ESBL-*E. coli* isolates, respectively. Lincosamide resistance genes, *lnu(F)*, were identified in 10.7% (25/233) of the isolates. Only one isolate was found to have the rifampicin (*arr-2*) and Fosfomycin (*fosA*) resistance genes.

Isolates originating from the same farm tend to display similar patterns of resistance genes. For example, all 14 ESBL-*E. coli* isolates obtained from Farm A exhibited an identical genotypic resistance pattern. They all harbored *bla*_{CTX-M-32}, a macrolide *mph(A)*, a fluoroquinolone (*qnrB19*), an aminoglycoside (*aadA1*), a tetracycline *tet(A)* and a chloramphenicol (*floR*) resistance genes and folate pathway inhibitor resistance genes (*sul1* and *dfrA1*), as well as point mutations in *parC* and *gyrA* genes.

4.3.11 Genetic Diversity of ESBL-*E. coli* and Their Spread Mechanisms in Dairy Farms

Multilocus sequence typing identified 47 distinct sequence types (STs), 13 unknown, and 4 novel STs of the ESBL-*E. coli* isolates. Of the STs identified, 20 were isolated from at least two samples, while the remaining 27 were only detected once. The most frequently detected STs were ST2325, ST9967, ST10, ST540, and ST1423. The 27 STs that were detected only once include ST23, ST46, ST48, ST69, ST101, ST156, ST165, ST192, ST225, ST306, ST683, ST747, ST1408, ST1415, ST2521, ST2717, ST5416, ST5708, ST5730, ST6732, ST8850, ST10269, and ST2600, as well as the four novel STs, which had distinct novel alleles of the housekeeping genes. Common ESBL-*E. coli* STs and *bla*_{CTX-M} gene, the most frequent *bla* gene variant detected in the ESBL-*E. coli* was presented in Table 4.13 (placed under the appendix).

Most ESBL-*E. coli* STs showed clustering within farms, but some were widespread and detected in multiple farms. Most of the isolates from Farms C, F, H, and K were dominated by specific STs of *E. coli* and *bla*_{CTX-M} variants. Furthermore, some STs (e.g., ST1011, ST5044, ST1423, and ST7585) were associated with specific *bla*_{CTX-M} variants and restricted to a single farm. On

the other hand, some *E. coli* STs, such as ST2325 and ST10, were detected in multiple farms. The genotypic resistance pattern of these STs is the same within a farm but tends to differ between farms. For instance, in Farm A, all ESBL-*E. coli* ST2325 (n=14 isolates) harbored the same *bla*_{CTX-M-32} variant and other resistance genes, but the same STs carried *bla*_{CTX-M-27} or both *bla*_{CTX-M-27} and -15 (n=6) in Farm M, and *bla*_{CTX-M-15} in farm N (n=4).

Interestingly, multiple distinct STs of ESBL-*E. coli* in a farm were also found to harbor the same or clonal variant of *bla*_{CTX-M}. In a few farms, such as C, the same STs, ST9967, were obtained from rectal fecal samples and water samples collected from water troughs.

The phylogenetic tree shows multiple clustering of isolates within farms. Some isolates from a different farm also clustered together, showing the possible within-farm spread of the bacteria or simply parallel evolution. Isolates labeled with the same colors were obtained from the same farm. The letters indicate the farm and county of its location, while the numbers represent the unique ID of individual animals on the farm (except manure, feed, and water samples) (Figure 4.2, placed under the appendix).

4.3.12 ESBL-*E. coli* Sequence Types Belonging to High-Risk Clones

Among 233 ESBL-*E. coli* strains circulating in the dairy farms, 27.5% (64/233) belonged to high-risk clonal lineages. The high-risk sequence types identified in this study belonged to 14 distinct sequence types. These includes ST10 (22 isolates), ST117 (7), ST224 (7), ST1011 (7), ST398 (6), ST155 (5), ST410 (2), ST58 (2), ST23, ST48, ST69, ST101, ST165, and ST156. All these STs were genotypically MDR. Among these strains, ST117 (n=5) and ST58 (n=1) had no *bla*_{CTX-M} gene. However, the ST117 co-carried *bla*_{TEM} and *bla*_{CMY-2}, whereas the ST58 carried chromosomal mutations in the promoter region of the *ampC* gene.

4.4 Discussion

In this study, all farms identified mastitis as the most frequent health problem of dairy cows and a primary driving factor for antibiotic use. This finding is consistent with other studies in the U.S. that also reported mastitis as the most common disease and the main reason for antibiotics use in dairy farms (72-76). Similar to previous studies in the U.S. (77, 78), most (86%, n=14) of

the farms in the present study used blanket dry cow therapy to manage mastitis. This practice may expose many animals and their gut commensal bacteria to antibiotics as portions of the administered dose or metabolites may enter the bloodstream and reach the gut (79).

Beta-lactam antibiotics such as cephalosporins (ceftiofur, cephalixin benzathine, and cephalixin sodium) and penicillin were the most frequently used antibiotics in the study farms. This finding concurs with a recent study by Nora et al. (19), who reported the consumption of large quantities of cephalosporins and penicillin in dairy cattle farms compared to other antibiotics. Similarly, the latest USDA survey reports (77, 80) and two other studies in the U.S. (72, 81) also showed that cephalosporins and penicillin are the most commonly used antibiotics to treat or prevent mastitis and other common diseases of dairy cattle. The previous studies demonstrated that frequent use of a given antibiotic leads to the emergence of resistance to antibiotics (82-87). Regularly using cephalosporins, particularly ceftiofur, in the study farms is concerning as it could lead to the emergence of ESBL-producing bacteria such as *E. coli* (88).

ESBL-*E. coli* was detected from fecal samples of at least one animal in all 14 study farms resulting in 100% herd-level prevalence. This is higher than previous studies from the U.S. that reported 20% (5/25 farms) from Ohio (53), 85% (18/21) from Washington (52), and 4% (3/80) from Pennsylvania (89) dairy herds. However, this comparison should be interpreted with caution as these studies may differ in the criteria that they used to define a herd as positive (use of pooled manure samples vs. rectal fecal sample), sample processing steps (use or not using enrichment steps, type of media used for bacterial isolation) involved to determine the herd level status of ESBL-*E. coli*, which has different detection sensitivity and specificity. The widespread occurrence of ESBL-*E. coli* across the present study farms could be related to the frequent use of beta-lactam antibiotics, such as ceftiofur, that may subject them to selection pressure and other farm management related factors that favor the spread of the ESBL-*E. coli* or their ESBL genes.

The weighted within-herd prevalence of fecal ESBL-*E. coli* ranged from 8.0% to 100%, and the difference between within-herd prevalence was statistically significant across the farms.

Detection of statistically significant variation in prevalence between different farms could be

related to the differences in the type and level of beta-lactam antibiotics use and the presence of other influencing farm management related factors that were not within this study's scope.

The within-herd prevalence of 8 – 100% in the present study is higher than a few previous studies conducted in the U.S., including 3.3 - 100% (53) in Washington state and 0 - 33% in Ohio (90). Studies from other countries also reported a within-herd prevalence ranging from 5.2 - 86.7% (91, 92). However, it should be noted that these studies differ from the present study and each other in the microbiological techniques they used to isolate and identify the bacteria and the study farms' selection criteria, which makes the comparison less plausible.

The prevalence of fecal ESBL-*E. coli* in the present study was 47.5% at an animal level, indicating a high level of colonization. The current prevalence report is higher than the previous report from Ohio dairy cattle (9.4%, 70/747) (53) but closely similar to a report from European dairy cattle, 41% (37/90) (92). The fecal prevalence of ESBL-*E. coli* in this study is higher than the recently reported prevalence of 4.6% in U.S. cow-calf operations (93). This variation could be related to the differences in the type and frequency of beta-lactam antibiotics use and other managerial factors that may favor the occurrence and spread of ESBL-*E. coli* in the farms.

The fecal ESBL-*E. coli* prevalence in dairy calves was significantly ($p < 0.001$) higher than in cows (61.7 % vs. 44%). This finding agrees with recent studies from North America (89, 93-98) and Europe (92, 99-101) that reported a higher proportion of ESBL-producing or -third-generation cephalosporins (3GC) resistant-*E. coli* in calves compared to older cattle. Conclusive evidence as to why the higher prevalence of resistant bacteria in calves is not available and needs further study. It is hypothesized that feeding medicated milk replacers, colostrum, or milk from cows treated with beta-lactam antibiotics might have exerted selective pressure that led to the increased fecal carriage of ESBL-*E. coli* in calves (92, 94, 96, 99, 102). However, a recent study in the U.S. and Canada dairy calves showed a high prevalence of antimicrobial resistant *E. coli* in young calves that were not exposed to antimicrobials-containing milk replacers or did not feed milk from cows treated with antibiotics (96). Another alternative hypothesis for a higher prevalence of ESBL-*E. coli* in calves is related to the fitness of the resistant bacteria due to less bacterial diversity in the calves' gut compared to adult dairy cattle with more diverse gut bacteria

where competition is strong for ESBL-*E. coli* to thrive (101, 103, 104). A controlled study is needed to identify factors that promote a higher fecal ESBL-*E. coli* carriage in dairy calves than in adult cattle. Whatever the driving factor may be, this study suggests that calves may be an important source or reservoir of ESBL-*E. coli* and can be considered as sentinel animals for surveillance.

The detection of a higher proportion of ESBL-*E. coli* in manure is not surprising as manure contains a mixture of fecal samples, bedding, and wastewater pooled from different animal sources and has a high chance of containing the bacteria. Previous studies in the U.S. also reported a comparable level (53.5%; 62/116) of ESBL-*E. coli* from pooled manure samples (90). Most dairy farms in this study use manure as a fertilizer on pasture fields, which can contaminate the environment and water. Thus, the presence of ESBL-*E. coli* in the manure sample has significant public and environmental health implications. In our previous study, we detected a higher proportion of 3GC-resistant *E. coli* in dairy manure-amended soils compared to prairie soils not impacted by dairy cattle suggesting the need for proper management of manure to reduce the spread of resistant bacteria (105). The detection of ESBL-*E. coli* in feed (33.3%) and water (26%) samples in this study suggest that the bacteria may remain circulating in dairy farms through oral-fecal transmission via contaminated feed and water (106). Frequent cleaning of water and feed troughs may help to reduce the chance of maintenance of ESBL-*E. coli* in the dairy farm (97, 107).

This study also assessed animal-level factors that may influence the fecal prevalence of ESBL-*E. coli*. Recent use of ceftiofur (for treatment or prophylaxis), parity, and lactation status of the cow were significant predictors for the probability of ESBL-*E. coli* fecal carriage ($P < 0.05$). Cows recently receiving ceftiofur treatment (up to six months before sample collection) were 35% more likely (OR=1.35) to carry ESBL-*E. coli* in their feces compared to cows not exposed to this antibiotic during the same period. Previous studies (51, 102, 108-111) also showed ceftiofur use leads to an increased probability of ESBL-*E. coli* detection or decreased susceptibility to 3GC, such as ceftriaxone in dairy cattle. Controlled studies in the U.S. (110) and Europe (112) showed a significant increase in ESBL mediating genes and mobile genetic elements (plasmids and

prophages) in ceftiofur treated compared to non-treated dairy cows. In addition, a European study showed a restrictive use of ceftiofur and cefquinome (4th generation cephalosporin) in cattle significantly decreased the prevalence of fecal ESBL-*E. coli* in cattle (101).

Nevertheless, existing literature does not fully agree on the association between ceftiofur use and the probability of detection of *E. coli* with reduced susceptibility to 3GC. Some authors reported a lack of association between the two (53, 98), and others reported a decreased chance of recovering resistant *E. coli* following treatment with ceftiofur (102). However, most controlled and observational studies (including the present study) indicated a stronger association between ceftiofur use and the probability of detecting ESBL-*E. coli*, considering ways to reduce ceftiofur use (e.g., using only when sensitivity test result justifies) may help to reduce the ESBL-*E. coli* emergence and spread.

The likelihood of lactating cows having ESBL-*E. coli* in their feces were more than three times greater (OR=3.42) than that of dry cows. The difference in the prevalence of fecal ESBL-*E. coli* may be related to the increased risk of mastitis and other diseases in lactating cows, prompting antibiotic use and favoring the emergence of ESBL-*E. coli* strains. This finding is significant because lactating cows are more likely to come into contact with humans than dry cows, and they also produce milk that can be contaminated. This increases the risk of transmission of ESBL-*E. coli* from cows to humans during the lactation period.

Cows with higher parity (≥ 3) were associated with an increased probability (OR=1.33) of fecal ESBL-*E. coli* recovery compared to those cows with lower parity (≤ 2). This could be related to frequent exposure to beta-lactam antibiotics for managing mastitis and other diseases whose prevalence likely increases with age. Previous studies (113-115) showed a strong association between the prevalence or incidence of mastitis and parity (higher risk in older cows), which is also correlated with increased use of antibiotics that promote selection pressure on commensal *E. coli*.

Almost all (99.6%, 230/231) ESBL- *E. coli* tested for AST exhibited resistance to at least one of the 14 antimicrobial agents tested. The isolates displayed the highest level of resistance to

ampicillin (99.1%, n=231) and ceftriaxone (98.7%) which is expected as the isolates produce ESBL-enzymes that hydrolyze penicillin and extended-spectrum cephalosporins (21, 116). Three ESBL-*E. coli* isolates were identified as ESBL-producers by the selective chromogenic media and were unexpectedly susceptible to ceftriaxone, a 3GC. One isolate was sensitive to all the tested antimicrobial agents. This finding was unexpected as the selective media used in this study contains a supplement that is supposed to select isolates resistant to beta-lactam antibiotics such as penicillin and 3GC. This could be related to the reduced specificity of the screening media used to isolate ESBL-producing *E. coli* or later loss of expression of the ESBL gene (117).

A high prevalence of co-resistance was detected between ceftriaxone and older antibiotics such as ampicillin (97.8%, n=231), tetracycline (79.7%), sulfisoxazole (59.7%), and streptomycin (54.5%). This resistance trend and prevalence are consistent with previous studies from the U.S. dairy cattle (23, 52, 89, 105). Recently, Masse *et al.*(95) also reported a similar trend but a lower prevalence of co-resistance to these antibiotics in Canada. However, some authors used different initial screening steps (selective media) to isolate *E. coli*, making the comparison challenging. Some of these antibiotics (streptomycin and sulfisoxazole) were not mentioned by farmers as predominantly used antibiotics in their farms, suggesting the high level of resistance detected may not be related to their utilization. Further investigation is important to explain if these resistance phenotypes were co-selected with ESBL phenotypes, common resistance phenotypes reported in this study.

ESBL-*E. coli* exhibited low resistance to amoxicillin-clavulanic acid (8.7%, n=231) and cefoxitin (10%). Generally, the growth of ESBL-*E. coli* is expected to be inhibited by both amoxicillin-clavulanic acid and cefoxitin, a cephamycin (21, 116, 118). As reported in other studies, the detection of resistance to these two antibiotics suggests the possible expression of multiple resistance phenotypes in the same *E. coli* isolates (52, 95, 107).

ESBL-*E. coli* displayed a higher level of concurrent resistance between ceftriaxone and other members of CIAs, such as streptomycin (55%), ciprofloxacin (18.6%), and azithromycin (13.4%). The prevalence of concurrent resistance is similar to a previous study from the U.S. (52) but 5 to 20 times higher than the reported prevalence from Canadian dairy farms (95). Other

previous studies reported ESBL-*E. coli* often exhibit co-resistance to multiple CIAs (98, 105, 119). Detection of a relatively higher prevalence of concurrent resistance of *E. coli* to CIAs antibiotics may pose a significant public health threat as very limited treatment options are available in the case of opportunistic ESBL-*E. coli* or their resistance genes are transferred to human pathogens (120).

The overall prevalence of MDR ESBL-*E. coli* isolates in this study were 94.4% (218/231), suggesting an association between MDR and ESBL-phenotypes in *E. coli*. This finding is consistent with previous studies that reported a high prevalence of MDR phenotype¹ in ESBL-*E. coli* isolates from cattle rectal fecal and farm manure samples (52, 93, 98). About 42.6% of MDR *E. coli* isolates in this study showed concurrent resistance to at least six classes of antibiotics, suggesting the ESBL-*E. coli* is a host of resistance genes which may complicate treatment if these bacteria cause infection or transfer the resistance genes to pathogenic bacteria. Multidrug resistant ESBL-*E. coli* was detected from all farms and all sample types, suggesting widespread occurrence across farms. Unlike recent studies from Canadian dairy farms (95), no difference was detected in the prevalence of the MDR across the five sample sources and study farms. The lack of detection of variation indicates that ESBL phenotype is a phenotypic marker of the MDR pattern in *E. coli*, irrespective of sample sources (121, 122).

The study found two specific patterns of multidrug resistance ESBL-*E. coli*, AXO-AMP-STR-TET (52.8%), and AXO-AMP-FIS-TET (48.5%) were prevalent and detected in all sample types and study farms. The widespread occurrence of these patterns is a cause for concern regarding the spread of antibiotic resistance. The study calls for further research to understand the mechanisms behind the spread of these patterns, and the potential impact on animal and human health to identify strategies for reducing their prevalence in dairy farms.

The study found that 6.5% of ESBL-*E. coli* had concurrent resistance to at least one antibiotic in all CIAs tested in this study. This is a serious cause for concern as these antibiotics are of the highest importance for human health, and their efficacy could be compromised. However, concurrent resistance was detected from only three study farms, highlighting the need for continuous monitoring with targeted and localized interventions. Another study in the U.S.

reported that CIAs such as macrolides and fluoroquinolones are commonly used to treat diseases in dairy calves and other non-lactating dairy cattle under 20 years of age, which could possibly drive the concurrent presence of resistance to these antibiotics (123). Other studies in humans reported the frequent use of 3GC as a risk factor for the co-occurrence of resistance to CIAs such as fluoroquinolones (124-126) which could be the case as two of these farms were frequently using ceftiofur, a 3GC.

Identification of at least one kind of β -lactamase encoding gene in 98.7% of the ESBL-*E. coli* genomes are expected as the isolates were first screened on selective media, which promotes the growth of β -lactamase producers. This genotypic result generally concurs with the phenotypic antibiotic resistance test. The *bla*_{CTX-M} type was the most prevalent β -lactamase variant detected in 95.4% of the ESBL-*E. coli* genomes. This finding supports the previous studies that *bla*_{CTX-M}-type genes are rapidly rising and responsible for most of the resistance to extended-spectrum cephalosporins (ESC), including ceftriaxone among *Enterobacteriaceae*, including *E. coli* (127, 128). A recent study in the U.S. dairy farms also showed the predominance of the *bla*_{CTX-M} gene variant in *E. coli* isolates resistant to ESC (23, 52, 105). The reason for the prevalence of the CTX-M variant of ESBL over other beta-lactamase variants is not well defined. Still, it can be related to its fast spread via horizontal transfer mediated by plasmids (23, 129). The most frequently detected *bla*_{CTX-M} variants identified in this study, *bla*_{CTX-M} (-65, -32, -15, -1, and -27), were also previously reported from the U.S. dairy farms in *E. coli* isolates (52, 98, 119, 130). However, the frequency of each *bla*_{CTX-M}-variant reported may be different. For instance, Afema *et al.* reported (52) *bla*_{CTX-M-15} and *bla*_{CTX-M-27} as the most frequent and *bla*_{CTX-M-65} as the less common variant in Washington states dairy farms. This difference could be attributed to the difference in local epidemiology and strains of ESBL-*E. coli* carrying the genes and the number of isolates tested.

The *bla*_{TEM} variants were the second most frequent β -lactamase genes detected in 52% of the ESBL-*E. coli* genomes and had the most diverse variant and complex phenotypes. Only a handful of studies reported *bla*_{TEM} using PCR without variant description from the U.S. dairy farms (53, 130, 131). Consistent with this study, the National Antimicrobial Resistance

Monitoring Program reported *bla*_{TEM-1} from *E. coli* isolates from dairy farms. The co-occurrence of one or more *bla*_{CTX-M} and *bla*_{TEM} genes was detected in nearly half (48%) of the *E. coli* isolates.

The presence of multiple β -lactamase genes in a single isolate may help the host bacteria maintain β -lactamase resistance phenotypes even if one or more genes have reduced expression (132). Furthermore, this could raise the chances of vertical transmission of at least one of these genes to subsequent generations and increases the odds of horizontal spread to other bacteria. Similar trends were reported in human clinical isolates of *E. coli* (133).

The *bla*_{SHV} was not detected in this study. The lack of detection of the *bla*_{SHV} gene in ESBL-*E. coli* in this study supports the past few years' research findings that indicated the *bla*_{CTX-M} variant gradually replaced the previously common variants, including the *bla*_{SHV}, especially in *E. coli* (134, 135). In addition to a few inhibitor-resistant variants of *bla*_{TEM}, three other kinds of beta-lactamases, *bla*_{CMY-2}, *bla*_{SED-1}, and *bla*_{OXA-10}, were detected in ESBL-*E. coli* isolates. In this study, the *bla*_{CMY-2} was detected in 18 ESBL-*E. coli* isolates, eight of which do not harbor ESBL genes. The presence of a non-ESBL gene, *bla*_{CMY-2}, on a media supposed to select only ESBL-producers could be attributed to the limited diagnostic specificity of the ChromAgar ESBL media used in this study and /or the co-presence of chromosomally encoded AmpC β -Lactamases (117).

The *bla*_{SED-1} gene co-occurred with *bla*_{CTX-M} and other beta-lactamase variants in four isolates obtained from three farms. The co-occurrence of the *bla*_{SED-1} gene with different ESBL variants is concerning, as bacteria carrying this gene have been identified as resistant to most beta-lactamase inhibitors. The bacteria carrying the combination of genes may spread horizontally to potentially pathogenic strains, resulting in infections that are difficult to treat with currently available extended-spectrum cephalosporins (136). Although the *bla*_{SED-1} gene is not frequently detected in *E. coli*, previous research has revealed its natural occurrence in *Citrobacter sedlakii* (137, 138). Consequently, it is hypothesized that *E. coli*, the most frequently identified ESBL-producing bacterial species in this study, may have acquired the gene from *C. sedlakii*. In the

U.S., *bla*_{SED-1} was previously detected in *Citrobacter freundii* obtained from a rectal swab of human patients (https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes:blaSED).

The *bla*_{OXA-10}, oxacillin-hydrolyzing class D beta-lactamase detected in this study mediates resistance against penicillin and potentiated penicillin (26, 139). This specific variant was not previously reported from the U.S. dairy cattle. Still, the same class but a different variant of this gene, *bla*_{OXA-27}, was previously reported in an *E. coli* isolate obtained from a dairy calf in the U.S. (131). However, there has been a recent surge in the detection of the *bla*_{OXA-10} gene among pathogenic strains of *Pseudomonas aeruginosa* in humans. The report on the NCBI database showed that the gene is detected in 443 of these bacteria isolates from bronchoalveolar lavage samples of human patients across various hospitals in the U.S.

(https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes:blaOXA).

The *bla*_{LAP-2}, a narrow-spectrum β -lactamase detected in this study, was considered a rare type of class A β -lactamases as it is one of its only two variants reported and archived in β -lactamases databases before (<http://bldb.eu/BLDB.php?prot=A>). The *bla*_{LAP-2} was first identified in China from human clinical isolates of *Enterobacter cloacae* (140) and recently from pandemic lineages of *K. pneumoniae* in Italy (141). The progenitor of this gene, *bla*_{LAP-1}, was previously identified in France and Vietnam from human clinical isolates of *Enterobacter cloacae* (142, 143). To the author's knowledge, *bla*_{LAP-2} was not previously reported from *E. coli* or other members of the *Enterobacteriaceae* family in the U.S. dairy farms. However, the same gene variant was reported from *K. pneumoniae* and *Enterobacter cloacae* isolated from human clinical samples in the U.S. (https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes:blaLAP). Further studies are needed to monitor the emergence and spread of this gene as it might already be spreading in human pathogenic species of *Enterobacteriaceae* in the U.S.

In this investigation, mutations were found in the promoter regions of the chromosomal *ampC* gene of *E. coli* in 39% of the ESBL-*E. coli* isolates, including isolates that had no ESBL gene. Previous studies showed that modifications of the promoter region of the *ampC* gene lead to constitutive overexpression of native AmpC beta-lactamase enzyme that leads to resistance to extended-spectrum cephalosporins (131, 144). A previous study suggested that this mutation

could be associated with exposure to β -lactam antibiotics (144). The high frequency of mutation detected in this study may indicate significant usage of expanded-spectrum cephalosporins in dairy farms.

Furthermore, AmpC beta-lactamases reportedly resist widely used beta-lactamase inhibitors, such as clavulanic acid, tazobactam, and sulbactam (144-146). Thus, the presence of the *ampC* gene in a significant proportion of the ESBL-*E. coli* genomes are concerning since these genes could be mobilized and potentially disseminated to other pathogenic bacterial strains via mobile genetic elements (144).

The study found that ESBL-*E. coli* isolates have resistance genes against nine different classes of antibiotics in addition to beta-lactamase genes. Almost all (97%) *E. coli* isolates were genotypically MDR, with resistance genes for up to eight categories of antibiotics, including critically important ones. These genotypic findings are generally consistent with the multidrug resistance patterns observed in the phenotypic testing of the isolates. This also confirms the previous studies that reported the frequent co-occurrence of multiple resistance genes and ESBL genes in ESBL-producing *E. coli* in dairy farms and other food-producing animals (52, 98, 105, 130, 147-149).

Most ESBL-*E. coli* isolates co-harbored ESBL-genes with other resistance genes to CIAs such as plasmid-mediated fluoroquinolone genes (*qnrB* and *qnrS*), macrolide resistance gene, *mph(A)*, and various kinds of aminoglycoside modifying genes. This finding is consistent with the previous studies that reported frequent co-occurrence of these genes in ESBL-*E. coli* isolates from the U.S. dairy cattle and farm environments (98, 105, 119). The identification of a large proportion of ESBL-*E. coli* strains co-carrying resistance genes to multiple classes of CIAs are of significant health concern. These genes are often associated with MGEs that favor their co-transfer and spread to other bacteria, including human pathogenic strains resulting in cross-resistance to similar antibiotics used for treating human infections (23, 98, 150, 151). However, it is interesting to note a considerable discrepancy between the prevalence of resistance gene and resistance phenotypes to fluoroquinolones, with *qnr* detected in 180 (77.3%) isolates and one or more mutations in QRDRs detected in 229 (98.3%) of the isolates. Still, phenotypic resistance

was seen in 47 ESBL-*E. coli* isolates. Here two questions require investigation. First, most isolates containing resistance genes exhibiting phenotypic resistance were obtained from adult dairy cows, where fluoroquinolone antibiotics are not used. Previous studies on *E. coli* from dairy cattle (52, 119) and human clinical *E. coli* isolates found a possible connection between ESBL production and fluoroquinolone resistance (131, 152, 153). However, their findings were inconclusive. Therefore, it is crucial to identify the mechanism and why the fluoroquinolone resistance gene is maintained in the ESBL-*E. coli* population without antibiotic selection pressure. Is selecting ESBL-producing *E. coli* linked to the simultaneous co-selection of the resistance gene to fluoroquinolones? Second, why do most isolates containing fluoroquinolone resistance genes did not exhibit corresponding resistance phenotypes? Do fluoroquinolone resistance genes lose their function (expression) without exposure to fluoroquinolones?

In addition to the resistance gene against CIAs, there is a high frequency of co-occurrence of one or more variants of the resistance gene against other medically important classes of antibiotics, including tetracycline, folate pathway inhibitors, and chloramphenicol which were detected in 80%, 75%, and 51.5% of in ESBL-*E. coli*, respectively. Previous studies have also reported the frequent co-occurrence of these resistance genes in ESBL-*E. coli*, which suggests that the presence of ESBL genes signals the presence of multiple resistance genotypes to unrelated classes of antibiotics (29, 52, 105, 154-158).

The Multilocus Sequence Typing of ESBL-*E. coli* strains revealed diverse sequence types (STs) circulating in dairy farms. The presence of unknown STs indicated that there are ESBL-*E. coli* strains are circulating in dairy farms that have not yet been fully characterized. The discovery of novel STs in dairy farms suggests that the ESBL-*E. coli* population constantly evolves and poses a potential challenge for controlling its spread. The detection of unknown and novel STs of ESBL-*E. coli* in dairy farms underscores the need for further investigation to better understand these emerging strains' genetic and phenotypic characteristics. This can help identify emerging antibiotic resistance trends and guide the development of strategies to control their spread.

The high degree of clustering (genetic relatedness) of specific ESBL-*E. coli* STs with specific resistance genotypes within most farms identified in this study suggest that animal-to-animal

transmission (clonal expansion) or exposure to a common source could be an important route of ESBL-*E. coli* distribution within dairy farms. The finding that multiple distinct STs within a farm harbored the same or clonal variant of *bla*_{CTX-M} suggests that the spread of resistance genes may occur within farms between different genotypes of ESBL-*E. coli* via mobile genetic elements (159). Furthermore, the finding that identical sequence types (STs) of ESBL-*E. coli* strains in some farms share the same resistance gene pattern suggesting the possible farm-to-farm spread of the bacteria or parallel micro-evolution (160).

This study revealed that 64 genotypically MDR ESBL-*E. coli* strains belonging to fourteen STs were classified as high-risk clones linked to community and healthcare-associated infections in humans (161, 162). Among these, ST10 (22 isolates) was the most frequent high-risk clone detected in 50% of the study farms. On a global scale, this ST was ranked as the third most prevalent or widespread high-risk clone, following ST131 and ST69 (163, 164). The ST10 was previously reported from dairy and swine farms (52, 159) and human clinical samples in the U.S. (https://pubmlst.org/bigdb?db=pubmlst_escherichia_isolates). The detection of many ESBL-producing MDR ST10 in dairy farms indicated that this strain could be well-adapted to dairy cattle and may cross to humans.

ST69 was detected in a cow in this study and was considered the second most widespread successful high-risk clone of *E. coli* (164). The ST69 was initially isolated over two decades ago from urine samples from hundreds of women suffering from community-associated urinary tract infections in California (165, 166). Some previous studies reported that ST69 is an infrequent producer of the ESBL gene (52, 161, 167). However, in this study, the ST69 carried *bla*_{CTX-M-15} along with several other resistance genes (*bla*_{TEM-1B}, *qnrS1*, *aph*(6)-Id, *aph*(3'')-Ib, *tet*(A), *sul2*, *dfrA14*, *fosA*, *parC*, *gyrA*, *gyrB*, and *ampC*). If ST69 is a rare ESBL producer, our study may have overlooked its presence because we utilized a media that only selected ESBL-producers, leading to a potential underestimation of the prevalence of this strain in our study. Thus, it highlights the importance of closely monitoring these bacteria in dairy farms.

Other high-risk ESBL-*E. coli* clones harboring several resistance genes detected in this study include ST1011(7 isolates), ST224 (7), ST398 (6), ST155 (5), ST410 (2), ST23, ST101, ST48,

ST165, and ST156. These sequence types were frequently reported from food animal species and human clinical cases (hospital and community-associated infections) in different parts of the world (159, 163, 168-179) and some of which were also reported from the USA (https://pubmlst.org/bigsdb?db=pubmlst_escherichia_isolates). Some studies showed evidence of transmission of one or more of these STs between animals and humans (33, 160, 171, 180).

Unlike other high-risk clones, one of the two ST58 detected in this study had only two resistance determinants, a mutation in the promoter region of the *ampC* gene and *parC*. The ST58 and ST101 were previously considered environmental strains. Still, recently, they are frequently reported as among emerging potential high-risk clones associated with invasive urinary tract and bloodstream infections in humans (163, 181, 182). Similarly, five of the seven ST117 detected in this study had no ESBL genes but harbored *bla*_{TEM-1B} and several other resistance genes against six classes of antibiotics. This ST was often identified as avian pathogenic *E. coli* and has been frequently isolated from animal and human clinical samples in the U.S. (https://pubmlst.org/bigsdb?db=pubmlst_escherichia_isolates) and across the globe, suggesting potential zoonotic importance (164, 174, 176, 183).

Detecting several high-risk clones of ESBL-*E. coli* in dairy cattle and farms can have significant implications for human health. These bacteria can be transmitted from animals to humans indirectly by consuming contaminated animal-source food or directly through contact with carrier animals, particularly by farm workers or their environment. Hence, the possibility of direct exposure to dairy cattle and farms serving as a route for transmitting these high-risk clones from dairy farms to the community cannot be ruled out (23, 184). The spread of these bacteria to the community can increase morbidity and mortality rates due to their ability to cause extraintestinal infections that are difficult to treat with most antibiotics (164, 185, 186).

Conclusion

This study showed a high prevalence of ESBL-*E. coli* in dairy cattle farms, with almost half of the tested animals positive for the bacteria. The prevalence of ESBL-*E. coli* varied significantly within each farm. The study found that recent treatment with third-generation cephalosporins,

cows with higher parity, and being calves was linked to an increased risk of fecal carriage of ESBL-*E. coli*. These findings suggest that interventions should target these identified variables. Almost all the ESBL-*E. coli* isolates were MDR with the highest resistance to ampicillin and ceftriaxone. Over 40 % of ESBL-*E. coli* showed concurrent resistance to at least six classes of antibiotics. Concurrent resistance to ceftriaxone, ampicillin, tetracycline, sulfisoxazole, streptomycin, and chloramphenicol was high and widespread across all farms. The study also identified six beta-lactamase genes, *bla*_{CTX-M}, and *bla*_{TEM}, being the most prevalent. The study also identified a range of antibiotic resistance genes (ARGs) co-harbored with the ESBL gene in *E. coli* isolates, including those conferring resistance to critically important classes of antibiotics such as fluoroquinolones, aminoglycosides, and macrolides. The study observed diverse genotypes of ESBL-*E. coli* with significant within-farm clustering. Nearly one-third of the detected STs belonged to high-risk clones indicating that dairy cattle could serve as a reservoir for high-risk clones of MDR ESBL-*E. coli*, which could pose a potential public health risk.

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Appendix

Table 4. 1. Farm location and samples collected

County	Farm	Rectal feces ^a	Manure	Water	Feed	Total
A1	A	14	2	1	1	18
A2	B	9	1	1	1	12
B2	C	63	2	1	1	67
B2	D	30	2	1	1	34
C1	E	13	2	1	1	17
C2	F	40	2	1	1	44
D	G	25	1	1	2	29
E	H	26	1	1	1	29
F1	I	30	2	1	1	34
G1	J	25	2	1	1	29
G2	K	45	2	1	1	49
F2	L	50	2	2	1	55
H	M	64	2	2	-	68
F3	N	74	7	4	2	87
All farms		508	30	19	15	572

^a Rectal fecal sample includes samples collected from dairy cows and calves; Farms obtained from counties designated by the same letters (e.g., as in A1 & A2) were obtained from the same county (e.g., Farm A & B are both from the same county).

Table 4. 2. Antimicrobial agents (CMV4AGNF Panels), concentration range, and CLSI breakpoints for ESBL-*E. coli* isolates

Antimicrobial Class (CLSI)	Antimicrobial Agent	Concentration (mg/mL)	Breakpoints (mg/mL) ^a		
			R	I	S
Beta-lactam/ Beta-lactamase inhibitor combinations	Amoxicillin/clavulanic acid	1/0.5–32/16	≥ 32/16	16/18	≤ 8/4
Penicillin	Ampicillin	1 – 32	≥ 32	16	≤ 8
Cephems	Cefoxitin	0.5 – 32	≥ 32	16	≤ 8
Cephalosporins	Ceftriaxone	0.25 – 64	≥ 4	2	≤ 1
Carbapenems	Meropenem	0.06 – 4	≥ 4	2	≤ 1
Macrolides	Azithromycin	0.25 – 32	≥ 32		≤ 16
Aminoglycosides	Gentamicin	0.25 – 16	≥ 16	8	≤ 4
	Streptomycin	2 – 64	≥ 32 ^b		
Quinolones	Ciprofloxacin	0.015 – 4	≥ 1	0.5	≤ 0.25
	Nalidixic acid	0.5 – 32	≥ 32		≤ 16
Phenicol	Chloramphenicol	2 – 32	≥ 32	16	≤ 8
Folate pathway inhibitors	Sulfisoxazole ^c	16 – 256	≥ 512		≤ 256
	Trimethoprim/sulfamethoxazole	0.12/2.38 – 4/76	≥ 4/76		≤ 2,38
Tetracyclines	Tetracycline	4 – 32	≥ 16	8	≤ 4

^a R: resistant; I: intermediate; S: susceptible. ^b The National Antimicrobial Resistance

Monitoring System (NARMS) gram-negative Sensititre plate interpretive breakpoint was used for streptomycin. All other breakpoints presented in Table 1 were adopted from the CLSI M100-32 edition (1).

Table 4. 3. Common diseases and antibiotics used in each dairy farm

Farm	Common Diseases on the Farms					Commonly Used Antibiotics ^a
	Mastitis	Hoof Problem	Metritis/ Endometritis	Resp. Disease	GIT Disease	
A	Yes	Yes	No	No	No	Ceftiofur, pirsue, and quartermaster
B	Yes	No	No	Yes	No	Tetracycline, pirsue, toDAY, ToMORROW
C	Yes	Yes	No	Yes	No	ToMORROW and ceftiofur
D	Yes	Yes	Yes	No	No	Ceftiofur, penicillin, and quartermaster
E	Yes	No	No	No	No	ToDAY and ToMORROW
F	Yes	Yes	No	No	No	Ceftiofur and quartermaster
G	Yes	Yes	No	No	No	Tetracycline, Penicillin ToMORROW, quartermaster
H	Yes	No	No	Yes	No	Ceftiofur, ampicillin
I	Yes	Yes	No	No	No	Pirsue, ToDAY, and ToMORROW
J	Yes	Yes	No	Yes	Yes	Ceftiofur, penicillin G, ampicillin, ToDAY, pirsue, and ToMORROW
K	Yes	Yes	Yes	Yes	Yes	Ceftiofur, Penicillin G, ampicillin, quartermaster, tulathromycin, and ToMORROW
L	Yes	Yes	No	No	No	Ceftiofur, ToMORROW, and quartermaster
M	Yes	Yes	Yes	Yes	No	Ceftiofur, penicillin G, and tetracycline
N	Yes	Yes	Yes	Yes	Yes	Ceftiofur, penicillin G, tetracycline, and tulathromycin

^a toMORROW: cephapirin benzathine; toDAY: cephapirin sodium; Pirsue: pirlimycin hydrochloride; Quartermaster: Procaine penicillin G and Dihydrostreptomycin sulfate; Endometr: Endometritis; Resp: Respiratory; GIT: Gastrointestinal

Table 4. 4. Within-herd prevalence of ESBL-*E. coli* across fourteen dairy farms

Farm	Herd Size ^a	Sample Size (n) ^b	Unweighted Frequency and Prevalence (%) ^c	95 % CI Unweighted Prevalence	Weighted Frequency and Prevalence (%) ^d	95 % CI of the Weighted Prevalence
A	100	14	14 (100)	76.8 - 100	100 (100)	96.4 -100
B	14	9	3 (33.3)	7.4 - 70	5 (35.7)	12.8 - 64.9
C	750	63	35 (55.6)	42.4 - 68	417 (55.6)	52.0 - 59.2
D	300	30	3 (10)	2.2 - 26.5	30 (10)	6.8 -13.9
E	50	13	1 (7.7)	0.2 - 36	4 (8)	2.2 -19.2
F	110	40	27 (67.5)	50.8 - 81.4	74 (67.3)	57.7 - 75.9
G	170	25	6 (24)	9.4 - 45.1	41 (24.1)	17.9 - 31.3
H	35	26	22 (84.6)	65.1- 95.6	30 (85.7)	69.7 - 95.2
I	61	30	4 (13.3)	3.8 - 30.7	8 (13.1)	5.8 - 24.2
J	60	25	18 (72)	50.6 - 87.9	43 (71.7)	58.6 - 82.5
K	1700	45	28 (62.2)	46.5 - 76.2	1058 (62.2)	59.9 - 64.5
L	250	50	21 (42)	28.2 - 56.8	105 (42)	35.6 - 48.4
M	450	64	21 (32.8)	3.0 - 16.8	148 (32.9)	28.6 - 37.4
N	350	74	6 (8.1)	3.0 -16.8	28 (8)	5.4 - 11.4
Total	4,400	508	209 (41.1%)	36.8 - 45.6	2090 (47.5%)	46.2 - 49.2

^a Herd size: the total number of animals on each farm; ^b: the actual number of animals sampled from each farm; ^c the number and proportion of animals from which ESBL-*E. coli* was isolated;

^d Frequency of the weighted sample size and the corresponding prevalence in the herd.

Table 4. 5. Comparison of prevalence of fecal ESBL-*E. coli* in dairy cattle

Herd Structure	Unweighted Sample size	Weighted size	Weighted frequency and [(Prevalence), 95 % CI]	OR (95 % CI)	P-value
Cows	424	3526	1551 [(44%), 42.3 - 45.6]	Ref	
Calves	84	874	539 [(61.7%), 58.4 - 64]	2.12 (1.78 -2.53)	< 0.001
Total	508	4400	2090 [(47.5%, 46.2 - 49.2]	NA	

Table 4. 6. Prevalence of ESBL-*E. coli* in dairy cattle farms by sample type

Sample type	Sample size (n) ^a	No. Positive ^b	Prevalence (95% CI)
Rectal feces	508	209	41.1% (36.8 - 45.6)
Manure	30	14	46.7% (28.3 - 65.7)
Water	19	5	26.3% (9.1- 51.2)
Feed	15	5	33.3% (11.8 - 61.6)
Total	572	233	40.7% (36.7- 44.9)

CI: confidence interval; ^a Number of samples tested from each source.

^b Number of samples that yielded ESBL-*E. coli*

Table 4. 7. Association between cow-level characteristics and weighted fecal ESBL-*E. coli* prevalence

Variable		Prevalence (Frequency)	Multivariable Analyses	
Name	Category		Adjusted OR (95% CI)	P-Value
Ceftiofur Treated	No	15.4% (254/1641)		
	Yes	20.5% (230/1124)	1.35 (1.1-1.65)	0.004
Reproductive Status	Dry	22.2% (180/812)		
	Lactating	50.5% (1371/2714)	3.42 (2.79 - 4.10)	< 0.001
Cow Parity	≤ 2	39.4% (840/2130)		
	≥ 3	50.8% (706/1391)	1.33 (1.13 - 1.56)	< 0.001

Table 4. 8. Number and percentage of resistance of ESBL-*E. coli* isolates (N=231) obtained from various sources

Antibiotic	Sources of <i>E. coli</i> Isolates and Proportion of Resistance to the Specific Antibacterial Agent					Overall Prevalence of Resistance to Specific Antibiotic n (% Resistant)
	Cows (N=180) ^a	Calf (N= 27)	Manure (N=14)	Feed (N= 5)	Water (N= 5)	
	n (% Resistant) ^b	n (% Resistant)	n (% Resistant)	n (% Resistant)	n (% Resistant)	
<i>AUG2</i>	8 (4.4)	11 (40.7)	0 (0)	1 (20)	0 (0)	20 (8.7)
<i>AMP</i>	178 (98.9)	27 (100)	14 (100)	5 (100)	5 (100)	229 (99.1)
<i>AZM</i>	25 (13.9)	4 (14.8)	0 (0)	2 (40)	0 (0)	31 (13.4)
<i>FOX</i>	11 (6.1)	11 (40.7)	1 (7.1)	0 (0)	0 (0)	23 (10)
<i>AXO</i>	178 (98.3)	26 (96.3)	14 (100)	5 (100)	5 (100)	228 (98.7)
<i>CHL</i>	83 (46.1)	12 (44.4)	7 (50)	3 (60)	3 (60)	108 (46.8)
<i>GEN</i>	9 (5)	12 (44.4)	1 (7.1)	0 (0)	0 (0)	22 (9.5)
<i>MERO</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>CIP</i>	36 (20)	0 (0)	2 (14.3)	3 (60)	2 (40)	43 (18.6)
<i>NAL</i>	16 (8.9)	4 (14.8)	0 (0)	1 (20)	0 (0)	21 (9.1)
<i>STR</i>	85 (47.2)	25 (92.6)	9 (64.3)	4 (80)	4 (80)	127 (55)
<i>FIS</i>	107 (59.4)	20 (74.1)	9 (64.3)	1 (20)	2 (40)	139 (60.2)
<i>TET</i>	138 (76.7)	25 (92.6)	14 (100)	4 (80)	4 (80)	185 (80.1)
<i>STX</i>	44 (24.4)	12 (44.4)	3 (21.4)	1 (20)	1 (20)	61 (26.4)

^a N: total number of isolates obtained from a given source; ^b n: number of isolates resistant to a specific antimicrobial agent; proportion: the percentage of isolates resistant to a particular antimicrobial in the given sample (n/N). AXO: ceftriaxone; FOX: ceftiofur; AUG2: amoxicillin-clavulanic acid; AMP: ampicillin; MERO: meropenem, AZM: azithromycin; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; TET: tetracycline; FIS: sulfisoxazole; STX: trimethoprim-sulfamethoxazole

Table 4. 9. Prevalence of multidrug resistant ESBL-*E. coli* across various sample sources

Sample Source	(No. of isolates) ^a	n (Prev. MDR Isolates) ^b	n (Prev.) of resistance to ≥ 6 Antimicrobial Classes
Cows	180	169 (93.9%)	66 (36.7%)
Calves	27	26(96.3%)	15 (55.6%)
Manure	14	14(100%)	6 (42.9%)
Water	5	5(100%)	2 (40%)
Feed	5	4 (80%)	4 (80%)
Total	231	218 (94.4%)	93 (42.6%)

^a Number of *E. coli* isolates from specific sources; No. MDR ^b: number and Prevalence of multidrug resistant *E. coli*; ‘n: frequency; Prev.: prevalence

Table 4. 10. Distribution of concurrent resistance of ESBL-*E. coli* to multiple classes of antibiotics across farms and sample sources

Concurrent Resistance Pattern	Sources and Number of Isolates				Prevalence and No. of Farms with the Resistance Pattern	
	Feces (N=207)	Manure (N =14)	Water (N =5)	Feed (N=5)	n (Prevalence) ^a	No. of Farms ^b
AXO-AMP-STR-TET	105	9	4	4	122 (52.8)	All (14)
AXO-AMP-FIS-TET	100	9	2	1	112 (48.5)	All (14)
AXO--AMP-TET-FIS-CHL	64	6	1	1	72 (31.2)	13
AXO-AMP-STR-FIS-CHL-TET	52	6	1	0	59 (25.5)	12
AXO-AMP-STR-FIS-CHL	48	6	0	1	55 (23.8)	11
AXO-AMP-STR-CIP	29	1	2	3	35 (15.2)	8
AXO-AMP-STR -AZM	20	0	0	2	22 (9.5)	6
AXO-AMP-FOX	20	1	0	0	21 (9.1%)	6
AXO-AMP-AUG2-FOX	16	0	0	1	17 (7.4)	6
AXO-AMP-STR-NAL-STX	19	0	0	0	19 (8.3)	6
AXO-AMP-STR-AZI-CIP	14	0	0	1	15 (6.5)	3

^a Frequency and proportion of isolates with a given concurrent resistant pattern; ^bNumber of farms having isolates with the given resistance pattern. AXO: ceftriaxone; FOX: ceftiofur; AUG2: amoxicillin-clavulanic acid; AMP: ampicillin; MERO: meropenem; AZM: azithromycin; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; TET: tetracycline; FIS: sulfisoxazole; STX: trimethoprim-sulfamethoxazole.

Table 4. 11. Beta-lactamase genes, their function, and distribution in ESBL-*E. coli* isolates in dairy farms

Beta-Lactamase Genes and Their Frequencies (n) ^a	Phenotype or Functional Information	Distribution Across Farms ^b
<i>bla</i> _{CTX-M-65} (n=67)	Class A ESBL	G, H, I, J, K and L
<i>bla</i> _{CTX-M-32} (n=61)	Class A ESBL	A, C, E, F, H, I, J, K, L and M
<i>bla</i> _{CTX-M-15} (n=49)	Class A ESBL	C, D, K, L, M, and N
<i>bla</i> _{CTX-M-1} (n=27)	Class A ESBL	F, G, H, I, J, K, L, M, and N
<i>bla</i> _{CTX-M-27} (n=25)	Class A ESBL	C, D, H, K, L, M, and N
<i>bla</i> _{CTX-M-55} (n=9)	Class A ESBL	G and M
<i>bla</i> _{CTX-M-196} (n=2)	Class A ESBL	C and N
<i>bla</i> _{CTX-M-174} (n=1)	Class A ESBL	N
<i>bla</i> _{CTX-M-105, -98, -90} (n=1)	Class A ESBL	H
<i>bla</i> _{CTX-M-61-138} (n=1)	Class A ESBL	K
<i>bla</i> _{CTX-M-163, -194, -186} (n=1)	Class A ESBL	J
<i>bla</i> _{CTX-M-104, -84, -129, -102, -192, -14, -38, -125, -130, -17, -134, -196, -24} (n= 1)	Class A ESBL	C
<i>bla</i> _{TEM-1A} (n=60)	2b Class A β -lactamase	B, C, D, E, F, G, H, J, K, L, M and N
<i>bla</i> _{TEM-1B} (n=55)	2b Class A β -lactamase	F, H, J, K, J, L, M and N
<i>bla</i> _{TEM-1C} (n=8)	2b Class A β -lactamase	B, G, H, L, M and N
<i>bla</i> _{TEM-13, -55, -135} (n=1)	2b Class A β -lactamase	N
<i>bla</i> _{TEM-29} (n=1)	2be Class A β -lactamase	N
<i>bla</i> _{TEM-33 and -34} (n=1)	2br Class A β -lactamase	C
<i>bla</i> _{TEM-122} (n=1)	2br Class A β -lactamase	N
<i>bla</i> _{TEM-40} (n=2)	2br Class A β -lactamase	B and H
<i>bla</i> _{TEM-57} (n=2)	2b Class A β -lactamase	N and C
<i>bla</i> _{TEM-82-97} (n=1)	2br Class A β -lactamase	B
<i>bla</i> _{TEM-98} (n=1)	Class A β -lactamase	B
<i>bla</i> _{TEM-141-206} (n=2)	2b Class A β -lactamase	C and H
<i>bla</i> _{TEM-150} (n=3)	Class A β -lactamase	B, K and H
<i>bla</i> _{TEM-141} (n=2)	2b Class A β -lactamase	C and N
<i>bla</i> _{TEM-171} (n=2)	Class A β -lactamase	B and H
<i>bla</i> _{TEM-208-209, -210, -213, -214, -216} (n=1)	Class A β -lactamase	C
<i>bla</i> _{TEM (-30, -99, -122, -163)} ^c	2br Class A β -lactamase	H
<i>bla</i> _{TEM (-104, -198, -201, -209, -214, -217, -234)} ^c	Class A β -lactamase	
<i>bla</i> _{TEM-164} ^c	2be Class A β -lactamase	
<i>bla</i> _{CMY-2} (n=18)	Class C β -lactamase	E, F, I, K and N
<i>bla</i> _{SED-1} (n=4)	2b Class A β -lactamase	A, K and N
<i>bla</i> _{LAP-2} (n=1)	Class A β -lactamase	M
<i>bla</i> _{OXA-10} (n=1)	Class D β -lactamase	G

^a The number of *E. coli* isolates harboring the specific beta-lactamase gene variant(s); ^b Letters (A to N) represent names of farms where a specific beta-lactamase gene variant is detected in *E. coli*. ESBL: extended-spectrum beta-lactamases; 2b: broad-spectrum; 2be: extended-spectrum; 2br: Inhibitor-resistant broad-spectrum. The phenotypic classification or functional information is adapted from https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes and beta-lactamase database (<http://bldb.eu/BLDB.php?prot=A>).

Table 4. 12. Resistance genes co-harbored with beta-lactamase genes and their function in ESBL-*E. coli*

Target Antibiotic Class	Resistance Gene and Frequency (n) ^a	Gene Product Description	Resistance Mechanism
Aminoglycosides	<i>aadA</i> (A1, A2, A5, or 17) (n= 82)	Aminoglycoside adenylyl transferase	IBM
	<i>aph(6)</i> -Id(n= 95)	Aminoglycoside-6-Phosphotransferase-Id	IBM
	<i>aph(3'')</i> -Ib(n=91)	Aminoglycoside-3''-phosphotransferase-Ib	IBM
	<i>aph(3'')</i> -Ia(n=27)	Aminoglycoside-3''-phosphotransferase-Ia	IBM
	<i>aac(3)</i> -VIa(n=19)	Aminoglycoside acetyltransferase-3-VI	IBM
	<i>aac(3)</i> -IIId(n=3)	Aminoglycoside-3-N-acetyltransferase- IId	IBM
Macrolides	<i>rmtE</i> (n=5)	16S rRNA methyltransferase	TM
Fluoroquinolones	<i>mph(A)</i> (n=70)	Macrolide phosphotransferase	IBM
	<i>qnrB19</i> (n=168)	FQ resistance protein B19	Target Protection
	<i>qnrS</i> (S1, S3, S4, S7,S8, S10, S11, S12, or S13), (n=54)	FQ resistance protein S	Target Protection
	<i>qnrB5</i> (n=15)	FQ resistance protein B5	Target Protection
	<i>qnrB81</i> (n=15)	FQ resistance protein B81	Target Protection
	<i>ParC*</i> (n=229)	DNA topoisomerase IV	TM
	<i>GyrA*</i> (n=109)	Altered GyrA protein	TM
	<i>ParE*</i> (n=32)	Altered DNA topoisomerase IV	TM
	<i>GyrB*</i> (n=14)	Altered GyrB protein	TM
	<i>tet(A)</i> (n=178)	TTC resistance protein	Antibiotic efflux
Tetracyclines	<i>tet(B)</i> (n=35)		Antibiotic efflux
	<i>tet(M)</i> (n=5)		Target Protection
Chloramphenicol	<i>floR</i> (n=120)	FLO/CHL export protein	Antibiotic efflux
	<i>cmlA1</i> (n=4)	CmlA1 protein	IBM
Folate pathway antagonist	<i>Sul</i> (<i>sul1</i> , <i>sul2</i> , <i>sul3</i>) (n=167)	Dihydropteroate synthetase	TM
	<i>dfrA</i> (A1, A8, A12, A14, A17, A23, A24) (n=79)	Dihydrofolate reductase	TM
Lincosamides	<i>Inu(F)</i> (n=25)	Lincosamide nucleotidyl transferase	IBM
Rifamycin	<i>arr-2</i> (n=1)	Rifampin ADP-ribosyl transferase	IBM
Fosfomycin	<i>fosA</i> (n=1)	Fos modifying protein	IBM

^a The number of *E. coli* isolates harboring the specific resistance gene. FQ.: fluoroquinolones;

CHL: Chloramphenicol; FLO: Florfenicol; Fos; Fosfomycin; TTC: tetracycline; IBM:

inactivation by drug modification; TM: target modification;*shows a chromosomal mutation that leads to target modification potential causing resistance to fluoroquinolones.

Table 4. 13. The distribution of frequent ESBL-*E. coli* sequence types (STs) and the *bla*_{CTX-M} gene variant associated with each clone

STs and Frequency (n) ^a	<i>bla</i> _{CTX-M} Gene Variant and Frequency (n) ^b	Farm and Frequency of the STs (n) ^c
ST2325 (n=37)	CTX-M-32 (n=22), CTX-M-27(n=3), CTX-M-15 (n=8), CTX-M-15 &-27 (n=2)	A (n=14), B (=2), H (n=1), K (n=10), M (n=6), N (n=4)
ST9967 (n=25)	CTX-M-15 (n=24), CTX-M-1 (n=1),	C (n=24), J (n=1)
ST10 (n=22)	CTX-M-15 (n=2), CTX-M-1 (N=5), CTX-M-65 (n=11), CTX-M-27 (n=4), CTX-M-32 (n=1)	D (n=2); F (n=2), H (n=3), J (n=4), K(n=2), L (n=5), M (n=4)
ST540 (n=16)	CTX-M-32 (n=16)	F (n=15), K (n=1)
ST1423 (n=11)	CTX-M-65 (n=11)	H (n=11)
ST1588 (n=8)	CTX-M-15 (n=8)	C (n=7), D (n=1)
ST117 (n=7)	CTX-M-27 (n=1), CTX-M-65 (n=1)	D (n=1), F (n=5) ^d , I (n=1)
ST224 (n=7)	CTX-M-55, & -27 (n=3), CTX-M-55 (n=4)	G (n=3), M (n=4)
ST1011 (n=7)	CTX-M-65 (n=7)	K (n=7)
ST398 (n=6)	CTX-M-32 (n=6)	F (n=5), L (n=1)
ST4089 (n=5)	CTX-M-27 (n=5)	C (n=4), K (n=1)
ST155 (n=5)	CTX-M-1 (n=3), CTX-M-27 (n=2)	N (n=3), M (n=1), J (n=1)
ST515 (n=4)	CTX-M-32 (n=4)	C (n=1), F (n=1), I (n=2)
ST5044 (n=4)	CTX-M-65 (n=4)	L (n=4)
ST580 (n=5)	CTX-M-32 (n=2), CTX-M-1 (n=3)	K (n=2), N (n=3)
ST1122 (n=3)	CTX-M-32 (n=2), CTX-M-65 (n=1)	L (n=3)
ST7585 (n=3)	CTX-M-1 (n=3)	J (n=3)
ST297 (n=3)	CTX-M-65 (n=3)	K (n=3)
ST5265 (n=2)	CTX-M-1 (n=2)	L (n=2)
ST8323 (n=2) ^e	-	I (n=2)
ST410 (n=2)	CTX-M-27 (n=2)	M (n=2)
ST58 (n=2)	CTX-M-65 (n=1)	I (n=1), J (n=1)
ST278 (n=2)	CTX-M-65 (n=2)	J (n=2)
ST345 (n=2)	CTX-M-65 (n=2)	J (n=1), K (n=1)
ST216 (n=2)	CTX-M-65 (n=2)	J (n=2)
ST164 (n=2)	CTX-M-65 (n=2)	H (n=2)

^a The number of isolates with the specific sequence types; ^b Number of ESBL-*E. coli* isolates or sequence types harboring a given *bla*_{CTX-M} gene variant; ^c Letters (A to N) represent farms, and the number in brackets shows the number of sequence types obtained from a farm; ^d The five ST117 isolated from Farm F do not contain the *bla*_{CTX-M} gene. ^e The two isolates do not harbor *bla*_{CTX-M} genes and other resistance genes except a single mutation in the *ampC* gene promoter region and *parC* gene. STs: Sequence types

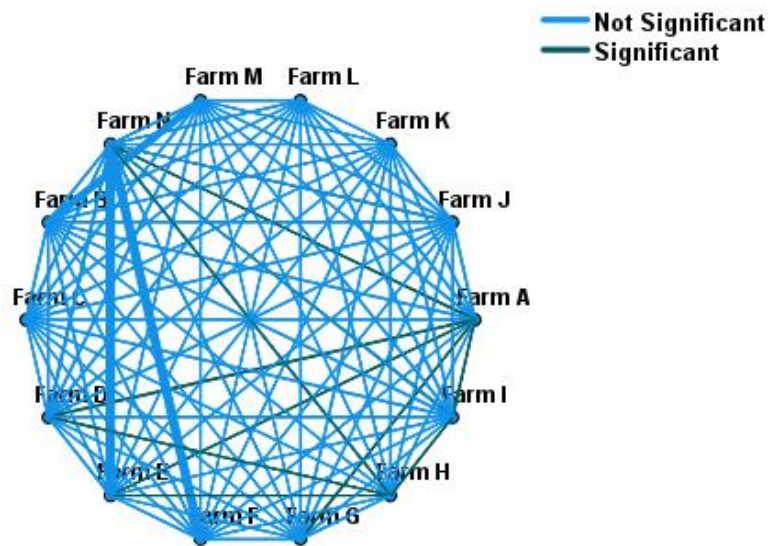


Figure 4.1. Pairwise comparison of within-herd fecal ESBL-*E. coli* prevalence across 14 farms
Farms connected with green light show statistically significant variation in their within-herd prevalence, whereas no difference for those farms connected with light blue lines

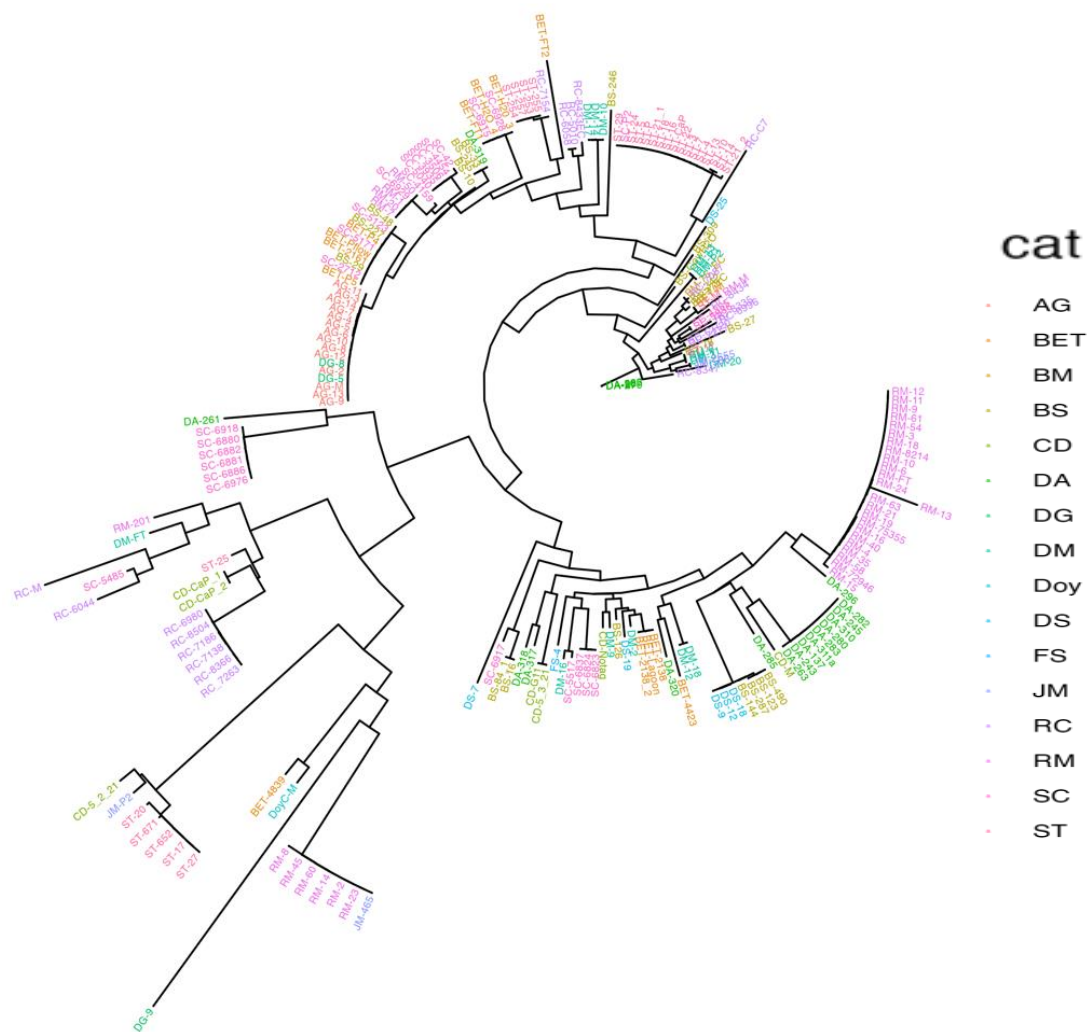


Figure 4.2. Phylogenetic tree of extended-spectrum beta-lactamases-producing *E. coli* Isolates sharing the same color coding were collected from the same farm. The letters indicate the farm and county, while the numbers represent the individual animal's ID within the farm (except manure, feed, and water samples). Typically, isolates originating from the same farm tend to cluster together, although there are also instances where isolates from different farms (isolates with different colors) cluster together.

**Chapter 5. Antimicrobial Resistance and Molecular Epidemiology of
Extended-Spectrum Beta-Lactamase-Producing-*Klebsiella* Species in East
Tennessee Dairy Cattle Farms**

Abstract

Klebsiella species commonly inhabit the gut of dairy cattle. Frequent use of ceftiofur in dairy farms may impose selection pressure and result in the emergence of extended-spectrum beta-lactamase (ESBL)-producing strains. However, information on the status and spread mechanism of ESBL-*Klebsiella* spp. in dairy farms is largely unknown in the U.S. dairy farms. This study aimed to determine the prevalence, antimicrobial resistance, and spread mechanisms of ESBL-*Klebsiella* spp. in dairy cattle farms. Rectal fecal samples (n= 508) and manure, feed, and water samples (n=64) were collected from 14 dairy farms in East Tennessee. The samples were processed and plated directly on CHROMagar™ ESBL. Presumptive ESBL-*Klebsiella* spp. were confirmed using MADLI-TOF MS. The isolates were subjected to antimicrobial susceptibility testing (AST) and whole genome sequencing (WGS). From 572 samples, 57 (10%) were positive for ESBL-*Klebsiella* spp. The prevalence of fecal ESBL-*Klebsiella* spp. was 7.2 % (95% CI: 6.5 - 8.0). Most (96.5%, n=57) ESBL-*Klebsiella* spp. were resistant to ceftriaxone. About 19% of ESBL-*Klebsiella* spp. were multidrug resistant (MDR; resistance to ≥ 3 antimicrobial classes). Six families of beta-lactamase (*bla*) genes (*bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXY}, *bla*_{OXA}, and *bla*_{SED}) were detected in ESBL-*Klebsiella* spp. genomes. Most (93%) isolates harbored two or more *bla* genes. The isolates were genotypically MDR, with 26 distinct types of antibiotic resistance genes (ARGs) and point mutation in *gyrA*, *gyrB*, and *parC* genes. The *Klebsiella* genomes also harbored 22 different plasmid replicon types. The IncFII and Col440I plasmids were most frequent and were associated with *bla*_{CTX-M-27} and *qnrB19* genes, respectively. Fifteen distinct sequence types (STs) and ten unknown STs of *K. pneumoniae* were detected. Clusters of ESBL-*Klebsiella* strains with identical STs, identical plasmids, and ARGs were detected in multiple farms indicating possible animal-to-animal transmission and inter-farm spread. The same ESBL variant was linked to identical plasmids in different *Klebsiella* STs, suggesting a possible horizontal spread of the ESBL genes. The high burden of ESBL genes per *Klebsiella* spp. genome coupled with the dual spread mechanism could rapidly increase the prevalence of ESBL-*Klebsiella* and pose a risk to the health of humans, animals, and the environment.

Keywords: *Klebsiella* species, Extended-spectrum beta-lactamase, prevalence, Whole genome sequence, dairy farm, plasmids, transmission

5.1 Introduction

Klebsiella spp. belong to the genus *Klebsiella* in the family of *Enterobacteriaceae*. *Klebsiella* is an opportunistic pathogen and a natural inhabitant of the gastrointestinal tract of healthy animals and humans (2). *Klebsiella* spp. is identified as one of the most concerning bacteria involved in carrying and disseminating genes mediating resistance to the highest priority and critically important classes of antibiotics (HPCIA), such as third-generation cephalosporins (3GCs) (3, 4). Studies conducted in human health settings documented that *Klebsiella* spp. is one of the most effective bacteria in disseminating extended-spectrum beta-lactamase (ESBL) genes, which confer resistance to 3GC. The bacteria transfer ESBL genes vertically to their subsequent generation and horizontally by acting as a donor via mobile genetic elements (MGEs (5, 6).

Klebsiella spp. is recognized as one of the most frequent causes of community and hospital-associated infections caused by ESBL-producing bacteria in Europe (3) and the U.S. (7). The U.S. Centers for Disease Control and Prevention (CDC) recently reported a 50% increase in ESBL-producing *Enterobacteriaceae* infections over six years from 2012 to 2017. Surprisingly, nearly half of these cases were community-associated and had no prior history of healthcare exposure. This rapid increase has led the CDC to designate ESBL-producing *Enterobacteriaceae*, including *Klebsiella* spp., as a "serious threat" to public health (7). However, as almost all previous studies on ESBL-producing *Klebsiella* spp. focused on humans [1, 7, 8], the cause for the rapid increase in ESBL infections in community settings is not yet understood. Thus, further research is needed to identify potential non-human reservoirs of ESBL-producing *Klebsiella* spp. to design appropriate control measures to prevent their spread.

Klebsiella spp. is a common member of dairy cattle's normal gut microbiota, and a carriage rate as high as 100 % has been reported in the U.S. (8). In addition, *Klebsiella* spp. are frequently isolated from dairy cattle's normal gut and other body parts, including teat-end (9, 10), indicating contamination of the udder and teat with bacteria shed through feces into the environment. In addition to being a common colonizer of animal gut, some *Klebsiella* spp. are among the primary cause of coliform mastitis in dairy cattle. *Klebsiella pneumoniae* and *K. oxytoca* are among the most frequent causes of environmental mastitis and mastitis-related culling (8-10).

As a common colonizer of dairy cattle's gastrointestinal tract (GIT) and cause of coliform mastitis, *Klebsiella* spp. is frequently exposed to beta-lactam antibiotics, especially to 3GCs, which is often used for prophylactic and therapeutic purposes in dairy cattle. Previous studies showed that the prevalence of ceftiofur, a veterinary 3GC-resistant *Enterobacteriaceae* is significantly higher in dairy cattle than in humans and other food animals, such as pigs and chickens (11). A recent molecular study indicated a similarity between *K. pneumoniae* isolates from animals and humans, suggesting inter-species transmission of the bacteria (12).

A recent study on *Klebsiella* isolates from mastitic milk in Wisconsin reported a significant resistance to ceftiofur, a common antibiotic used to treat and prevent mastitis caused by non-coliform bacteria (13). However, the authors reported no significant changes in resistance trends against ceftiofur over the years. Another recent whole genome sequencing-based study on mastitic milk from Iowa State reported the isolation of ESBL-*K. pneumoniae* from mastitic milk in dairy farms (14).

Despite the widespread presence of *Klebsiella* spp. in dairy cattle and the extensive use of ceftiofur in dairy farms that may exert selection pressure, this bacterium has received no to very little attention regarding its status as an ESBL producer. For example, *Klebsiella* spp. is not included in the National Antibiotic Resistance Monitoring System (NARMS). As a result, information on the burden of ESBL-*Klebsiella* spp. on U.S. dairy farms and the possible mechanisms of its transmission within and between farms is unknown. Without understanding the status of ESBL-*Klebsiella* spp. and possible means of spreading these bacteria within and between farms, it is challenging to effectively control its spread from dairy farms to humans through different routes. Therefore, this study aimed to determine the prevalence, antimicrobial resistance, and genomic epidemiology of ESBL-*Klebsiella* spp.

The widespread presence of *Klebsiella* spp. in the GIT of dairy cattle, combined with the frequent use of third-generation cephalosporins (3GCs) in dairy farms, has led to the hypothesis that dairy cattle may serve as a potential reservoir for ESBL-producing *Klebsiella* spp.; and ESBL genes that spread vertically and horizontally in the dairy production system.

5.2 Materials and Methods

5.2.1 Sample Collection and Laboratory Analyses

The study design and detailed laboratory analysis methods for this part are already described in Chapter 4, section 4.2. Briefly, this cross-sectional study investigated the prevalence of ESBL-*Klebsiella* spp. in dairy cattle farms and identified ESBL genes and their spread mechanisms through clonal expansion or horizontal transfer of the resistance determinants. Individual animal rectal fecal samples (n= 424 dairy cows, n=84 calves) and farm environmental samples (n= 30 manure, n= 15 feed, and n=19 water) were collected from 14 dairy farms in East Tennessee. In addition, a brief survey on general farm management, antibiotic usage, and animal-level information was collected.

The collected samples were processed and plated directly on CHROMagar™ ESBL (DRG International, Inc., Springfield, NJ, USA). Presumptive ESBL-*Klebsiella* spp. were confirmed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). MALDI-TOF confirmed isolates were tested for antimicrobial susceptibility against 14 antimicrobial agents belonging to 10 classes by minimum inhibitory concentration (MIC) using Sensititre plates (Sensititre™ CMV4AGNF) (Thermo Fisher Scientific, CA, USA) following the manufacturer's protocol and the Clinical and Laboratory Standards Institute guidelines (CLSI M100: *Enterobacteriaceae* (1)). Chromosomal DNA was extracted from all antibiotic-resistant ESBL-*Klebsiella* spp. using the Qiagen MagAttract HMW DNA kit (Qiagen, Germantown, MD, USA). The genomic DNA libraries were prepared using the Nextera XT DNA Library prep kit from Illumina Corporation (Illumina Inc., San Diego, CA, USA), following the manufacturer's instructions. Whole genome sequencing (WGS) was conducted on *Klebsiella* isolates (n=56). using Illumina NovaSeq PE250 at the University of Tennessee Genomics Core (Knoxville, TN, USA). The detailed procedures for each technique are described in detail under Chapter 4, sections 4.2.3 to 4.2.7.

5.2.2 Statistical Data Analyses

The study collected raw data and imported it into Microsoft Excel for Windows 10 (Microsoft Corp., Redmond, WA) before transferring it to SPSS (SPSS Statistics for Windows, Version 27.0, IBM Corp, Armonk, NY) for statistical analysis. The unit of analysis was the sample, dairy cattle, farm, and bacterial isolate. The prevalence of ESBL-*Klebsiella* spp. in different sources (units) was determined. Farms were classified as ESBL-positive if the bacteria were detected on CHROMagar™ ESBL from the fecal sample of at least one dairy cattle. Descriptive (frequency) statistics were used to summarize the collected data, such as proportions across various categories. Inferential (mixed-effects logistic regression) statistics were used to analyze the data and evaluate the association between selected predictors and the odds of ESBL-*Klebsiella* spp. fecal carriage. The significance level for all statistical analyses was set at 0.05. The details of the data analysis are described in Chapter 4, section 4.2.9.

5.2.3 Whole Genome Sequence Data Processing and Analyses

The whole genome sequence data analysis involved demultiplexing of the sequence data, followed by quality assessment using FastQC v0.11.9 (15) and multiQC v1.14 (16). Trimmomatic v0.39 was used for removing adapters, filtering, and trimming raw reads that had low quality (17). Preprocessed reads of each isolate were then subjected to de novo assembly using the default parameters of the SPAdes genome assembler v3.15.5 (18). For downstream analysis, PlasmidSPAdes modes were used to assemble only the plasmids. Several web-based online bioinformatics tools were used to analyze the genetic characteristics of bacterial isolate To confirm bacterial spp. KmerFinder 3.2 (<https://cge.food.dtu.dk/services/KmerFinder/>) (19-21) and https://pubmlst.org/bigsdb?db=pubmlst_rmlst_seqdef_kiosk) were used. Antibiotic resistance genes (ARG) and mutations conferring resistance were detected using (<https://cge.food.dtu.dk/services/ResFinder/>) (22, 23) and CARD (https://card.mcmaster.ca/analyze/rgi_) (24). Plasmids associated with ARGs were identified using plasmid finder (<https://cge.food.dtu.dk/services/PlasmidFinder/>) (25, 26), and their sequence types were characterized using pMLST 2.0 (<https://cge.food.dtu.dk/services/pMLST/>). Mobile genetic elements (including plasmids) associated with ARGs were identified using

MobileElementFinder (<https://cge.food.dtu.dk/services/MobileElementFinder/>) (26-28). The ESBL-*Klebsiella* spp. sequence type (ST) was determined based on seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) as described in Diancourt et al. (29). The ST of the *Klebsiella* spp. were assigned using tools at the Center for Genomic epidemiology (<https://cge.food.dtu.dk/services/MLST/>) and the *Klebsiella* Pasteur MLST sequence database (https://bigsd.b.pasteur.fr/cgi-bin/bigsd/bigsd.pl?db=pubmlst_klebsiella_seqdef&page=sequenceQuery). The combination of best matching allele was selected to determine the sequence type of each *Klebsiella* spp.

The same methods and tools used to construct *E. coli* phylogenetic tree were used to create a phylogenetic tree of *K. pneumoniae* isolates. Briefly, GToTree (version 1.7.08) was used to analyze 172 single-copy genes from the genome assemblies (30). Gene lengths that varied from the median length by 20% or more were excluded on a per-sample basis. IQ-TREE (version 2.2.0.3) was then used to generate a consensus tree with 1000 bootstrap replicates (31), and ggtree was used to produce tree visualizations (32).

5.3 Results

5.3.1 Microbiological Results

Based on the results of the phenotypic screening of samples on the chromogenic agar (CHROMagar™ ESBL), 221 metallic blue colonies were recovered from rectal fecal, manure, water, and feed samples. Out of 221 isolates, 25.8 % (n=57 isolates) were confirmed to be *Klebsiella* spp. by MALDI-TOF MS. The rest were *Citrobacter sedlakii* (n=132 isolates), *Citrobacter amalonaticus* (n=7), *Citrobacter koseri* (n=5), *Citrobacter freundii* (n=3), *Enterobacter cloacae* (n=8), *Enterobacter bugandensis* (n=3), *Enterobacter asburiae* (n=2), , and *Proteus vulgaris* (n=4). From 57 *Klebsiella* spp. the majority, 87.7% (50/57) of isolates, were isolated from rectal feces, whereas the remaining 12.3% (7 isolates) comprised two isolates each from feed and water and three isolates from pooled manure samples. We identified four different *Klebsiella* spp. using MALDI-TOF MS. These include *K. pneumoniae* (91%; n=57), *K. variicola* (2 isolates), *K. oxytoca* (2), and *K. aerogenes* (1).

5.3.2 Fecal Prevalence of *Klebsiella* Species at Farm Level

The weighted fecal prevalence of ESBL-*Klebsiella* spp. in dairy cattle was 7.2% (95% CI: 6.5 - 8.0). *Klebsiella* spp. were recovered from at least one fecal sample of cattle in five farms (n=14), resulting in herd-level fecal prevalence of 35.7% (95% CI: 12.7–64.8). The within-herd prevalence of fecal ESBL-*Klebsiella* among positive herds ranged from 4% to 62.4%. Eighty percent (n=57) of fecal and 73.6% of all *Klebsiella* spp. isolates were obtained from a single farm, Farm M (Table 5.1, placed under the appendix).

ESBL-*Klebsiella* spp. were recovered from all sample types (Table 5.2, placed under appendix). Furthermore, the prevalence of ESBL-*Klebsiella* spp. in combined samples of manure, water, and feed) (10.9%, n=64) was not significantly different ($P=0.22$) from that of rectal fecal samples (9.8%), as determined by mixed effects logistic regression analysis (result not shown).

5.3.3 Factors affecting Fecal ESBL-*Klebsiella* species Prevalence in Dairy Cattle

Parity was a risk factor for fecal carriage of ESBL-*Klebsiella* spp. The odds of cows with parity greater than or equal to three were about five times [$OR_{adj} = 4.9$ (95% CI: 3.0 - 8.03), $p < 0.001$] of cows with parity ≤ 2 to carry ESBL-*Klebsiella* spp. in their feces. The age and parity of cows were strongly correlated ($R=0.73$; $p < 0.001$); thus, the age of the cows was not included in the analysis.

The weighted fecal prevalence of ESBL-*Klebsiella* spp. was 13.3% (95% CI: 11.1 - 15.7) in calves and 6.2% (95% CI: 5.4 - 7.1) in adult cows. The odds of recovering ESBL-*Klebsiella* spp. from calf feces were about ten times higher than that of cows [$OR_{crude} = 10.4$ (5.7 - 18.8), $p < 0.001$] (Table 5.3, placed under appendix). Among dairy cattle, ESBL-*Klebsiella* spp. was isolated from only lactating cows and none from dry cows. Similarly, all ESBL-*Klebsiella* isolates were obtained from animals with no recent treatment history with 3GC cephalosporins. Thus, further analysis was not necessary on these two variables.

5.3.4 Antimicrobial Resistance Profile of ESBL-*Klebsiella* species

As expected, all 57 (100%) of ESBL-*Klebsiella* spp. isolates were resistant to ampicillin. All but two isolates (96.5%; 55/57) from five sample sources were resistant to ceftriaxone. Out of the 14 antimicrobial agents evaluated in the study, the two isolates exhibited resistance only to ampicillin and sulfisoxazole. Both ceftriaxone-susceptible isolates were obtained from the same farm, Farm J. The highest level of acquired co-resistance among ceftriaxone-resistant *Klebsiella* spp. was seen with sulfisoxazole (66.7%; 38/57), followed by tetracycline (15.8%; 9/57) and amoxicillin-clavulanic acid (10.5%; 6/57). Only one isolate, retrieved from a cow fecal sample, exhibited co-resistance to cefoxitin. *Klebsiella* isolates recovered from calves showed the highest level of concurrent resistance against most of the tested antibiotics (Table 5.4, placed under the appendix).

Among the critically important antimicrobials (CIAs), ceftriaxone resistant *Klebsiella* spp. displayed the highest levels of concurrent resistance to streptomycin (10.5%) and gentamycin (8.8%). Among sample sources, ceftriaxone resistant *Klebsiella* spp. isolated from calves showed the highest level of concurrent resistance to the highest priority CIAs; streptomycin (26.3%), azithromycin (15.8%), and gentamycin (10.5%). Acquired concurrent resistance was relatively low for ceftriaxone and fluoroquinolones (3.5%). Importantly, all *Klebsiella* spp. isolates were susceptible to meropenem.

5.3.5 Multidrug Resistant (MDR) *Klebsiella* species Prevalence in Dairy Farms

The overall prevalence of MDR (acquired resistance to ≥ 3 classes of antibiotics) *Klebsiella* spp. isolates were 19.3% (11/57). The prevalence of MDR *Klebsiella* spp. recovered from calves (31.6%; 6/19) was higher than those retrieved from cows (12.9%; 4/31) though the difference was not statistically significant after controlling for the farm effect ($p > 0.05$) (data not shown). The prevalence of MDR *Klebsiella* isolates resistant to at least five classes of antibiotics was 36.4% (4/11), and all of them were recovered from rectal fecal samples collected from the same farm (Farm M). Three-fourths of *Klebsiella* spp. isolated from calves were resistant to at least five classes of antibiotics. Most MRD (90%; 10/11) *Klebsiella* spp. were isolated from rectal

fecal samples, and most of them (80%) were from the same farm (Farm M). No MDR *Klebsiella* spp. was detected in the water and feed sample (Figure 5.1, placed under the appendix).

5.3.6 Distribution of Antimicrobial Resistant ESBL-*Klebsiella* species Across Farms

Klebsiella spp. from Farm M were resistant to 11 out of 14 antimicrobial agents tested in this study, and some resistance patterns were limited to this farm. MDR ESBL-*Klebsiella* spp. with concurrent resistance to six classes of antibiotics (ceftriaxone, azithromycin, cefoxitin, chloramphenicol, trimethoprim-sulfamethoxazole, and gentamicin) were obtained from farm M alone. Ceftriaxone-resistant *Klebsiella* spp. with co-resistance to sulfisoxazole was widespread and detected in all six farms (Table 5.5; placed under appendix).

5.3.7 Whole Genome Sequence (WGS) Result of *Klebsiella* species

Of the 57 *Klebsiella* isolates identified, 56 were subjected to WGS, and one *K. pneumoniae* was not sequenced. The WGS confirmed 51 *K. pneumoniae* and one *K. aerogenes*, as also identified by MALD-TOF. Initially, the MALDI-TOF MS test misidentified two *Klebsiella* isolates as *K. variicola*, but upon WGS analysis, they were indeed *K. pneumoniae*. Therefore, the total number of *K. pneumoniae* isolates was 53. Additionally, two isolates from the same farm were identified as *K. oxytoca* by MALDI-TOF MS and were found to be *K. michiganensis* by WGS analysis. Unfortunately, one *K. michiganensis* genome was contaminated with the *E. coli* genome (43%) and excluded from further downstream WGS analysis. Thus, further genomic analysis was carried out on the remaining 55 *Klebsiella* spp. (53 *K. pneumoniae* and one each of *K. michiganensis* and *K. aerogenes*).

5.3.8 Genetic Determinants Conferring Resistance to Beta-lactam Antibiotics

All sequenced isolates had one or more kinds of ESBL-encoding genes. This study detected six families of beta-lactamase genes from the ESBL-*Klebsiella* spp. genomes. These include *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXY}, *bla*_{OXA}, and *bla*_{SED}. About 93% (51/55) of the isolates had at least two of these gene families. Among the 55 whole genome sequenced isolates, the majority (73%; 40/55) harbored three types of beta-lactamase genes, namely *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM}, in their genomes. Three isolates had four types of beta-lactamase genes, including *bla*_{CTX-M}, *bla*_{SHV},

*bla*_{TEM}, and either *bla*_{OXY} or *bla*_{SED}. Only five isolates carried a single type of beta-lactamase gene, four of which had *bla*_{SHV-2}, and the remaining one had *bla*_{CTX-M}. The remaining ten isolates had two beta-lactamase genes in their genomes.

Interestingly, thirteen isolates of *K. pneumonia* had inhibitor-resistant *bla*_{SHV} genes. Among these, the *bla*_{SHV} variant inhibitor-resistant beta-lactamases, specifically *bla*_{SHV-56} and *bla*_{SHV-26}, were detected in five and seven isolates, respectively. In addition, inhibitor-resistant *bla*_{TEM} variant beta-lactamases, including *bla*_{TEM-33}, *bla*_{TEM-34}, *bla*_{TEM-35}, *bla*_{TEM-36}, and *bla*_{TEM-122}, were each detected in five separate isolates.

Regarding distribution within isolates, the *bla*_{SHV} was the most frequent and was detected in 53 isolates, followed by *bla*_{CTX-M}, and *bla*_{TEM}, which were detected in the genomes of 51 and 40 isolates, respectively. Furthermore, *bla*_{SHV} exhibited the highest level of diversity with 30 identified variants, surpassing the number of variants detected in *bla*_{TEM} and *bla*_{CTX-M}, which had 24 and 8 variants, respectively. In most cases (41 out of 53 isolates), multiple variants of *bla*_{SHV} were found clustered together on the same contigs within a genome of an isolate creating a resistance island. Thirteen isolates from four farms had five different variants of *bla*_{SHV} (*bla*_{SHV-56}, *bla*_{SHV-79}, *bla*_{SHV-40}, *bla*_{SHV-89}, and *bla*_{SHV-85}) co-occurring in the same contigs within their genomes. Eight isolates from three farms that carry the five distinct SHV variants exhibited the same sequence type, ST353.

Similarly, seven isolates had seven different SHV variants (*bla*_{SHV-145}, *bla*_{SHV-78}, *bla*_{SHV-199}, *bla*_{SHV-98}, *bla*_{SHV-194}, *bla*_{SHV-179}, and *bla*_{SHV-26}) in the same contigs within their genomes. The six isolates were obtained solely from farm M, and five belonged to the same sequence type, ST687. Furthermore, eleven isolates had a combination of two to three *bla*_{SHV} variants in their genomes, while the remaining twelve isolates had only a single variant of *bla*_{SHV} in their genomes.

Of the 51 isolates containing *bla*_{CTX-M}, only three had more than one variant of the *bla*_{CTX-M} gene, while 48 had only one variant. However, two *Klebsiella* isolates from the same farm harbored six distinct variants of the *bla*_{CTX-M} genes per genome. These variants include *bla*_{CTX-M-27}, *bla*_{CTX-M-146}, *bla*_{CTX-M-32}, *bla*_{CTX-M-61}, *bla*_{CTX-M-138}, and *bla*_{CTX-M-1}. All these genes were located

on the same contigs except for *bla*_{CTX-M-27}. One of these isolates was obtained from water, while the other was from a calf. The water isolate had an additional seven variants of the *bla*_{SHV} gene, whereas the calf isolate had three *bla*_{SHV} variants. In this study, *bla*_{CTX-M-27} was the most frequent *bla*_{CTX-M} allele, followed by *bla*_{CTX-M-1} and *bla*_{CTX-M-65} (Table 5.6, placed under appendix). Four isolates (all from Farm J) did not possess *bla*_{CTX-M}. However, three of them carry *bla*_{SHV-2} variants, while the fourth isolate had five different variants of *bla*_{SHV}, namely *bla*_{SHV-79}, *bla*_{SHV-40}, *bla*_{SHV-89}, *bla*_{SHV-56}, and *bla*_{SHV-85}. All isolates from Farm A, B, and G; two isolates from Farm M and one from Farm L harbored *bla*_{CTX-M-1}. Among the two rare *Klebsiella* spp. reported in this study, *K. Michiganensis* had *bla*_{CTX-M-1} and *bla*_{OXY-1-7} genes, while *K. aerogenes* had *bla*_{CTX-M-65}. Neither species had *bla*_{SHV} or *bla*_{TEM} genes in their genome.

Among 24 different variants of *bla*_{TEM}, the *bla*_{TEM-1B} was detected in 97.6% (40/41) of *Klebsiella* isolates carrying this gene, making it the most prevalent variant. Only two isolates possessed *bla*_{TEM-1c}. The remaining 22 *bla*_{TEM} variants were found in just one *Klebsiella* isolates each. Unlike SHV variants, most *Klebsiella* isolates contained only one variant of *bla*_{SHV} in their genomes. Nonetheless, two isolates had an unexpectedly high number of *bla*_{SHV} variants in their genomes. One isolate harbored seventeen *bla*_{TEM} variants, including four inhibitor-resistant beta-lactamases (*bla*_{TEM-33}, *bla*_{TEM-34}, *bla*_{TEM-35}, and *bla*_{TEM-36}) and fourteen other variants (indicated with the superscript "e" in table 5.6) on a contig. Similarly, other *Klebsiella* isolates from the same farm harbored nine *bla*_{TEM} variants; all co-located on the same contigs (the genes are indicated with the superscript "f" in Table 5.6).

5.3.9 Horizontally Transferable Resistance Genes in ESBL-*Klebsiella* Isolates

Apart from beta-lactamase genes, *Klebsiella* spp. harbored resistance genes against eight different classes of antibiotics in their genomes (Table 5.7, placed under the appendix). All 55 *Klebsiella* isolates were genotypically multidrug resistant, carrying resistance genes to as high as seven classes of antibiotics in their genomes. In addition to the beta-lactamase genes, about 26 resistance genes mediating resistance to about eight classes of antibiotics were detected. These include resistance genes against (fluoro)quinolones, macrolides, aminoglycosides, folate pathway inhibitors, tetracycline, chloramphenicol, lincosamides, and phosphonic acids.

Multiple variants of resistance genes against specific classes of antibiotics were detected, such as five variants of each of the fosfomycin and sulfonamide resistance genes and four variants of each of the aminoglycoside and quinolone resistance genes. Among these genes, the most frequently occurring ones were the Fosfomycin resistance gene (*fosA*) and multidrug efflux pump encoding genes (*oqxAB*), present in the genome of 53 isolates. The plasmid-mediated fluoroquinolone resistance gene, *qnrB19*, was detected in 36 *Klebsiella* isolates.

Isolates from the same farm exhibited similar resistance gene patterns. For instance, all isolates from Farm B had the same resistance pattern, with respect to the *bla*_{CTX-M} variant type (*bla*_{CTX-M-1}), the presence of macrolide resistance gene (*mph(A)*), and sulfonamide resistance gene (*dfrA1*). Similarly, all isolates from Farm J had *bla*_{SHV-2} genes (no *bla*_{CTX-M} or *dfrA1*). In contrast, identical sequence types between farms showed the same resistance pattern. For example, ST353 detected in three farms (Farm A, B, and J) carried the same gene variants, including *bla*_{CTX-M-1}, *mph(A)*, *bla*_{SHV}, *dfrA1*, *qnrB19*, and *fosA*.

5.3.10 Frequent Chromosomal Mutation Detected in ESBL-*Klebsiella* species

The most frequent chromosomal mutation detected in ESBL-*Klebsiella* genomes were DNA gyrases (*gyrA* and *gyrB*) and topoisomerase IV (*parC*). Multiple mutations that mediate fluoroquinolone resistance, *gyrA*, and *parC*, were detected in all 55 *Klebsiella* spp. genomes. However, mutations in the *gyrB* gene were relatively rare, with only six *Klebsiella* spp. genomes showing these mutations.

5.3.11 Plasmid Replicon Types and Their Association with Resistance Genes

From the WGS data of 55 ESBL-*Klebsiella* isolates obtained from six farms, Plasmid Finder identified 22 different plasmid replicon types. On average, each ESBL-*Klebsiella* isolate harbored five different types of replicons, with a minimum of two and a maximum of nine replicon types. Isolates with similar genotypes tend to have similar numbers and replicon types of plasmids. The most frequently detected replicon types in this study were IncF family plasmids (IncFII, IncFIA, and FIB) and Col plasmids such as Col440I, Col(IMGS31), Col(MG828), ColRNAI, Col(pHAD28), ColpVC, Col(BS512), and Col156. Additionally, a limited number of

isolates were found to harbor broad-host-range plasmids, including IncH plasmids (such as ncHI2A and IncHI2), IncN, and IncR.

Most (96.4%) of the *Klebsiella* isolates' genomes harbored the Col440I plasmid. Among the narrow host range, IncF family plasmids, including FII, IncFIA, and FIB replicon types, were highly prevalent, with up to four subtypes of these plasmids present in each isolate. Among the IncF family plasmids, the IncFII plasmid replicon types were the most frequently detected (89%), followed by IncFIB (85%). Notably, all IncFII plasmid replicon types were found to have the same sequence type, ST F2:A⁻:B⁻.

The broad host range plasmids IncN was detected in all nine *K. pneumoniae* with identical STs obtained from three farms (Farms A, B, and G) and *K. michiganensis* strain obtained from Farm L. The IncN plasmid was identified as ST1 in all these isolates and was primarily linked with *bla*_{CTXM-1}. On the other hand, the IncH plasmids (IncHI2A and IncHI2) were detected in only three *Klebsiella* isolates with identical genotypes (ST111), which carried *bla*_{CTXM-65}. The IncN plasmid was not detected in the remaining 45 isolates obtained from the other farms. The IncR plasmids were identified in only two *K. pneumoniae* isolates in a single farm but were not associated with any of the resistance genes identified in this study. Three Novel IncF RSTs (due to novel FIA allele) were detected in three *Klebsiella* isolates obtained from the same farm. Two of them have the same nearest Sequence type, *K4*: A⁻:B⁻, and the ST closest to the third novel allele was *K7*:A⁻:B⁻.

5.3.12 Association Between Resistance Genes and Mobile Genetic Elements

Most isolates (88.5%; 46/52) containing *bla*_{CTX-M} genes were found to have these genes associated with mobile genetic elements (MGEs). In 80% (37/46) of the isolates, *bla*_{CTX-M} was associated with plasmids, while in the remaining 20% of isolates (9 out of 46), it was associated with insertion sequences (IS) of different types. In 67.4% (31/46) of the isolates, the *bla*_{CTX-M-27} and *bla*_{TEM-1B} genes co-occurred and were associated with epidemic resistance plasmids of the kind of IncFII, which had identical plasmid sequence types, STF2:A⁻:B⁻. In six isolates obtained from three different farms, the genes *bla*_{CTX-M-1} and *mph*(A) were both associated with broad host

range plasmids of the IncN type, which were subtyped as ST1. The gene *bla*_{CTX-M-65}, often detected in ST111, was associated with IncHI2 ST3 or IS102, or IS26. In addition to *bla*_{CTX-M}, multiple variants of *bla*_{SHV} were seen to be associated with MGEs, such as Integrative Conjugative Element (ICEKp1). Other resistance genes, such as *aph*(6)-Id, *aph*(3'')-Ib, and *sul2*, were also co-harbored with different variants of *bla*_{CTX-M} on the same MGEs. Similarly, other resistance genes were often associated with MGEs (for example, *qnrB19* was associated with col440I plasmids in 73% of the isolates containing the resistance genes). Additionally, the *tet*(A) gene was often found to be associated with broad host range plasmids of the IncHI2A type with ST3 (Table 5.8, placed under the appendix).

5.3.13 Genetic Diversity and Spread Mechanisms of ESBL-*Klebsiella* species in the Dairy Farms

Fifteen distinct sequence types (STs) of *K. pneumoniae* were identified, along with ten unknown STs. Six STs were found multiple times, while the remaining seven were only found once. The most frequently occurring STs were ST353 (n=8), ST469 (n=6), ST687 (n=5), ST322 (n=4), ST111 (n=4), ST2239 (n=4), ST867 (n=3), and ST225 (n=2). The other seven STs were observed only once, including ST34, ST6250, ST2703, ST36, ST294, ST661, and ST419. The ST of *K. michiganensis* was unknown, but its ST is nearest to ST446, while *K. aerogenes* was identified as ST57.

ESBL-producing *Klebsiella* spp. were found to spread both clonally through specific *Klebsiella* spp. sequence types, and horizontally, via plasmids between genetically unrelated *Klebsiella* spp. Some of the *Klebsiella* STs showed both within-farm and between-farm similarity. ST353, for example, was found in three different farms: three isolates, each from farms A and B, and two isolates from farm G. All these isolates carry the same ESBL variant (*bla*_{CTX-M-1}) associated with plasmids with identical sequence types, IncN (ST1). Farm A and B are in the same county, but different cities, and Farm G is in a separate county.

On the other hand, some STs were only detected within a single farm. For instance, all four ST322 isolates were found exclusively in farm J and had the same *bla*_{SHV-2} variants. One of these STs was isolated from a water sample collected from water troughs.

Farm M had the largest number of sequenced *Klebsiella* isolates (76.4%; 42/55) and displayed the greatest diversity regarding *Klebsiella* STs. It contained 13 distinct sequence types of *K. pneumoniae* and ten unknown STs. The most common STs in this farm include ST469 (n=6), and ST687 (n=5), followed by ST111 and ST2239, each occurring four times in the farm. Despite the genetic diversity, most isolates in farm M harbored indistinguishable plasmid (e.g., IncFII STF2:A⁺B⁻) associated with the same variants of ESBL (*bla*_{CTX-M-27}). The same ESBL variant was also detected associated with the same plasmid sequence types among diverse STs of *Klebsiella* spp. A similar pattern was also observed for other resistance genes, such as *qnrB19* and *tet*(A) (Table 5.8). In addition, the phylogenetic tree displayed several clusters of *Klebsiella* isolates within a farm, and some isolates from a different farm were also clustered together (Figure 5.2, placed under the appendix)).

5.4 Discussion

This study identified ESBL-producing *Klebsiella* spp. in 10 % (57/572) of the collected samples and from all sample types (fecal samples, pooled manure, animal feed, and water from watering troughs). The presence of ESBL-*Klebsiella* spp. in animal feed and watering troughs could play a crucial epidemiological role in maintaining and spreading the bacteria on farms and serving as a critical point source of transmission (12). Detection of ESBL-*Klebsiella* in all samples confirms the previous studies that reported the pervasive presence of the bacteria in dairy cattle environments (8-10, 34).

The herd-level prevalence of ESBL-*Klebsiella* spp. was 35.7%. This result is higher than a study from Japan that reported a lower herd-level prevalence of 15% (3/20) (35). In this study, the within-farm fecal prevalence of the ESBL-*Klebsiella* spp. ranged from 4% to 62.4%, highlighting the variability in the ESBL-*Klebsiella* spp. prevalence across farms. This variation may be related to the difference in farm management practices, hygienic farm measures in keeping clean and dry, and antibiotic use patterns that may affect the emergence and spread of ESBL-*Klebsiella* spp. A previous study conducted in New York found a high within-herd fecal prevalence of 8 -100% of *Klebsiella* spp. in most of the studied dairy farms (36). However, it is

less plausible to directly compare the current study and the New York study since the latter did not investigate the ESBL status of the bacteria.

The prevalence of ESBL-*Klebsiella* spp. in fecal samples, adjusted for sampling weight, was 7.2% (95% CI: 6.5 - 8.0%). Previous studies have reported as high as 84% prevalence of fecal *Klebsiella* spp. in the U.S. and 27% in China from healthy adult dairy cattle (8, 36, 37).

However, there is a lack of published research on the prevalence of ESBL-*Klebsiella* spp. in the rectal feces of dairy cattle. The fecal prevalence of ESBL-*Klebsiella* spp. in the present study is similar to a previous report (10%; 3/28) from Japanese dairy farms (35).

While the fecal prevalence of ESBL-*Klebsiella* spp. may seem lower than that of ESBL-*E.coli* reported in the U.S. (38) and other countries (39-41). However, the occurrence of ESBL-*Klebsiella* in the tested samples is concerning because of their potential to cause hard-to-treat infections in humans if transmitted (42) and mastitis in dairy cattle (13). It also poses a significant risk of spreading to humans, animals on the farm, and the environment. This suggests that dairy cattle may be potential sources of ESBL-*Klebsiella* spp.

The study showed that the odds of carrying ESBL-*Klebsiella* spp. in their feces for cows with parity greater than or equal to three were about five times higher than those with parity ≤ 2 . The reason for this association could be related to the fact that cows with higher parity may have more lifetime exposure to beta-lactam antibiotics for treatment of mastitis and more prolonged exposure to the ESBL-*Klebsiella* contaminated farm environment and other animals, which in turn increases the risk of acquiring ESBL-producing bacteria. The results of this study align with that of earlier studies that indicated older cows have a higher risk of having mastitis, which is potentially linked to increased beta-lactam antibiotic use and selection pressure that may lead to the occurrence of ESBL-producing *Enterobacteriaceae* (43-45). This finding has important implications for controlling the spread of ESBL-*Klebsiella* spp., at the farm level. Farmers and veterinarians should be aware of the increased risk of fecal carriage of ESBL-*Klebsiella* spp. in cows with higher parity and take appropriate measures to prevent their spread to humans, animals, and environments.

The present study found that the odds of recovering ESBL-*Klebsiella* spp. from calf feces were approximately ten times higher than that of cows, with a crude odds ratio 10.4 (95% CI: 5.7-18.8). We did not find published studies comparing ESBL-*Klebsiella* spp. prevalence between dairy calves and adult dairy cattle. However, this finding is consistent with previous studies that reported a higher proportion of ESBL-producing *E. coli* in calves than in older cattle (41, 46-51). The reason for this higher prevalence remains speculative, but selective pressure from feeding medicated milk replacers or a potential fitness advantage in calves (40, 47, 48, 52, 53), as discussed in Chapter 4, might be responsible for this difference

Among 57 ESBL-*Klebsiella* spp. isolated from fecal and farm environments, 55 (96.5%) were resistant to ceftriaxone. This result was predictable since isolates were initially screened on ESBL selective media that select isolates resistant to third-generation cephalosporins. Resistance to ampicillin was observed in all ESBL-*Klebsiella* spp., as expected since *Klebsiella* spp. are intrinsically resistant to penicillin (54-56).

After ceftriaxone, ESBL-*Klebsiella* spp. showed the highest acquired co-resistance to sulfisoxazole, followed by tetracycline. This resistance pattern was widespread across six farms. This could be related to the increased usage of these antibiotics as both antibiotic classes are frequently used to treat dairy calves' digestive and respiratory diseases in the U.S. (57). This finding is consistent with previous studies from the U.S. (58, 59) and China (37) that reported high-frequency of co-resistance to sulfonamides and tetracycline in *Klebsiella* spp. A lower co-resistance frequency to sulphonamides in ESBL-*Klebsiella* spp. was reported from Brazil (9.5%) (60) and Canada (13%) (61), which could be related to the differences in the use of these antibiotics.

Detection of 6 (10.5%) ESBL-*Klebsiella* spp. resistant to amoxicillin-clavulanic acid is not expected as ESBL-producers should be (by definition) susceptible to beta-lactamase inhibitors (clavulanic acid) (62-64). However, this finding suggests that these isolates might carry additional resistance genes mediating resistance to beta-lactam inhibitors such as AmpC beta-lactamases, efflux pumps, or other beta-lactamase variants not inhibited by clavulanic acid (65). In addition, two *K. pneumoniae* isolates were susceptible to ceftriaxone despite being

presumptively identified as ESBL-producers expected to be resistant to ceftriaxone. This may be due to the limited specificity of the CHROMagar™ ESBL used to screen them (66).

Despite being lower than the prevalence of ESBL-*E. coli* reported in the previous chapter, a significant proportion (19.3%) of the ESBL-*Klebsiella* isolates were MDR-ESBL-producing strains. This finding aligns with previous results that reported ESBL phenotypes are often associated with MDR traits (51, 67-73). Concurrent resistance to at least six classes of antibiotics was observed in 36.4% of MDR-*Klebsiella* isolates, and 75% of them were recovered from calves. This may be related to the vulnerability of calves' microbiota to disruption by antibiotic use early in life, creating a selective pressure and conducive environment for the emergence and spread of MDR-bacteria (74-76). However, additional research is needed to fully understand the underlying causes of the observed pattern.

Some MDR patterns observed in some isolates were limited to a single farm, while others were detected in multiple farms. For example, ESBL-*Klebsiella* spp. with concurrent resistance to ceftriaxone, azithromycin, ceftiofur, chloramphenicol, trimethoprim-sulfamethoxazole, and gentamicin were obtained from farm M alone. This suggests that distinct factors could contribute to the emergence and spread of MDR bacteria in various farms. It could be due to the differences in antibiotic use and/or differences in farm management practices. For instance, using certain antibiotics in one farm but not in another could contribute to developing unique MDR patterns in the former. Similarly, varying levels of biosecurity measures (animal density or access to outside environments), waste milk use, disposal method, and medicated milk replacer for calves may affect the MDR patterns in ESBL-*Klebsiella* spp. (77, 78).

We found that ceftriaxone-resistant *Klebsiella* isolates from calves exhibited higher levels of concurrent resistance of 26.3%, 15.8%, and 10.5% to streptomycin, azithromycin, and gentamicin, respectively, than isolates from cows. This may be because of the higher exposure of calves to these antibiotics, leading to selective pressure and the development of resistance to these CIAs. For example, streptomycin and azithromycin are often used to treat digestive and respiratory diseases in dairy calves (57, 79). Compared to adult dairy cows which usually receive intramammary infusion for treatment and prevention of mastitis, dairy calves usually receive

antibiotic treatment orally for GIT related infections (e.g., diarrhea) and parenterally for other health issues (e.g., pneumonia), which has a higher chance of creating selection pressure on microbes in the GIT than intramammary infusion.

We found that all ESBL-*Klebsiella* isolates were susceptible to meropenem, a relatively new beta-lactam antibiotic in the carbapenem class of antibiotics, which is considered the last-line option for treating infections caused by MDR Gram-negative bacteria, including ESBL producers (80). This result is expected since carbapenem antibiotics are not licensed for use in dairy cattle or other food animals in the U.S. (81). Unlike the present study, carbapenem-resistant *Enterobacteriaceae* were previously isolated from the feces of dairy cattle in Texas and New Mexico (82) and swine operations in the Midwest region (83, 84). Thus, it is important to note that antibiotic susceptibility or resistance can vary by farm location, the species of farm animal population studied, and other specific factors involving each farm suggesting that the results of this study may not necessarily equally apply to other settings or populations.

Detection of at least one ESBL-encoding gene in all sequenced *Klebsiella* spp. generally concurs with the phenotypic result where all but two isolates were resistant to ceftriaxone. Two of the *Klebsiella* isolates which were susceptible to ceftriaxone harbored *bla*_{SHV-2} genes. The *bla*_{SHV-2} is the first beta-lactamase that exhibits an ESBL phenotype (3, 65, 85). The lack of phenotypic expression of these genes may be related to a mutation in the gene proper or their regulatory regions in the two isolates, as recent studies have shown that positions, where amino acid substitution occurs dictate the phenotypes of the gene in the isolates (65, 86, 87).

Interestingly, two other *K. pneumoniae* isolates from the same farm harbored only the *bla*_{SHV-2} variant but were resistant to ceftriaxone, suggesting the adequate expression of the gene in these isolates. WGS of the six ESBL-*Klebsiella* isolates resistant to potentiated amoxicillin (amoxicillin-clavulanic acid) revealed that the genome of five of these isolates had inhibitor-resistant (IR) beta-lactamases, four of them had *bla*_{SHV56}, and the remaining one had *bla*_{SHV26} which enable them resistant to beta-lactamase inhibitors. Studies have shown that the specific amino acid substitutions in these *bla*_{SHV} variants confer resistance to beta-lactamase inhibitors such as clavulanate and sulbactam (88-90). So, their resistance cannot be reversed by inhibitors

like clavulanic acid that target class A β -lactamase enzymes, as observed in this study (89). The detection of IR ESBL-producing *Klebsiella* spp. in dairy farms indicates a serious public health risk because of the possibility of their spread to humans through various routes.

Detection of six distinct beta-lactamase gene families (*bla*_{SHV}, *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{OXY}, *bla*_{OXA}, and *bla*_{SED}) in *Klebsiella* genomes in this study supports the idea that these bacteria harbor different types of beta-lactamase genes (3, 5). Detection of two beta-lactamase gene families (*bla*_{SHV}, *bla*_{CTX-M}) in about 93% and three families of beta-lactamase genes (*bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{TEM}) in 73% of ESBL-*Klebsiella* spp. genomes indicate that these bacteria are equipped with armaments of genes to inactivate beta-lactam antibiotics and can serve as a reservoir of these genes (3). This is particularly problematic given that these resistance genes can transfer from *Klebsiella* spp. to other pathogenic bacterial species that cause life-threatening infections in humans and animals that are difficult to treat with available antibiotics (5). A similar level of ESBL burden was reported from *Klebsiella* spp. isolated from a food animal in China (91).

We detected thirty (30) unique variants of *bla*_{SHV} genes in more than 96% of the *Klebsiella* spp. This observation concurs with previous studies that reported *Klebsiella* spp. predominantly harbored *bla*_{SHV} gene variants, which is a key beta-lactamase gene mediating intrinsic resistance to ampicillin (56, 65, 87). Interestingly, in 35 (66%) *Klebsiella* genomes bearing *bla*_{SHV}, multiple variants (ranging from two to seven) of this gene co-occurred in the same contigs. The presence of several *bla*_{SHV} gene clusters in the same contig of the bacterial genome may form a resistance gene island that could be mobilized together and transferred to other bacteria through MGEs, increasing the spread of the resistance genes (92). This finding agrees with the previous report that indicated *Klebsiella* spp. is a “key source and trafficker “of resistance genes, including ESBL genes (3, 5).

Among the 30 *bla*_{SHV} variants, *bla*_{SHV-27}, -11, -13, and -70 occurred frequently, and *bla*_{SHV-27} occurred alone, whereas the remaining three co-occurred, suggesting they were relatively widespread among the *Klebsiella* isolates in the farms. We did not find previous reports of *bla*_{SHV} ESBL variants from *Klebsiella* spp. in U.S. dairy farms. However, a study conducted in Oklahoma in 2005 detected *bla*_{SHV-1} from *K. pneumoniae* isolated from cattle farms. It is unclear whether these

were beef or dairy farms (93). In addition, *bla*_{SHV} variants have been reported in *Klebsiella* spp, isolated from humans in the U.S. (94, 95). Some *bla*_{SHV} variants identified in this study, such as *bla*_{SHV-11}, have previously been reported in *E. coli* and *K. pneumoniae* isolated from dairy farms in Japan (35). In contrast, others, like *bla*_{SHV-2}, have been reported in *E. coli* isolated from beef cattle in Canada (96).

The identification of the *bla*_{CTX-M} gene as the second most prevalent ESBL gene in the *Klebsiella* genomes (93%) supports the recent studies that showed this gene family is rapidly disseminating among ESBL-producing *Enterobacteriaceae* (72, 97-99). However, the result of this study contrasts with the idea that the *bla*_{CTX-M} is replacing other ESBL genes (67, 100) as *bla*_{SHV} was identified as most prevalent among *Klebsiella* isolates and had a greater number of its variants per genome.

Eight *bla*_{CTX-M} gene variants, including *bla*_{CTX-M-1}, *bla*_{CTX-M-15}, *bla*_{CTX-M-27}, *bla*_{CTX-M-32}, *bla*_{CTX-M-61}, *bla*_{CTX-M-65}, *bla*_{CTX-M-146}, and *bla*_{CTX-M-138}, were detected in this study. Most of these gene variants were reported previously from other members of *Enterobacteriaceae*, mainly from *E. coli* isolates from U.S. dairy farms. For instance, *bla*_{CTX-M-1} was reported from *K. pneumoniae* isolates from mastitic milk in Iowa and New York (14, 101). The *bla*_{CTX-M-1} was also detected from *E. coli* and *Salmonella* isolates retrieved from dairy cattle fecal samples obtained from Ohio, Washington, Texas, and the Southwestern region of the U.S., suggesting that it is a common *bla*_{CTX-M} variant in dairy farms (51, 102-104). Due to selective pressure, it is possible that *K. pneumoniae* acquired this gene through horizontal transfer from *E. coli* or other sources.

Similarly, *bla*_{CTX-M-15}, *bla*_{CTX-M-27}, *bla*_{CTX-M-32}, and *bla*_{CTX-M-65} were reported from *E. coli* isolated from fecal samples in different parts of the U.S., including Washington, Texas, and the Southwestern region of the country (51, 68, 72, 102, 104). The *bla*_{CTX-M-61}, *bla*_{CTX-M-146}, and *bla*_{CTX-M-138} were not previously reported from U.S. dairy farms. In addition, except *bla*_{CTX-M-1}, all other *bla*_{CTX-M} variants were not reported from *Klebsiella* isolates from the U.S. dairy farms.

Among the detected *bla*_{CTX-M} genes, *bla*_{CTX-M-27} was the most prevalent (detected in 67% of the *Klebsiella* spp. genomes) but limited to a single farm, whereas *bla*_{CTX-M-1} was relatively

widespread and detected in five farms. Unlike this study, an earlier study reported that *bla*_{CTX-M-15} is the dominant variant, followed by *bla*_{CTX-M-27} in ESBL-*E. coli* isolates from U.S. dairy farms (72). However, *bla*_{CTX-M-15}, which was detected only in one *Klebsiella* isolate in the current study, was reported as the most prevalent *bla*_{CTX-M} variant detected in *Klebsiella* isolates from humans in different parts of the U.S. (105), and elsewhere (100, 106). In addition, *bla*_{CTX-M-15} was also reported from ESBL-*Klebsiella pneumoniae* in France (107), ESBL-*K. ozaena* in Italy (108) and ESBL-*K. oxytoca* in Egypt (109) that were isolated from dairy cows.

The *bla*_{CTX-M-1}, detected in five farms in this study, seems to be the most widespread variant in ESBL-*E. coli* isolates from dairy farms in other countries. It was reported from 17 countries in Europe (108, 110), at least four countries in Asia (China, Japan, South Korea, and Indonesia) (111-114), and Canada (115, 116).

In this study, *bla*_{TEM} was another frequently detected beta-lactamase gene. Among ESBL-*Klebsiella* isolates carrying the *bla*_{TEM} gene, the narrow-spectrum *bla*_{TEM-1B} variant was found in 95% of cases. It is uncertain whether this variant provides a selective advantage to the host bacteria over other variants, necessitating further research to determine the underlying cause. In contrast with the *bla*_{SHV} variants, most *Klebsiella* isolates harbored only one variant of *bla*_{TEM} in their genome, except two isolates, both from the same farm, which had a high number of *bla*_{TEM} variants (one carrying 17 variants and the other one carrying nine variants). The two isolates with an unusually high number of *bla*_{TEM} variants in their genomes might have accumulated these genes via horizontal gene transfer or persistent exposure to antibiotics that led to a series of mutations leading to multiple *bla*_{TEM} variants. Furthermore, clustering multiple variants of the *bla*_{TEM} gene in one contig of a single isolate is concerning because it may facilitate the spread of these genes to other bacteria via MGEs (117).

To our knowledge, no previous studies in the U.S. reported the *bla*_{TEM} gene from *Klebsiella* spp. in dairy farms. However, a study from Pennsylvania reported *bla*_{TEM} in *E. coli*, but the variant specification was not provided (68). Various *bla*_{TEM} gene was also reported from *K. pneumoniae* isolates from humans in the U.S. (94, 95). An extensive European review revealed that several studies documented *bla*_{TEM} variants different from those observed in the current study in *E. coli*

isolates from dairy cattle (110). The lack of previous detection of *bla*_{TEM} and *bla*_{SHV} genes from *Klebsiella* isolates from U.S. dairy farms could be due to limited studies on these resistance genes than the actual absence of these resistance genes, which emphasizes the need for ongoing surveillance and monitoring of bacterial resistance in dairy cattle and other food production environments. It also sheds light on the importance of implementing measures to prevent the spread of this antibiotic-resistant bacteria in these settings.

Three rare β -lactamase genes, *bla*_{SED-1}, *bla*_{OXA-1}, and *bla*_{OXY 1-7}, were detected in *Klebsiella* isolates from dairy farms. Specifically, *bla*_{OXA-1} and *bla*_{SED-1} were detected in *K. pneumoniae*, whereas *bla*_{OXY 1-7} was in *K. Michiganensis*. To our knowledge, this is the first study identifying these resistance genes in *Klebsiella* spp. from U.S. dairy farms.

The *bla*_{SED-1} gene naturally occurs in *Citrobacter sedlakii* (118, 119). *C. sedlakii* constituted most ESBL-producing *Enterobacteriaceae* identified from dairy farms in this study. Thus, *K. pneumoniae* may have acquired this gene via horizontal gene transfer from *C. sedlakii* in the animal GIT. *K. michiganensis* is a recently described novel member of the *K. oxytoca* complex (120, 121). The detection of *bla*_{OXY 1-7} variants of ESBL aligns with previous studies that reported that *K. oxytoca* inherently carries *bla*_{OXY}, a chromosomally encoded class A beta-lactamase gene (122). But plasmid-associated *bla*_{OXY} was recently reported from *K. oxytoca* in Spain (123). But in this study, this gene was not associated with plasmid or any other MGEs, suggesting it is a chromosomal beta-lactamase gene

The genome of ESBL-*Klebsiella* isolates was genotypically MDR, carrying resistance genes for up to seven classes of antibiotics. In addition to beta-lactamase genes, eight horizontally transferable ARGs were detected in *Klebsiella* spp., including resistance against fluoroquinolones, aminoglycosides, macrolides, folate pathway inhibitors, tetracycline, chloramphenicol, fosfomycin, and lincomycin. This finding concurs with previous studies conducted in humans, animals, and environmental samples that showed ESBL-producing *Enterobacteriaceae* carried multiple resistance genes to structurally unrelated classes of antibiotics (67, 73, 80, 93, 124-126).

Like previous studies (51, 93, 102), genes mediating resistance to CIAs, such as aminoglycoside modifying genes, macrolide modifying genes, and plasmid-mediated quinolone resistance genes (PMQR), were detected in a significant number of isolates. The use of 3GC (ceftiofur), fluoroquinolones (enrofloxacin), and macrolides (erythromycin) in dairy calves might result in the co-selection and spread of these resistance genes in *Enterobacteriaceae* in dairy farms (51).

The detection of these ARGs against CIAs in many isolates is concerning, as the horizontal transfer of these genes from commensal *Klebsiella* spp. to other enteric pathogens may impede the treatment of infections caused by these pathogens (127-129). The PMQR, *qnrB*, was found in 36 (66%) isolates, making it the most frequent ARG to CIAs. This finding is consistent with previous studies that reported frequent co-occurrence of ESBL and fluoroquinolone resistance genes in *Enterobacteriaceae* (130-132).

The most frequently detected ARGs on the *Klebsiella* genome with the ESBL gene were the fosfomycin resistance gene (*fosA*) and a multidrug efflux pump (*oqxAB*) gene, each present in 53 (96%) of the isolates. Since its first report in 2004 in *E. coli* isolates from swine in Europe [60, 61], studies have shown that the *oqxAB* gene is increasing among *Enterobacteriaceae* (133, 134). According to an international report on ESBL-producing *K. pneumoniae*, the *oqxAB* gene was found in about 88% of isolates from various hospitals in different countries, including the U.S. (133). In a separate study of *K. pneumoniae* clinical isolates from Ohio, *oqxAB* was also detected in most isolates (133). The co-occurrence of the *oqxAB* and ESBL genes has significant veterinary and human health implications. This gene is often associated with MGE and encodes a multidrug efflux pump that confers resistance to multiple classes of antibiotics, including disinfectants (135).

The high prevalence of the *fosA* gene, which confers resistance to fosfomycin, in *Klebsiella* spp., is worrying, even though the gene's phenotypic expression was not tested in this study. Fosfomycin is a promising broad-spectrum antibiotic used in the U.S. to treat MDR Gram-negative organisms such as ESBL-producing *Enterobacteriaceae* and carbapenem-producing *K. pneumoniae* (136). This resistance gene is considered rare in the U.S. and was reported seven years ago from a clinical ESBL-*E. coli* from a women patient in Pennsylvania (137). Thus,

detecting fosfomycin resistance genes in 96% of *Klebsiella* spp. found in the GIT of dairy cattle and the farm environment is concerning because the same genes may spread to human pathogenic strains through MGEs, or the bacteria may spread to humans via the food chain or direct contact with carrier animals (138-140). Studies showed that *fosA* genes are relatively common in Eastern Asia, particularly China. They were detected from ESBL-*E. coli* isolates from food animals such as dairy cattle and pets (138, 140, 141). More investigation is required to determine the origin of these genes in commensal *Klebsiella* spp. since fosfomycin is not used in U.S. dairy farms. Furthermore, it is vital to examine the phenotypic expression of these genes since fosfomycin was not in the antibiotics panel tested in this study.

Apart from horizontally transferable genes, multiple chromosomal mutations that affect quinolone resistance-determining regions (QRDRs) (*gyrA*, *gyrB*, *parC*) were detected. All 55 *Klebsiella* spp. genomes had QRDRs, including *gyrA* and *parC*, the primary resistance mechanisms to (fluoro)quinolones in Gram-negative bacteria, including *Klebsiella* spp. (142). However, of all the *Klebsiella* isolates tested, only two were resistant to ciprofloxacin, and one of these isolates was obtained from a calf and was simultaneously resistant to ciprofloxacin and nalidixic acid. It is unclear and needs further investigation as to why most *Klebsiella* spp. were still susceptible to (fluoro)quinolones despite carrying both PMQRs determinants and mutations in QRDRs.

This study found that ESBL-*Klebsiella* isolates had diverse plasmids, with an average of five types of replicons per isolate. As noted in earlier studies, this could facilitate the spread of ESBL and other ARGs through horizontal gene transfer (117, 143). In addition, the study also identified three novel plasmid STs of the IncF family (FIA replicon types). The discovery of new plasmid STs can help understand the spread and evolution of ARGs or plasmids and control the spread of antibiotic resistance in different environments (144, 145).

In this study, both narrow-host range or epidemic resistance plasmids (ERPs) of the IncF family (IncFII, IncFIA, and FIB) and broad-host-range plasmids (IncHI2A, IncHI2, IncN, and IncR) were detected. Previous studies also reported similar replicon types of plasmids in ESBL-*E. coli* isolated from dairy farms in the USA (72).

After Col440I plasmid, which was detected in more than 96% of *Klebsiella* isolates' genomes, the conjugative narrow host-range, or ERPs of IncF families were highly prevalent (seen in 89% of the *Klebsiella* genomes) with up to four subtypes of this plasmid present in each isolate. The high prevalence of ERPs in *Klebsiella* spp. is concerning as previous studies showed that these families of plasmids could easily acquire resistance genes and rapidly spread throughout *Enterobacteriaceae*, particularly among specific genotypes (146, 147).

In this study, the IncFII replicon type with ST F2:A⁺:B⁻ was most frequent and was mainly associated with two co-occurring beta-lactamase genes, *bla*_{CTX-M-27} and *bla*_{TEM-1B}, in over 56% (31/55) of sequenced *Klebsiella* isolates. Interestingly, these IncFII replicon type-beta-lactamase variant combinations were detected in isolates obtained from the same farm, suggesting that this specific subtype of plasmid may contribute to the spread of the two genes in *Klebsiella* on the farm. A previous study from the US dairy farm also reported that IncF plasmid families were associated with *bla*_{CTX-M-27} in *E. coli* (72).

The study identified the IncN plasmid ST1 linked to *bla*_{CTX-M-1} in six clonally related *K. pneumoniae* isolates from three farms and one *K. michiganensis* strain from a fourth farm. In six *Klebsiella* isolates, *bla*_{CTX-M-1} co-occurred with the *mph*(A) gene on the identical plasmids. Similarly, a previous study from the New York dairy farms reported the co-occurrence of *bla*_{CTX-M-1} and *mph*(A) on IncN plasmids in *Klebsiella* spp. isolated from mastitic cows (101). The presence of the *bla*_{CTX-M-1} gene and other ARGs on identical plasmids can facilitate the emergence and persistence of MDR through co-selection (148). This finding contrasts the previous studies from Canada and Europe that showed IncI1, narrow-host range plasmids, is involved in carrying *bla*_{CTX-M-1} in *Enterobacteriaceae* (116, 149, 150). In addition, unlike the present study, a previous study on U.S. dairy cattle reported IncN plasmid in association with other *bla*_{CTX-M} genes (e.g., *bla*_{CTX-M-15,27}, and -65) in *E. coli* (72). Further study is needed to identify if various plasmid replicon types are adapted to carrying the *bla*_{CTX-M-1} in different members of *Enterobacteriaceae*.

Another broad-host-range plasmid, IncHI2(ST3), was associated with *bla*_{CTXM-65} and/or *tet*(A) genes in five *K. pneumoniae* isolates. Previous studies also reported that the IncHI2A plasmid is associated with multiple kinds of resistance genes in *Enterobacteriaceae* isolated from animals and humans and is responsible for inter-species transmission of ARGs (151, 152).

In this study, 75% of *qnrB19* detected in *Klebsiella* genomes was associated with small Col440I plasmids in all the farms suggesting its widespread occurrence. This concurs with a recent study from Germany that reported frequent association of the *qnrB* gene with Col440I plasmids in ESBL-producing *E. coli* isolated from livestock (153). The wide distribution of *qnrB*-carrying Col440I plasmids among different STs of *Klebsiella* spp. and other members of *Enterobacteriaceae* suggests that they may play a crucial role in maintaining and spreading *qnrB19* genes within *Enterobacteriaceae*. However, further investigation is necessary to understand the mechanisms responsible for the Col440I plasmid spread and maintenance among *Enterobacteriaceae* even in the absence of selection pressure, as most *Klebsiella* isolates in this study were obtained from adult dairy cattle that do not use (fluoro)quinolones. Specifically, further study is needed to determine plasmid types (e.g., conjugative, or it needs a helper plasmid for successful transfer or if its resistance gene is co-selected by different antibiotics (e.g., ceftiofur), and the benefits that the plasmid provides to the host bacteria, leading to its retention and widespread occurrence.

In this study, in addition to plasmids, variants of ESBL co-occurred with non-ESBL genes such as *aac*(3)-IId, *aph*(6)-Id, *aph*(3'')-Ib, *sul2* were associated with other MGEs such as Insertion sequences (IS), and Integrative Conjugative Element (ICEKp1). Previous studies widely reported the co-occurrence of ESBL and other unrelated resistance genes (65, 126, 154, 155). This will facilitate the mobility of diverse ARGs gene and the spread of MDR among the bacterial population. For instance, in three *Klebsiella* genomes, one or more ESBL variants co-occurred with *mph*(A) or *aac*(3)-IId on the same contigs with IS26. This has an important implication on the epidemiology of resistance genes, as previous studies reported that IS26 helps to capture and mobilize drug resistance genes found in both chromosomes (156, 157) and plasmids (158, 159).

Studies also showed that IS26 also aids in plasmid stability and persistence by facilitating the deletion of plasmid backbone sequences whose expression is burdensome for the cell (160).

The final objective of this study was to determine the genetic relatedness and spread mechanism of ESBL-producing *Klebsiella* spp. within and between farms. This study demonstrated that the ESBL-producing *Klebsiella* spp. spreads through two modes. These include 1) clones or STs with specific *Klebsiella* spp.-plasmid-ESBL gene variant combinations were disseminated widely within and between farms, 2) plasmids harboring ESBL genes were detected in dissimilar *Klebsiella* spp. STs suggest possible horizontal transfer of the genes that might contribute to the successful spread of the bacteria in the study farms.

The extent of the two modes of ESBL transmission appeared to be different between the farms. In four farms (Farm A, B, G, and J), the spread of the ESBL genes within the farms might be primarily attributed to clonal expansion. This was evident by clusters of genetically similar isolates with the same STs-ESBL gene variant-plasmid type combinations and the same chromosomal mutations in QRDRs, indicating the clonal transmission of specific resistant *Klebsiella* clones throughout the dairy herd. For example, all *K. pneumoniae* from farms A, B, and G were identified as ST353, and they all carry bla_{CTX-M-1} on the same plasmid, IncN (ST1). The detection of the identical ST of *K. pneumoniae* harboring the same ESBL variant on indistinguishable plasmids among animals on the same farm indicates potential animal-to-animal transmission of the bacteria or exposure to a common source (161).

The detection of the same ESBL-producing *K. pneumoniae* STs, with the same ESBL genes and plasmid profile, in three separate farms suggests that there may be a clonal spread of the bacteria between the farms through feed, contaminated vehicles, movement of dairy cattle, and humans as described in previous studies (38, 72, 162). However, further study is needed to determine whether the spread of the bacteria is due to clonal dissemination via the mechanisms mentioned above or if there is a unique adaptation between the bacterial STs, the plasmid, and the ESBL gene.

Similarly, all *Klebsiella* isolates were identified as ST322 in Farm J and harbored the same *bla*_{SHV-2} variants. The isolates also carried identical epidemic resistance plasmids, IncFIIK (K7:A⁻:B⁻), and novel alleles for IncFIA replicon types suggesting the possible clonal spread of the ESBL-producing *Klebsiella* spp. in the farm. Since one of the isolates in this farm was obtained from a water sample taken from the watering troughs, the animals on the farm might have independently acquired the same ST of *K. pneumoniae* by consuming the contaminated water from the same trough.

In farm M, evidence of clonal and horizontal spread of *bla*_{CTX-M} genes was observed. However, horizontal spread appeared to be the primary factor in disseminating *bla*_{CTX-M} genes on the farm. This was supported by identifying the same predominant *bla*_{CTX-M} variants, specifically the *bla*_{CTX-M-27} gene, associated with the same epidemic-resistant plasmid, IncFII(ST F2:A⁻:B⁻).

About 31 (74%) of *Klebsiella* spp. obtained from this farm, including nine distinct STs and ten Unknown STs, contained *bla*_{CTX-M-27} associated with IncFII (ST F2:A⁻:B⁻) plasmids. This indicates a potential horizontal spread of this ESBL variant among diverse *Klebsiella* spp. genotypes in the farm. This concurs with the previous studies that showed narrow host range plasmids of the IncF group, FII, FIA, and FIB, can acquire resistance genes and quickly spread among specific members of *Enterobacteriaceae* (146, 147). In addition, some *Klebsiella* spp. with identical STs (e.g., ST469, n=6; ST 687, n=4; and ST239, n=3) had the same *bla*_{CTX-M-27} on IncFII (ST F2:A⁻:B⁻), suggesting a possible clonal spread of these STs within the animals in the farm.

Conclusion

ESBL-*Klebsiella* spp was identified in 10% of the tested samples and fecal samples of 35 % of dairy cattle. The prevalence of fecal ESBL-*Klebsiella* spp. was 7.2%. The fecal prevalence of ESBL-*Klebsiella* spp. was significantly higher in the calves than in the cows and higher in cows with higher parity. ESBL-*Klebsiella* spp. displayed high phenotypic co-resistance to ceftriaxone and sulfisoxazole, with almost 20% of the isolates being MDR. The study identified six families of beta-lactamase genes and 26 distinct types of horizontally transferable antibiotic resistance

genes. Multiple mutations in fluoroquinolone resistance regions were detected in all isolates though the corresponding phenotypic expression is low. Most *Klebsiella* spp. carried numerous variants of β -lactamase and other ARGs, suggesting that these bacteria are a reservoir of resistance genes. The *Klebsiella* genomes harbored 22 different plasmid replicon types, with IncFII and Col440I plasmids being the most frequently detected associated with *bla*_{CTX-M} and *qnrB19*, respectively. The presence of multiple variants of beta-lactamase, other antibiotic resistance genes, and multiple plasmid replicon types in *Klebsiella* spp. indicates that it could be a reservoir of antibiotic resistance genes.

We identified 15 distinct sequence types of *K. pneumoniae*, including ten unknown STs. The most frequently occurring STs were ST353, ST469, and ST687. In most farms, the identified ESBL-*K. pneumoniae* strains were genetically identical and carried identical plasmids and ESBL genes, indicating possible clonal expansion (animal-to-animal transmission). ESBL-*Klebsiella* isolates with the same STs and resistance gene patterns and MGEs were obtained from two farms in the same county, suggesting a possibility of inter-farm spread of the bacteria. In addition, the identical ESBL variant was found in conjunction with the same plasmid sequence types in unrelated STs of *Klebsiella* spp. suggesting the possible spread of the ESBL gene via the horizontal transfer of plasmids. The dual mechanism of the spread of ESBL genes in *Klebsiella* spp. can rapidly increase the prevalence of ESBL-*Klebsiella* spp. This can pose significant risks to human health, highlighting the urgent need for prevention and control measures to limit its spread.

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Appendix

Table 5. 1. Within-herd prevalence of fecal ESBL-*Klebsiella* species

County*	Farm	Herd Size	Sample Size (n)	Unweighted Frequency and Prevalence: n (%)	95 % CI	Weighted Frequency and Prevalence (%): n/N (%)	95 % CI
A1	A	100	14	2 (14.3)	1.8-42.8	14 (14)	7.9-22.4
A2	B	14	9	3 (33.3)	7.5-70	5 (35.7)	12.7-64.9
B1	C	750	63	0 (0)	NA	0 (0)	NA
B2	D	300	30	0 (0)	NA	0 (0)	NA
C1	E	50	13	0 (0)	NA	0 (0)	NA
C2	F	110	40	0 (0)	NA	0 (0)	NA
D	G	170	25	0 (0)	NA	0 (0)	NA
E	H	35	26	0(0)	NA	0 (0)	NA
F1	I	61	30	0 (0)	NA	0 (0)	NA
G1	J	60	25	3 (12)	2.5-31.2	7 (11.7)	4.8-22.6
G2	K	1700	45	0 (0)	NA	0 (0)	NA
F2	L	250	50	2 (4)	0.49-13.7	10 (4)	1.9-7.2
H	M	450	64	40 (62.5)	49.5-74.3	281 (62.4)	57.8-66.9
F3	N	350	74	0 (0)	NA	0 (0)	NA
Total		4400	508	50 (9.8)	7.3-12.8	318 (7.2)	6.5-8.0

*Farms from counties designated by the same letter are in the same county. NA: not applicable

Table 5. 2. Prevalence of ESBL-*Klebsiella* species in dairy cattle farms according to sample sources

Sample Source	(No. Tested) ^a	(No. of Positives) ^b	Prevalence (95 % CI)
Rectal feces	508	50	9.8(7.4-12.8)
Manure	30	3	10% (2-26.5)
Water	19	2	10.5% (1.1-33.1)
Feed	15	2	13.3% (1.7-40.5)
Total	572	57	10% (7.6-12.7)

^aNumber of samples tested for ESBL-*Klebsiella* species from each sample source

^bNumber of ESBL-*Klebsiella* isolates obtained from each sample sources

Table 5. 3. Relationship between ESBL-*Klebsiella* species and animal-level variables

Variable	Category	Weighted Frequency	Prevalence (95% CI)	OR (95%CI)	P-value
Parity	≤ 2	114	5.4% (4.4-6.3)	Ref.	<0.001
	> 2	88	6.3% (5.1-7.7)	4.91 (3.0-8.03)	
Herd structure	Cow	202	6.2% (5.4-7.1)	Ref.	<0.001
	Calf	116	13.3% (11.1-15.7)	10.43 (5.7-18.8)	
Total		318	7.2% (6.5-8.0)	NA	

Table 5. 4. Frequency of resistant ESBL-*Klebsiella* species (n=57) to 14 antimicrobial agents

Antibiotic	Sources of <i>Klebsiella</i> Isolates and Proportion of Resistance to the Specific Antibacterial Agent					Overall n (% Rest.)
	Cows (N=31) ^a n (% Rest.) ^b	Calf (N= 19) n (% Rest.)	Manure (N=3) n (% Rest.)	Feed (N= 2) n (% Rest.)	Water (N= 2) n (% Rest.)	
<i>AUG2</i>	2 (6.5)	2 (10.5)	1 (33.3)	1 (50)	0	6 (10.5)
<i>AMP</i>	31 (100)	19 (100)	3 (100)	2 (100)	2 (100)	57 (100)
<i>AZM</i>	0	3 (15.8)	0	0	0	3 (5.3)
<i>FOX</i>	1 (3.2)	0	0	0	0	1(1.8)
<i>AXO</i>	31 (100)	19 (100)	2 (66.7)	2 (100)	1 (50)	55 (96.5)
<i>CHL</i>	0	3 (15.8)	0	0	0	3 (5.3)
<i>CIP</i>	1 (3.2)	1 (5.3)	0	0	0	2 (3.5)
<i>MERO</i>	0	0	0	0	0	0 (0)
<i>GEN</i>	2 (6.5)	2 (10.5)	1 (33.3)	0	0	5 (8.8)
<i>NAL</i>	0	1 (5.3)	0	0	0	1 (1.8)
<i>STR</i>	1 (3.2)	5 (26.3)	0	0	0	6 (10.5)
<i>FIS</i>	21 (67.7)	14 (73.7)	2 (66.7)	1 (50)	2 (100)	40 (70.2)
<i>TET</i>	3 (9.7)	5 (26.3)	1 (33.3)	0	0	9 (15.8)
<i>STX</i>	1 (3.2)	3 (15.8)	0	0	0	4 (7)

^aThe total number of *Klebsiella* isolates obtained from a given source; ^b number and proportion of isolates resistant to a given antibiotic from a specific source. Rest: resistant. AXO: ceftriaxone; FOX: cefoxitin; AUG2: amoxicillin-clavulanic acid; AMP: ampicillin; MERO: meropenem, AZM: azithromycin; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; TET: tetracycline; FIS: sulfisoxazole; STX: trimethoprim-sulfamethoxazole.

Table 5. 5. Distribution of antimicrobial resistant ESBL-*Klebsiella* species (n=57) across six farms

Antibiotic	Number of Isolates (n) Resistant to a Specific Antibiotic in Each Farm						Total
	Farm A (n=3) ^a	Farm B (n=3)	Farm G (n=2)	Farm J (n=5)	Farm L (n=2)	Farm M (n=42)	
AUG2	2	0	1	0	0	3	6
AMP	3	3	2	5	2	42	57
AZM	0	0	0	0	0	3	3
FOX	0	0	0	0	0	1	1
AXO	3	3	2	3	2	42	55
CHL	0	0	0	0	0	3	3
CIP	0	0	0	0	1	1	2
MERO	0	0	0	0	0	0	0
GEN	0	0	0	0	0	5	5
NAL	0	0	0	0	1	0	1
STR	0	0	0	0	1	5	6
FIS	2	3	2	3	2	28	40
TET	0	0	0	1	0	8	9
STX	0	0	0	0	0	4	4

AXO: ceftriaxone; FOX: ceftiofur; AUG2: amoxicillin-clavulanic acid; AMP: ampicillin;

MERO: meropenem, AZM: azithromycin; CIP: ciprofloxacin; NAL: nalidixic acid; CHL:

chloramphenicol; GEN: gentamicin; STR: streptomycin; TET: tetracycline; FIS: sulfisoxazole;

STX: trimethoprim-sulfamethoxazole.

Table 5. 6. Beta-lactamase genes, their function, and frequency in ESBL-*Klebsiella* species

Farm	β -Lactamase Gene Variant	Functional Information	Frequency
A, B, G, L, M	<i>bla</i> _{CTX-M-1}	Class A ESBL	12
M	<i>bla</i> _{CTX-M-15}	Class A ESBL	1
M	<i>bla</i> _{CTX-M-27}	Class A ESBL	37
M	<i>bla</i> _{CTX-M-32}	Class A ESBL	2
M	<i>bla</i> _{CTX-M-61}	Class A ESBL	2
M	<i>bla</i> _{CTX-M-65}	Class A ESBL	5
M	<i>bla</i> _{CTX-M-138}	Class A ESBL	2
M	<i>bla</i> _{CTX-M-146}	Class A ESBL	2
M	<i>bla</i> _{SHV-1}	2b Class A β -lactamase	1
J, M	<i>bla</i> _{SHV-2}	2be Class A β -lactamase	4
M	<i>bla</i> _{SHV-11} ^c	2b Class A β -lactamase	8
M	<i>bla</i> _{SHV-13} ^c	2be Class A β -lactamase	8
M	<i>bla</i> _{SHV-110}	Class A β -lactamase	1
A B, G, M	<i>bla</i> _{SHV-85} ^a	2b Class A β -lactamase	5
A, B, G, M	<i>bla</i> _{SHV-40} ^a	2be Class A β -lactamase	5
A, B, G, M	<i>bla</i> _{SHV-56} ^a	2br Class A β -lactamase	5
A, B, G, M	<i>bla</i> _{SHV-79} ^a	2b Class A β -lactamase	6
A, B, G, M	<i>bla</i> _{SHV-89} ^a	2b Class A β -lactamase	5
M	<i>bla</i> _{SHV-59} ^d	2b Class A β -lactamase	4
M	<i>bla</i> _{SHV-70} ^c	2be Class A β -lactamase	8
M	<i>bla</i> _{SHV-71}	2b Class A β -lactamase	1
M	<i>bla</i> _{SHV-25}	2b Class A β -lactamase	1
M	<i>bla</i> _{SHV-26} ^b	2br Class A β -lactamase	7
M	<i>bla</i> _{SHV-27}	2be Class A β -lactamase	9
M	<i>bla</i> _{SHV-62}	2b Class A β -lactamase	4
M	<i>bla</i> _{SHV-78} ^b	2b Class A β -lactamase	7
M	<i>bla</i> _{SHV-81}	2b Class A β -lactamase	1
M	<i>bla</i> _{SHV-145} ^b	2b Class A β -lactamase	7
M	<i>bla</i> _{SHV-172}	Class A β -lactamase	1
M	<i>bla</i> _{SHV-157}	Class A β -lactamase	1
M	<i>bla</i> _{SHV-187}	Class A β -lactamase	1
M	<i>bla</i> _{SHV-164} ^d	2b Class A β -lactamase	4
M	<i>bla</i> _{SHV-98} ^b	2be*Class A β -lactamase	7
M	<i>bla</i> _{SHV-199} ^b	Class A β -lactamase	7
M	<i>bla</i> _{SHV-103}	2b Class A β -lactamase	1
M	<i>bla</i> _{SHV-78}	2b Class A β -lactamase	7
M	<i>bla</i> _{SHV-179} ^b	Class A β -lactamase	7

Table 5. 6. Continued.

Farm	β -Lactamase Gene Variant	Functional Information	Frequency
M	<i>bla</i> _{SHV-194} ^b	Class β -lactamase	7
M	<i>bla</i> _{TEM-1B} ^{e, f}	2b Class A β -lactamase	40
M, G	<i>bla</i> _{TEM-1C} ^f	2b Class A β -lactamase	2
M	<i>bla</i> _{TEM-16} ^e	2be Class A β -lactamase	1
M	<i>bla</i> _{TEM-29} ^f	2be Class A β -lactamase	1
M	<i>bla</i> _{TEM-33} ^e	2br Class A β -lactamase	1
M	<i>bla</i> _{TEM-34} ^e	2br Class A β -lactamase	1
M	<i>bla</i> _{TEM-35} ^e	2br Class A β -lactamase	1
M	<i>bla</i> _{TEM-36} ^e	2br Class A β -lactamase	1
M	<i>bla</i> _{TEM-55} ^f	2b Class A β -lactamase	1
M	<i>bla</i> _{TEM-57} ^f	2b Class A β -lactamase	1
M	<i>bla</i> _{TEM-104} ^e	Class A β -lactamase	1
M	<i>bla</i> _{TEM-122} ^{e, f}	2br Class A β -lactamase	2
M	<i>bla</i> _{TEM-135} ^f	2b Class A β -lactamase	1
M	<i>bla</i> _{TEM-141} ^{e, f}	2b Class A β -lactamase	2
M	<i>bla</i> _{TEM-163} ^e	2be Class A β -lactamase	1
M	<i>bla</i> _{TEM-164} ^e	2b Class A β -lactamase	1
M	<i>bla</i> _{TEM-198} ^e	Class A β -lactamase	1
M	<i>bla</i> _{TEM-206} ^e	Class A β -lactamase	1
M	<i>bla</i> _{TEM-209} ^{ef}	Class A β -lactamase	2
M	<i>bla</i> _{TEM-210} ^e	Class A β -lactamase	1
M	<i>bla</i> _{TEM-214} ^e	Class A β -lactamase	1
M	<i>bla</i> _{TEM-216} ^e	Class A β -lactamase	1
M	<i>bla</i> _{OXA-1}	Class D β -lactamase	1
M	<i>bla</i> _{OXY-1-7}	Class A ESBL	1
M	<i>bla</i> _{SED-1}	2b Class A β -lactamase	1

^{a-f} beta-lactamase genes with the same superscript co-occur in the same isolates and are usually found on the same contigs. 2b: broad-spectrum; 2be: extended-spectrum; 2br: Inhibitor-resistant broad-spectrum.

The phenotype or functional information of the gene in the table was adopted from the NCBI (https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes), beta-lactamase database (<http://bldb.eu/BLDB.php?prot=A>) and <https://www.lahey.org/Studies>.

Table 5. 7. Horizontally transferable resistance genes co-harbored with beta-lactamase genes and in *Klebsiella* species

Target Antibiotic Class	Resistance Gene and Frequency	Gene Description	Resistance Mechanism
Beta-lactams	<i>bla</i> _{CTX-M} (51)	Class A ESBL	IBH
	<i>bla</i> _{SHV} (53)	Class A beta-lactamase	IBH
	<i>bla</i> _{TEM} (40)	Class A beta-lactamase	IBH
	<i>bla</i> _{OXY} (1)	Class A ESBL	IBH
	<i>bla</i> _{OXA} (1)	Class D β -lactamase	IBH
	<i>bla</i> _{SED} (1)	Class A beta-lactamase	IBH
Aminoglycosides	<i>aph</i> (6)-Id(3)	Aminoglycoside-6- Phosphotransferase	IBM
	<i>aph</i> (3'')-Ia(1)	Aminoglycoside-3'' phosphotransferase	IBM
	<i>aac</i> (3)-IId(5)	Aminoglycoside3-N-acetyltransferase	IBM
	<i>aac</i> (6')-Ib-cr(1)	Aminoglycoside6'-N-acetyltransferase	IBM
Macrolide	<i>mph</i> (A) (9)	Macrolide 2'-phosphotransferase	IBM
(Fluoro)quinolone	<i>qnrB19</i> (36)	Fluoroquinolone resistance protein B19	TP
	<i>qnrB10</i> (2)	Fluoroquinolone resistance protein B10	TP
	<i>qnrB5</i> (2)	Fluoroquinolone resistance protein B5	TP
	<i>qnrB81</i> (2)	Fluoroquinolone resistance protein B81	TP
Folate pathway antagonist	<i>dfrA1</i> (10)	Dihydrofolate reductase type 1	TM
	<i>dfrA12</i> (1)	Dihydrofolate reductase type 12	TM
	<i>dfrA14</i> (1)	Dihydrofolate reductase type 14	TM
	<i>sul1</i> (3)	Dihydropteroate synthetase type 1	TM
	<i>sul2</i> (2)	Dihydropteroate synthetase type 2	TM
Tetracycline	<i>tet</i> (A) (6)	Tetracycline resistance protein A	EM
	<i>tet</i> (J) (1)	Tetracycline resistance protein J	EM
Chloramphenicol	<i>floR</i> (1)	Florfenicol/chloramphenicol export protein	EM
	<i>catB3</i> (1)	Chloramphenicol acetyltransferase (group B)	IBM
	<i>cat</i> (1)	Chloramphenicol acetyltransferase	IBM
Lincomycin	<i>lnu</i> (G) (1)	Lincosamide nucleotidyl transferase	IBM
Phosphonic acid	<i>fosA</i> (53)	Fosfomycin modifying enzyme	IBM
	<i>fosA5</i> (1)	Fosfomycin modifying enzyme type 5	IBM
	<i>fosA6</i> (5)	Fosfomycin modifying enzyme type 6	IBM
	<i>fosA7</i> (1)	Fosfomycin resistance protein type 7	IBM
	<i>fosA8</i> (1)	Fosfomycin resistance protein type 8	IBM
Multidrug class	<i>oqxAB</i> (53)	Efflux transporter	EM

EM: Efflux-mediated; IBH: Inactivation by hydrolysis; TM: drug target modification; IBM: Inactivation by modification; TP: Target Protection

Table 5. 8. Association between *Klebsiella* species sequence types, resistance genes, and mobile genetic elements

Farm	ST(Freq.) ^a	Resistance genes ^b	Mobile genetic elements	
			Plasmid replicon type (pMLST) ^c	Insertion sequence
A	ST353 (2)	<i>bla</i> _{CTX-M-1} , <i>mph</i> (A)	IncN (ST1)	-
B	ST353 (1)	<i>bla</i> _{CTX-M-1} , <i>mph</i> (A)	IncN (ST1)	-
G	ST353(2)	<i>bla</i> _{CTX-M-1} , <i>mph</i> (A)	IncN (ST1)	-
M	Unknown ST (10), ST 867(3), ST 687(4), ST 469(6), ST 2259(1), ST 239(3), ST 419(1), ST 36(1), ST 294(1)	<i>bla</i> _{CTX-M-27} , <i>bla</i> _{TEM-1B}	IncFII ([F2:A- :B-])	-
M	ST111(1)	<i>bla</i> _{CTX-M-65}	IncHI2 (ST3)	
M	ST34(1)	<i>bla</i> _{CTX-M-27}	IncFII ([F2:A- :B-])	
M	ST687(1), ST2239(1), ST2703(1), Unknown ST(1)	<i>bla</i> _{CTX-M-27} , <i>bla</i> _{TEM-1B}	-	IS102
M	ST111(1)	<i>bla</i> _{CTX-M-65}	-	IS102
M	ST469(1)	<i>bla</i> _{SHV-11} , <i>bla</i> _{SHV-13} , <i>bla</i> _{SHV-70}	-	IS102
A	ST353 (1)	<i>bla</i> _{CTX-M-1} , <i>mph</i> (A)	-	IS26
M	ST661(1)	<i>bla</i> _{CTX-M-27} , <i>bla</i> _{TEM-1B}	-	IS26
M	ST111(1)	<i>bla</i> _{CTX-M-65} , <i>aac</i> (3)-IId	-	IS26
M	ST2703(1)	<i>bla</i> _{SHV-199} , <i>bla</i> _{SHV-179} , <i>bla</i> _{SHV-45} , <i>bla</i> _{SHV-194} , <i>bla</i> _{SHV-26} , <i>bla</i> _{SHV-98} , <i>bla</i> _{SHV-78}	-	IS3
M	ST6250(1)	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{TEM-1B} , <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, and <i>sul2</i>	-	ISEc9
G	ST353 (1)	<i>bla</i> _{SHV-79} , <i>bla</i> _{SHV-40} , <i>bla</i> _{SHV-89} , <i>bla</i> _{SHV-56} , and <i>bla</i> _{SHV-85}	-	ICEKp1
All farms	ST353(8), ST322(4), unknown (6), ST867(2), ST469(5), ST111(1) ST225(1)	<i>qnrB19</i>	Col440I	-
M	ST111(3), ST57(1)	<i>tet</i> (A)	IncHI2A(ST3)	-
M	ST2239 (1)	<i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul2</i> , <i>dfrA1</i> , <i>floR</i> , and <i>lnu</i> (G) Dihydropteroate synthetase type 2,	-	IS91

^a ST: Sequence type; Freq.: Number of isolates with the specific sequence type; ^b genes co-occurred on the same mobile genetic elements; ^c pMLST: plasmid multilocus sequence type

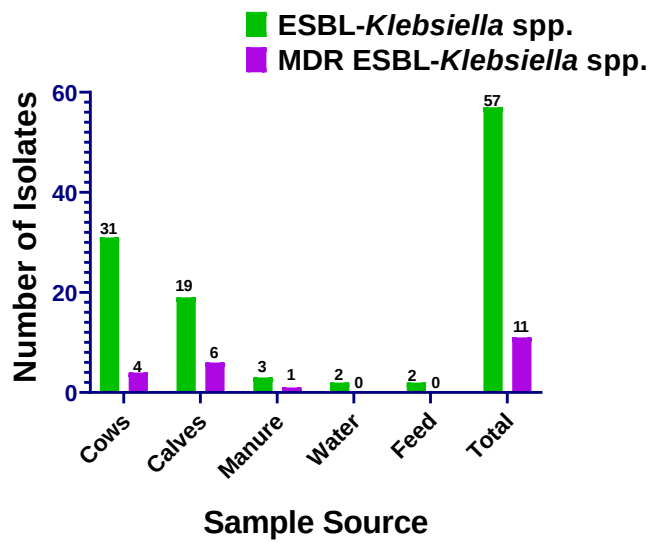


Figure 5. 1. Distribution of multidrug-resistant ESBL-*Klebsiella* species across the five sample sources

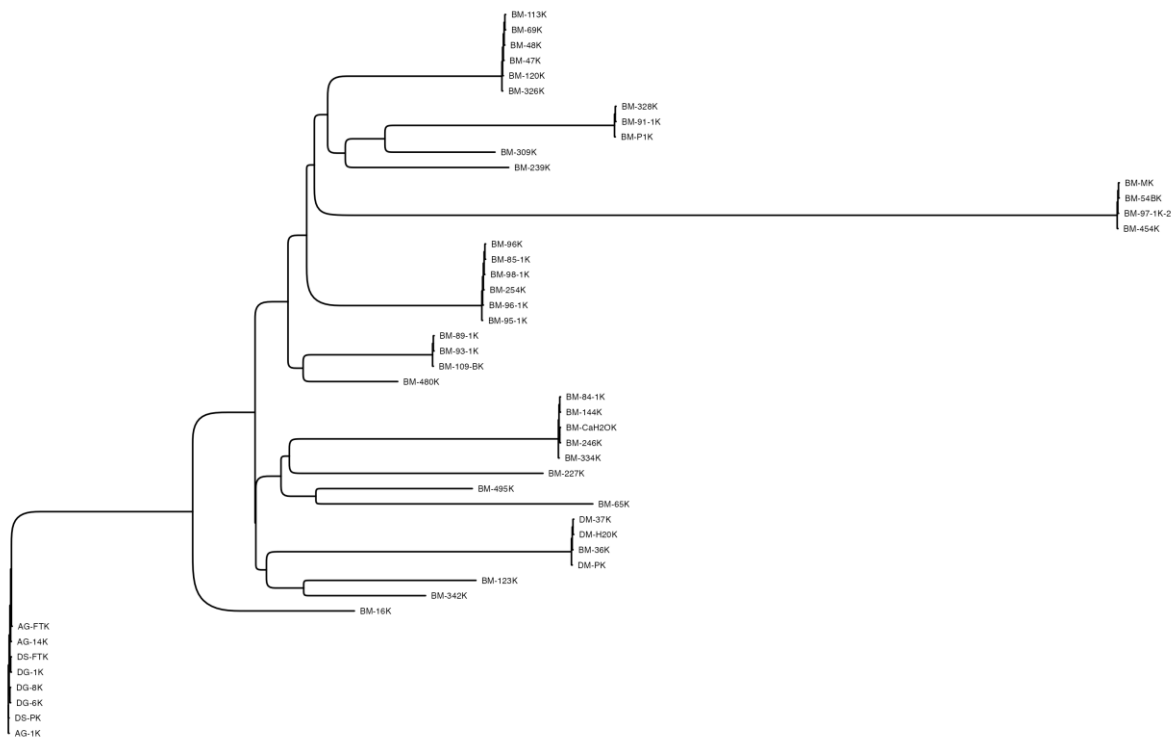


Figure 5. 2. Phylogenetic tree of ESBL-*Klebsiella pneumoniae*

Isolates sharing the same first two letters were collected from the same farm. The letters indicate the farm and county, while the numbers represent the individual animal's ID within the farm (except manure, feed, and water samples). Most of the isolates were obtained from the same farm, and they showed multiple clustering on vertical lines where isolates in each cluster had identical sequence types. The isolates clustered in the lower left part of the phylogenetic tree were collected from three farms (AG, DG, and DS), suggesting a potential inter-farm spread of the ESBL-*K.pneumoniae* strain.

Chapter 6. Molecular Epidemiology and Pathogenomics of ESBL-*E. coli* and-*K. pneumoniae* Isolates From Bulk Tank Milk

Abstract

The rise in ESBL-producing *Enterobacteriaceae* in dairy farms poses a significant risk to human health as they can spread to humans through the food chain, such as raw milk consumption. The growing popularity of raw milk consumption in the U.S. and the presence of multidrug resistance (MDR) traits in these bacteria make it particularly concerning. This study was designed to determine the status and pathogenic potential of ESBL-*E. coli* and -*Klebsiella* spp. isolates from bulk tank milk (BTM). Thirty-three BTM samples were collected from 17 dairy farms and screened for ESBL-*E. coli* and -*Klebsiella* spp. on Chromogenic media. All isolates were confirmed by MALDI-TOF MS and subjected to antimicrobial susceptibility testing (AST) and whole genome sequencing (WGS). Ten presumptive ESBL-producing bacteria, eight *E. coli*, and two *K. pneumoniae* were isolated. The prevalence of ESBL-*E. coli* and -*K. pneumoniae* in BTM was 24.2% and 6.1%, respectively. The ESBL-*E. coli* were detected in 41.2% of the study farms, while only two tested positive for ESBL-*K. pneumoniae*. Seven of the ESBL-*E. coli* isolates were MDR. The two ESBL-producing *K. pneumoniae* isolates were resistant to ceftriaxone. Seven ESBL-*E. coli* strains carry the *bla*_{CTX-M} gene, and five of them co-harbored *bla*_{TEM-1}. Most of ESBL-*E. coli* co-harbored *bla*_{CTX-M} with several other resistance genes such as *qnrB19*, *tet(A)*, *aadA1* *aph(3'')-Ib*, *aph(6)-Id*, *floR*, *sul2*, and mutations (*gyrA*, *gyrB*, *parC*, *parE* and *pmrB*). Most *E. coli* resistance genes were associated with mobile genetic elements, mainly plasmids. Six sequence types (STs) of *E. coli* were detected. All ESBL-*E. coli* were predicted to be a human pathogen. Four STs (three ST10 and ST69) were high-risk clones of *E. coli*. Up to 40 virulence markers were detected in all eight *E. coli* isolates. One of the *K. pneumoniae* was ST867; the other was novel. Each *K. pneumoniae* isolates carried three types of beta-lactamase genes, *qnrB19* and *fosA*. The novel ST also carried a novel IncFII(K) ST. In isolates recovered from fecal samples collected from the same farm where BTM was collected, matching STs and resistance patterns were discovered. Detection of high-risk clones of MDR ESBL-*E. coli* and the presence of ESBL-*K. pneumoniae* in BTM indicated that raw milk could be a reservoir of potentially zoonotic ESBL-*E. coli* and -*K. pneumoniae*. Thus, consuming raw milk and its products can be a significant concern for public health safety.

Keywords: ESBL, *E. coli*, *K. Pneumoniae*, virulence gene, High-risk clones, Bulk Tank Milk,

6.1 Introduction

In the United States of America (U.S.), beta-lactam antibiotics, specifically ceftiofur, are frequently used for prophylactic and therapeutic purposes in dairy cattle farms (1-4). This heavy use can drive the emergence of extended-spectrum beta-lactamases (ESBL) producing bacteria, which confers resistance to beta-lactam antibiotics, including third-generation cephalosporins (3GC) (5). As a result, multi-drug resistant (MDR) ESBL-producing bacteria such as *E. coli* and *Klebsiella* spp., the main trafficators of ESBL genes (*bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}), are becoming increasingly prevalent in dairy farms (4-6). The prevalent ESBL-*E. coli* and *Klebsiella* spp. can enter the bulk tank milk from subclinically infected mammary glands, contaminated udders, milking machines, and the dairy farm environment (7). The ESBL-*E. coli* and *Klebsiella* spp. in dairy farms may spill over to humans indirectly by consuming contaminated, unprocessed dairy products such as raw milk (8).

In recent years, there has been a growing trend towards more natural products and the promotion of the "probiotic effects" of raw milk, leading to an increase in raw milk consumption in the U.S. Some reports estimated that more than 3% of the population in the U.S. consumes raw milk, and at least 30 states in the U.S. legally accepted the sale of unpasteurized milk for human consumption (9-11). This highlights the increased risk of infection with multidrug-resistant bacteria that are difficult to treat and need to pasteurize milk to ensure the safety of milk to reduce the risk of multidrug resistant foodborne pathogens.

Consuming raw dairy products can increase the risk of ESBL-producing bacteria, such as *E. coli* and *Klebsiella* spp., spreading to humans through the food chain, especially milk, and colonizing the gastrointestinal tract (12). These bacteria can spread ESBL genes commonly associated with the mobile genetic element (MGEs) to other more harmful human pathogenic bacteria via horizontal gene transfer, creating a reservoir of antibiotic-resistant bacteria that threatens public health (5, 13). In addition to transferring ESBL genes, some *E. coli* and *Klebsiella pneumoniae* strains are highly pathogenic and can cause various diseases in humans on their own (14).

Some studies reported a possible convergence among carrying ESBL genes, multidrug resistance, and virulence in *E. coli* and *K. pneumoniae* isolates from dairy cattle and other farm environmental samples (15-18). This further increases public health risks if these bacteria end up in raw milk meant for human consumption. Although there is limited evidence linking dairy products as a source of ESBL-*Enterobacteriaceae* for humans, the high prevalence of ESBL-*Enterobacteriaceae* in dairy farms combined with the growing demand for unpasteurized milk suggests that raw milk may serve as a potential source and exit route for ESBL-*E. coli* and -*Klebsiella* spp. (19) from dairy farms to humans. This could be a potential source of the rising incidence of community-associated ESBL-*Enterobacteriaceae* infections in the U.S. (20).

Previous studies on raw milk in the U.S. were focused on microbiological identification of the commonly reported milk borne pathogens, such as *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, and *E. coli* O157:H7 (4, 21-24). Few other studies in Asia (25, 26) and Europe (27) reported the detection of ESBL-*E. coli* from mastitic milk samples. A 12-year retrospective study on mastitic milk in Wisconsin also identified a significant number of *Klebsiella* spp. resistant to ceftiofur, a 3GC whose resistance is mainly mediated by the production of ESBL genes (28). This study was solely based on phenotypic detection of resistance and did not examine the genetic characteristic of the isolates. Another recent research on *Klebsiella* isolates from mastitic milk reported several resistance genes that mediate resistance to critically important antibiotics (CIAs), including ESBL genes (29). Milk from cows with mastitis is usually discarded and not used for human consumption. Thus, detecting ESBL-producing bacteria in such milk may not entirely indicate public health risks posed by raw milk consumption.

In our recent study, we detected ESBL-*E. coli* co-carrying resistance genes to multiple classes of antibiotics in bulk tank milk obtained from dairy farms (6). In that study, we tested a limited number of farms and detected ESBL-*E. coli* using PCR which is less informative. To the best of our knowledge, published reports on the detection of ESBL-*K. pneumoniae* from BTM in the U.S. and elsewhere are unavailable. Thus, further studies with larger sample sizes and high-resolution molecular tools such as WGS are necessary to understand the status and distribution of

ESBL-*E. coli* and -*Klebsiella* spp. in dairy farms and the risk for human health. This information can help to increase public awareness about the potential health risks of raw milk consumption. Therefore, the objective of this study was to determine the prevalence of ESBL-*E. coli* and -*Klebsiella* spp. in bulk tank milk and characterize their antimicrobial resistance phenotypes, genotypes, virulence genes, and potential pathogenicity for humans.

6.2 Materials and Methods

6.2.1 Isolation and Identification of ESBL-*E. coli* and -*K. pneumoniae* from Bulk Tank Milk

Approximately 40 mL of bulk tank milk samples were collected into sterile 50 mL falcon tubes (mostly in duplicates) from 17 farms (14 farms described in chapter two with three additional farms). The samples were transported to the laboratory on ice. A 20 mL of the collected milk samples were suspended in 80 mL TSB-PO4 (MG Scientific, Pleasant Prairie, WI, USA). A 100 μ L of the suspension was streaked on CHROMagar ESBL plates (DRG International, Inc., Springfield, NJ, USA). to isolate ESBL-*E. coli* and -*Klebsiella* spp. Plates were aerobically incubated for 24 h at 37°C. From each plate, two presumptive ESBL-*E. coli* (dark pink to reddish) and *Klebsiella* spp. (metallic blue) colonies were subcultured into new CHROMagar ESBL plates (DRG International Inc.) and incubated for 24 h at 37°C to obtain a pure culture. Up to two well-isolated colonies were selected and inoculated into 5 mL of Luria Bertani broth (LBB) (Thermo Fischer Scientific, Waltham, MA, USA) and grown overnight at 37°C while shaking. A 0.5 mL overnight culture was added to a 2 mL serum block, mixed with an equal volume of 80% sterile glycerol, and stored at -80°C for further tests.

Presumptive ESBL-*E. coli* isolates stored at -80°C were thawed and plated on CHROMagar ESBL plates and incubated at 37°C for about 18 h. A single colony of ESBL-*E. coli* or -*Klebsiella* spp. was picked and subcultured on blood agar (Thermo Fischer Scientific) at 37°C for about 18 h. The ESBL-*E. coli* were identified using MALDI-TOF MS following the manufacturer's instructions (Bruker Daltonics, Billerica, MA, USA) at the University of Tennessee College of Veterinary Medicine Diagnostic Bacteriology and Mycology Lab as described under Chapter 4, section 4.2.4.

6.2.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed on MALDI-TOF MS-confirmed *E. coli* isolates against 14 antimicrobials representing ten classes of antimicrobials using the broth microdilution method. Commercially available 96-well microtiter plates containing the 14 antimicrobial panels (Sensitre™ CMV4AGNF) (Thermo Fisher Scientific) were used. The 14-antimicrobials include β -lactams (ampicillin, ceftriaxone, cefoxitin, amoxicillin-clavulanic acid, meropenem), Aminoglycosides (gentamicin and streptomycin), sulfonamides (sulfisoxazole, trimethoprim/sulfamethoxazole), macrolides (azithromycin), phenicols (chloramphenicol), quinolones (ciprofloxacin and nalidixic acid), and tetracyclines. The minimum inhibitory concentrations (MIC) of the 14 antimicrobials were determined following the manufacturer's recommended protocol and following CLSI M100 Clinical Laboratory Standards Institute guidelines (CLSI M100: *Enterobacteriaceae*) (30). The detail procedures followed for AST are described in Chapter 4, section 4.2.5.

6.2.3 DNA Extraction

The MALDI-TOF MS confirmed *E. coli* isolates stored at -80°C were thawed and plated on CHROMagar ESBL plates (DRG International Inc.). After overnight incubation, 2-3 colonies were picked with a sterile inoculating wire loop, subcultured into 5 mL LBB (Thermo Fisher Scientific) and incubated at 37°C with shaking (at 200 rpm) for 18 - 24 h. Bacterial DNA was extracted using the Qiagen MagAttract HMW DNA kit (Qiagen, Germantown, MD, US) following the manufacturer's procedure with slight modifications described in Chapter 4, section 4.2.6.

6.2.4 Library Preparation and Whole-Genome Sequencing of ESBL- *E. coli* and -*K. pneumoniae*

Genomic libraries were prepared using the Nextera XT DNA library prep kit (Illumina Corporation, San Diego, CA, USA) following the manufacturer's instructions. Final concentrations were confirmed on a Qubit fluorometer (Thermo Fisher Scientific). Some libraries checked for size and concentration on a high-sensitivity Bioanalyzer (Agilent

Technologies, Santa Clara, CA, USA). Samples were normalized and pooled, and a final quality confirmation analysis was performed on a Bioanalyzer (Agilent Technologies). The pooled samples were diluted to a final loading concentration of 0.5 nM and combined with 5% PhiX control (Illumina Corporation). The sample pool and PhiX were then loaded on one lane of an SP flow cell reading 250 nucleotides paired-end (2X 250) on the Illumina NovaSeq (Illumina Corporation). The DNA library was sequenced at the University of Tennessee Genomics Core (Knoxville, TN, USA).

6.2.5 Quality Control and Assembly of Whole Genome Sequence Data

Samples were demultiplexed, and quality assessment was done via FastQC v0.11.9 (31) and multiQC v1.14 (32). Adapter removal, filtering, and trimming of low-quality raw reads were done using Trimmomatic v0.39 (33). Preprocessed reads of each isolate were *de novo* assembled using SPAdes genome assembler v3.15.5 (34) with default parameters. The coverage, N50, number of scaffolds, maximum scaffold length, and percentage of predicted genome size were calculated for each assembled genome. Accordingly, the coverage (the mean depth) varies from 40 to 146, the mean total scaffold count was 264, the mean scaffold sequence total (bp) was 5004428, and the maximum scaffold length (bp) ranged from 346424 to 648826. PlasmidSPAdes modes were used for the *de novo* assembly of the ‘plasmids only’ for downstream analysis.

6.2.6 Analyses of Whole Genome Sequence Data

To further confirm the species of bacteria identified by MALDI-TOF, KmerFinder 3.2 (<https://cge.food.dtu.dk/services/KmerFinder/>) was used to predict bacterial species using a fast K-mer algorithm from the assembled genome (35-37). The WGS data were used to identify variants of ESBL genes and detect virtually all known antibiotic resistance genes (ARGs) or mutations co-harbored with ESBL genes. To determine the presence of acquired antibiotic resistance genes and chromosomal point mutations in assembled genomes of the isolates, ResFinder v4, online bioinformatics tools at the Center for Genomic Epidemiology (<https://cge.food.dtu.dk/services/ResFinder/>) (38, 39) and Comprehensive Antimicrobial Resistance Database (CARD) (<https://card.mcmaster.ca/analyze/rgi>) (40) were employed using *E. coli* and *Klebsiella* spp. specific panels. ESBL-encoding genes and other resistance

determinants were considered present when at least 60% of the length of the best-matching gene in the ResFinder database was covered with sequence identity greater than or equal to 95% (41).

To identify plasmids and other mobile genetic elements (MGE) associated with ARGs, draft assemblies of ESBL-*E. coli* and *Klebsiella* spp. genome was analyzed using PlasmidFinder 2.1 (<https://cge.food.dtu.dk/services/PlasmidFinder/>) and MGE Finder v1.0.3 (<https://cge.food.dtu.dk/services/MobileElementFinder/>). The PlasmidFinder detects plasmids and assigns them to incompatibility groups (42, 43). At the same time, MGE Finder was used to identify plasmids and other MGEs, such as transposon (Tn) and Insertion Sequences (IS) associated with ARGs and virulence genes (43-45). The MGEs were identified with a minimum 98% sequence identity threshold with the closest-matching hits and minimum 90% coverage (46). The virulence gene associated with MGE in each genome was identified with 98% sequence identity and a minimum of 95% coverage using virulenceFinder. The virulence markers in the isolates were identified using virulenceFinder (<https://cge.food.dtu.dk/services/VirulenceFinder/>) as described before (47, 48).

To investigate genetic relatedness, the WGS of isolates were analyzed using in silico sequence serotyping (*E. coli* only), multilocus sequence types (MLST), and Core genome Multi Locus Sequence Type (cgMLST) for both *E. coli* and *Klebsiella pneumoniae*. Multilocus sequence types (MLST) of *E. coli* were identified according to the Achtman seven-locus MLST approach, which is based on sequences of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (49-51). For *Klebsiella* spp., MLST was identified based on seven housekeeping genes (*gapA*, *InfB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) described in other studies (51, 52). The serotype of *E. coli* isolates was predicted from the draft assembly using SerotypeFinder 2.0.1 (<https://cge.food.dtu.dk/services/SerotypeFinder/>) with 95% threshold identity and 80% minimum length (35, 43). The MLST of both species of bacteria was predicted from the assembled draft genome using MLST 2.0 2.0 (<https://cge.food.dtu.dk/services/MLST/>) as described in (51, 53, 54). The assignment of cgMLST of *E. coli* was done using cgMLSTFinder 1.2 (<https://cge.food.dtu.dk/services/cgMLSTFinder/>) as described in previous studies (35, 55). The pathogenicity of both species of bacteria was predicted from their genome based on gene

families that correlate with pathogenicity using PathogenFinder 1.1 (<https://cge.food.dtu.dk/services/PathogenFinder/>) as described in (56). Plasmid Multilocus Sequence Typing (pMLST) was carried out on draft assembled plasmids on incompatibility groups for which a pMLST scheme is available using (<https://cge.food.dtu.dk/services/pMLST/>) as described in (42).

6.3 Results

6.3.1 Prevalence and Antimicrobial Resistance Profile of ESBL-*E. coli* and -*K. pneumoniae* in Bulk Tank Milk

A total of eight ESBL-*E. coli* and two ESBL-*K. pneumoniae* strains were isolated from 33 milk samples collected from 17 farms. The isolates were detected from nine farms; one *E. coli* and *K. pneumoniae* were isolated from the same farm, whereas the others were detected from 8 different farms. From the eight *E. coli* isolates identified with ESBL phenotype, one isolate, CD-M, was later confirmed to be susceptible to all beta-lactam antibiotics and genotypically did not have any signature of ESBL genes. Thus, the sample level prevalence of ESBL-*E. coli* was 24.2% (7/33), whereas that of *Klebsiella pneumoniae* was 6.1% (2/33). At the farm level, ESBL-*E. coli* and -*K. pneumoniae* prevalence was 41.2% (7/17) and 11.8% (2/17), respectively.

All eight *E. coli* isolates identified in this study were tested for antimicrobial susceptibility against 14 antimicrobial agents. All *E. coli* isolates with ESBL phenotype (n=7) were co-resistant to ampicillin, ceftriaxone, and tetracycline, and all of them were multidrug resistant (resistant to three or more antimicrobial classes). More than half of the ESBL-*E. coli* isolates (n=4) were phenotypically resistant to five or more antimicrobials, including those considered the highest priority critically essential classes (HPCI). The ESBL phenotype co-existed with other resistant phenotypes to CIAs, such as ceftriaxone, streptomycin, and fluoroquinolones (nalidixic acid or ciprofloxacin) in four *E. coli* strains. One *E. coli* isolates (AG-M) was resistant to nine antimicrobial agents belonging to 7 classes. All *E. coli* isolates were susceptible to meropenem, ceftiofur, and amoxicillin-clavulanic acid. One *E. coli* isolates (CD-M) was susceptible to all the tested antibiotics (Table 6.1, placed under the appendix).

6.3.2 Resistance Genes and Their Association with Mobile Genetic Elements in *E. coli*

Whole genome sequencing analysis confirmed that eight bacterial strains identified by MALDI-TOF as ESBL-*E. coli* were confirmed to be ESBL-*E. coli* by WGS. One of these is ESBL-*E. coli* isolates (CD-M) were found to have no resistance genes to beta-lactam antibiotics or many other classes of antibiotics. Genome sequence analysis did not find the presence of ESBL or other ARGs in *E. coli* isolates that were phenotypically susceptible to all tested antibiotics.

All seven ESBL-*E. coli* strains carry the *bla*_{CTX-M} variant and five isolates co-carry *bla*_{TEM-1}. Among ESBL genes, *bla*_{CTX-M-32} was a common variant and was detected in five of the isolates. All ESBL-*E. coli* strains co-harbored *bla*_{CTX-M} with plasmid-mediated quinolone resistance gene (*qnrB19*), and a tetracycline resistance gene, *tet*(A).

Resistance genes to aminoglycosides (*aadA1* or *aph*(3'')-Ib, *aph*(6)-Id)), quinolones (plasmid-mediated and point mutations), phenicol (*floR*), and sulphonamides (*sul2*) were concurrently detected in four ESBL-*E. coli* strains that harbored the *bla*_{CTX-M-32} variant. The macrolide phosphotransferases (*mph*(A)), aminoglycoside nucleotidyltransferase (*aadA1*), dihydrofolate reductase (*dfrA*), and dihydropteroate synthase (*sul1*) resistance genes were detected in a single isolate, AG-M. As expected, *bla*_{AmpCs} and carbapenemase-associated genes were not identified.

Mutation in the *ampC* gene promoter region was detected in three isolates (CD-M, DA-M, and DC-M) but was not expressed. Double or triple mutations in Quinolone-Resistance Determining Regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* were detected in six isolates. These multiple chromosomal mutations were (*gyrA*-S83L, D87Y, D741E, D87G, D87N, D678E, Q454H, and D741E; *parC*- E62K or S80I; *parE*- L416F and *gyrB*-Y184D). A point mutation that potentially confers resistance to colistin, *pmrB*, was detected in four of the isolates. For one isolate (DM-M), the genotypes that mediate the phenotypic resistance, such as aminoglycosides and chloramphenicol, were not detected in the Center of Genomic Epidemiology database (<https://cge.food.dtu.dk/services/ResFinder/>).

Further in-depth analysis of WGS in the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/analyze/rgi/>) identified *marA* and *evgA*, genes that encode

efflux pumps mediate resistance to several classes of antibiotics, including aminoglycosides and chloramphenicol. A quinolone resistance gene, *qnrB19*, was consistently associated with Col440I plasmid replicon types in six ESBL-*E. coli* isolates. All except one *bla*_{CTX-M} gene were associated with MGEs alone or with other resistance genes. In some of the ESBL-*E. coli* isolates, multiple resistance genes are co-located on the same contigs associated with the same or different MGEs (Table 6.1, placed under appendix). In two of the ESBL-*E. coli* strains, BET-M, and RM-M, six other resistance genes, (*aph*(3'')-Ib, *aph*(6)-Id, *tet*(A), *floR*, *sul2*, *bla*_{TEM-1A}), mediating resistance to five different classes of antibiotics were all found on the same contigs associated with IS91. All except one *bla*_{CTX-M} and almost all other co-harbored acquired ARGs were associated with MGEs such as plasmids, Insertion sequences (IS), or transposons (Tn) on the same genetic fragment.

6.3.3 Genetic Diversity, Plasmid Types, and Pathogenomics of ESBL-*E. coli* Isolates

The *E. coli* isolates were assigned to seven distinct serotypes, six multilocus sequence types (STs), and eight-core genome sequence types (cgSTs) based on in silico sequence typing of the WGS data. Two isolates from different farms, RM-M and BET-M, had the same serotype (O101:H9) and sequence type (ST10). All eight strains were distinct according to their core genome sequences, based on comparing nearly all the genes shared by *E. coli*. The ST10 type was relatively common and found in three farms. Four *E. coli* isolates (3 ST10 and 1 ST69) were associated with human disease. Three isolates, AG-M, ST-M, and DA-M, had identical genotypes (ST and resistance gene pattern) as *E. coli* isolates from fecal samples collected from dairy cattle in the same farms where BTM was also obtained.

PlasmidFinder identified 13 different plasmid replicon types from the WGS data of the seven ESBL-*E. coli* isolates. Each ESBL-*E. coli* isolate had at least two plasmid replicon types. The IncF family plasmids (FII, FIA, and FIB replicon types) were common and found in four ESBL-*E. coli* isolates. Colicinogenic plasmids (Col440I) were the most frequent and detected in all seven ESBL-*E. coli* isolates. The ST10 type carried the largest number (6 to 8) and types of plasmids. Two ST10 and ST69 had epidemic resistance plasmids of the IncF family. No plasmid

was detected in the WGS of one *E. coli* isolate, CD-M, which was susceptible to all tested antibiotics.

Several virulence genes were detected in all eight *E. coli* isolates. Each isolate had multiple virulence genes involved in adhesion (adhesin), invasion (invasins for bacterial fitness), and effectors or toxins (Table 6. 2, placed under the appendix). The highest number of virulence genes (40) were detected in the ST69 isolate, and the lowest number (8) in the ST5891 isolate, susceptible to all tested antibiotics. Widespread virulence genes, such as *aslA*, *fimH*, *yeh*, *treC*, *gad*, *csgA*, *terM*, *hylF*, *iss*, and *traT*, were found in the *E. coli* genomes. No hits for the Shiga toxin-producing gene were detected in the WGS of the isolates. The draft sequences of all *E. coli* isolates were submitted to PathogenFinder (<https://cge.food.dtu.dk/services/PathogenFinder>), and all were predicted as human pathogens with a probability greater than 0.92.

6.3.4 Co-Localization of Virulence Genes and Mobile Genetic Elements

Several virulence genes were detected on ESBL-*E. coli* were co-located on the same contigs with the MGEs (plasmids, insertion sequences, and transposons). In addition, multiple virulence genes were often co-located with or on the same MGEs. In some instances, copies of the same virulence genes were associated with different MGEs (e.g., plasmids and transposon). Most virulence genes related to MGE are those involved in the invasion and adhesion of mammalian cells. Only two isolates have effector (protease) encoding genes associated with MGEs. Table 6.3, placed under appendix) showed several virulence genes associated with one or more MGEs.

6.3.5 Resistance Phenotypes and Genotypes in ESBL-*K. pneumoniae*

The two *K. pneumoniae* isolates were resistant to ceftriaxone and ampicillin. In addition to the two antibiotics, one of the isolates, DM-MK, was resistant to sufisoxazole. Each *K. pneumoniae* isolate carried two distinct types and variants of ESBL-genes, the *bla*_{CTX-M}, and *bla*_{SHV}. In addition, both isolates had two different types of narrow-spectrum beta-lactamase genes.

The *K. pneumoniae* isolates contain a multidrug efflux pump encoding gene, *oqxAB*. Both *oqxB* and *oqxA* genes were found in one of the isolates, and this isolate was resistant to sufisoxazole.

The other isolate only had the *oqx*A gene and was susceptible to the same antibiotic. Both isolates are considered genotypically multidrug resistant, carrying at least eight resistant determinants against multiple classes of antibiotics. However, the observed phenotypic resistance was low (Table 6. 4, placed under the appendix).

6.3.6 Genetic Diversity, Plasmid Types, and Pathogenomics of *K. pneumoniae*

The WGS data were used for in silico sequence typing on the two *K. pneumonia* isolates. One of the isolates (BM-MK) was assigned as ST867, while the remaining one was novel and could not be assigned. The ST867 is identical to three other STs identified from fecal samples from the same farm where BTM was obtained. Both *K. pneumoniae* obtained from BTM were identified as new serotypes of *K. pneumoniae*. Both strains contain IncF family plasmids with replicon types FII. The ST867 strain only had the FII replicon. The novel strain had seven plasmids, including two Col plasmids (Col440I and II), four epidemic resistance plasmids with IncF plasmid families [FII, FII(K), FIA, and FIB replicons], and one IncN plasmid, which is a broad host range plasmid. This study identified a new allele type for IncFIA (HI1) plasmid using MLST. The WGS data also revealed the presence of multiple virulence genes such as *fimH* (type 1 fimbriae), *fyuA* (siderophore receptor), *ituA* (ferric aerobactin receptor), *traT* (outer membrane protein complement resistance), and *mrkA* (type 3 Fimbrial Shaft) in ST867. More number of virulence genes were detected in the novel *K. pneumoniae* ST. The virulence genes detected in this ST include *fimH*, *traT*, *fyuA*, *iutA*, *mrkA*, *Irp2* (non-ribosomal peptide synthetase), *ccI* (Cloacin), *clpK1* (thermal stress survival ATPase). Both *K. pneumoniae* were predicted to be human pathogens with a probability greater than 0.82.

6.4 Discussion

This study found a high prevalence of ESBL-producing *E. coli* in BTM from dairy farms. About 41% of the farms were found to have ESBL-*E. coli* in their BTM, and 24% of the BTM samples tested positive for these bacteria. Additionally, ESBL-producing *K. pneumoniae* was detected in two BTM samples from two farms. Since no similar studies have been conducted in the U.S. or elsewhere, a comparison was not possible in the current study. However, in our earlier study that

used different screening techniques, ESBL-*E. coli* was detected in at least one of the BTM samples collected from four farms (6).

Similarly, studies conducted on mastitic milk in New York (17), South Korea (26), Greece (27), and China (25) detected ESBL-*E. coli*. A comparison of the prevalence of ESBL-*E. coli* between the current study and these studies are not plausible as the sample source, and the initial screening methods used were different. This suggests the prevalence of ESBL-*E. coli* in BTM may be higher than previously thought, highlighting the need for continuous monitoring and evaluation of associated risk and developing innovative control tools at farm levels.

The study also indicated that all ESBL-*E. coli* were MDR, with most of them displaying concurrent resistance to five or more antibiotic classes. These findings are consistent with previous studies in animals and humans that showed ESBL-*Enterobacteriaceae* frequently showed co-resistance to multiple classes of antibiotics (4, 6, 57, 58). Four ESBL-*E. coli* also showed concurrent resistance to CIAs, such as ceftriaxone, fluoroquinolone (ciprofloxacin and/or nalidixic acid), and aminoglycosides (streptomycin and/or gentamycin). This result also agrees with our recent study and several other studies that reported ESBL-producers frequently exhibit co-resistance to fluoroquinolones, aminoglycosides, chloramphenicol, and tetracycline (4-6, 59-62). However, the study farms did not report aminoglycosides, tetracyclines, and fluoroquinolones as frequently used antibiotics. In addition, fluoroquinolones and macrolide are not allowed to use in the U.S. dairy cattle of more than 20 months of age (63). Thus, the detection of fluoroquinolone resistance in half of the isolates and azithromycin resistance in one of the isolates from BTM pooled from adult cows may not be related to antibiotic use. Further investigation is needed to identify if the use of these antibiotics at a young age on calves has an impact during adult age or if ceftiofur uses in dairy cattle co-select for resistance determinants against these two CIAs, as previously hypothesized (64). In addition, it is also vital to examine if resistance genes against these two classes of antibiotics are maintained through bacterial generation via MGEs that confer a fitness advantage to the host.

In line with this thought, WGS analysis of the isolates identified that *qnrB19* (a gene mediating resistance to ciprofloxacin) was consistently associated with Col440I plasmids. A previous study

showed that ColE1-derived plasmids, including Col440I, contain genes that encode for bacteriocins, proteins that can kill other bacteria and provide a competitive advantage to the host bacterium (65). This suggests that the association of *qnrB19* with Col440I might be responsible for its maintenance in the absence of selection pressure. A recent study in Germany also reported frequent association of *qnrB19* with Col440I plasmids in ESBL-producing *E. coli* from livestock (66).

All isolates were susceptible to meropenem, one of the last resort antibacterial agents whose use is typically reserved for severe infections caused by pathogenic members of ESBL-*Enterobacteriaceae* (67). This finding is not unexpected, as carbapenems are not approved for animal use in the U.S. due to the risk of promoting the emergence of carbapenemase-producing *Enterobacteriaceae*. Detection of ESBL-*E. coli* with phenotypic resistance to multiple classes of antibiotics, particularly to those CIAs, makes consuming raw milk a dangerous practice for public health as only limited therapeutic options are available to cure the infection caused by the bacteria with these phenotypes (68).

The WGS analysis showed that the observed phenotypic resistance patterns are generally congruent with genotypic results, as reported in a previous study suggesting that the genotypes of the bacteria are a good indicator of their resistance to antibiotics (69). The *bla*_{CTX-M} was the only ESBL-variant observed from all ESBL-*E. coli* isolates phenotypically resistant to ceftriaxone, a 3GC. This finding concurs with our previous study, where we reported *bla*_{CTX-M} as the only ESBL variant from *E. coli* isolates retrieved from a dairy farm environment with phenotypic resistance to cefotaxime, another 3GC (6). Similarly, other studies in the U.S. and elsewhere also showed the predominance of the *bla*_{CTX-M} variant of ESBL over the others among the *Enterobacteriaceae* in humans and food animals, including dairy cattle (4, 5, 70-75). It is hypothesized that the frequent occurrence and the dominance of the *bla*_{CTX-M} variant over the others in ESBL-*E. coli* may be related to its low fitness cost to the host, successful dissemination through MGEs, and rapid response to beta-lactam antibiotics selection pressure (76). As we already discussed in our published review (4), further controlled study is needed to identify the cause of the spread and dominance of this variant of ESBL genes.

The *bla*_{CTX-M-32} was detected in five farms, whereas the remaining two, *bla*_{CTX-M-27} and *bla*_{CTX-M-65}, were obtained from ESBL-*E. coli* retrieved from two separate farms. These *bla*_{CTX-M-32} variants were previously reported from ESBL-*E. coli* isolated from fecal samples in the U.S. dairy farms (5, 64, 77). The frequent presence of the non-ESBL beta-lactamase gene (*bla*_{TEM-1}) along with the ESBL gene in *E. coli* may aid in maintaining and transmitting one or both genes to future generations clonally and to other bacteria horizontally via MGEs.

The fact that all ESBL-*E. coli* strains co-harbored *bla*_{CTX-M} with *qnrB19* (plasmid-mediated quinolone resistance gene), and *tet(A)* concurs with the phenotypic observation. But despite the presence of the *qnrB19*, no corresponding resistance to ciprofloxacin was observed in one of the isolates. This discrepancy may be due to a low or lack of gene expression in that isolate due to various factors, including mutation in the resistance gene and lack of activation of regulatory genes. Four ESBL-*E. coli* strains, co-harbored *bla*_{CTX-M-32} with resistance genes to aminoglycosides (*aadA1* or *aph(3'')-Ib*, *aph(6)-Id*), quinolones (*qnrB19*), and mutation in one or more of fluoroquinolone resistance determining regions (QRDRs), phenicol (*floR*), and sulfonamides (*sul2*). This observation is also consistent with the phenotypic result and previous molecular studies that detect frequent co-occurrence of ESBL genes with multiple other ARGs (5, 6, 78).

It is noteworthy that strains of ESBL-*E. coli* from two farms have the same six antibiotic resistance genes co-located on the same contigs with IS91, a mobile genetic element. Multilocus Sequence Typing (MLST) results showed that both strains belong to the same Sequence Type (ST10). However, the cgMLST typing identified both isolates as distinct strains. The two strains carry the same mutation in QRDRs (*gyrA*, *parC*, and *parE*) but differ in the *pmrB* gene presence and their plasmid replicon types. The detection of the same ARG with a similar genetic arrangement on the identical STs of *E. coli* needs further investigation. Still, a possible explanation could be that the strains may have a common ancestry and may have spread from one farm to another, parallel evolution between the two strains with the ARGs they carry, or contamination in the laboratory. However, contamination is less likely as the samples were collected and processed at different times.

All but one of the *bla*_{CTX-M} genes and most other ARGs co-harbored by the ESBL-*E. coli* were associated with mobile genetic elements (MGEs), such as plasmids, insertion sequences (IS), or transposons (Tn). The presence of ESBL and non-ESBL beta-lactamase genes, along with multiple ARGs on MGEs, is consistent with findings from previous studies on ESBL-*E. coli* isolates from dairy cattle and humans (4, 5, 25, 27, 79, 80). This suggests that MGEs may facilitate the spread of these ARGs. The presence of multiple ARGs located on the same MGEs could make the transfer of these genes to other bacteria of the same or different species easier. This could lead to the development of extensively drug-resistant bacteria, which may risk human health through indirect transmission (80, 81).

Some of the ESBL-*E. coli* strains, ST10 (n=3) and ST69 (n=1) reported in this study had the same STs as previously reported as "high-risk" clones. These two sequence types were identified globally as the most prevalent extra-intestinal pathogenic *E. coli* (ExPEC) lineages after ST131 (82). A study on clinical *E. coli* isolates from 1999 to 2017 in California also identified these two STs as the most common causes of urinary tract infection (83). The ST10 and ST69 carry IncFII and/or IncI1, plasmid families. This finding is consistent with previous studies that reported high-risk clones of *E. coli* are frequently harbored by easily transmissible narrow host range or epidemic plasmids such as IncF and/or IncI1 (5, 84, 85).

The detection of triple mutations in the QRDR region in two of the STs, ST10, and ST69, along with other ARGs (*qnrB19*, *floR*, *tet(A)*, and *sul2*), indicated a public health risk associated with these bacterial clones. These STs have been previously reported in fecal samples from dairy cattle and other food-producing animals in the U.S. (5, 86). The ST69 carried *bla*_{CTX-M-27} and *bla*_{TEM-1c} on IncFII (F2:A-B-), an epidemic resistance plasmids known by its ability to acquire resistance genes and easily and rapidly spreading within the same or related species of bacteria (84). This ability to acquire and spread resistance genes quickly increases the prevalence of ESBL-producing bacteria and the difficulty of treating infections and is a significant public health concern.

In this study, the *pmrB* gene, which is commonly associated with colistin resistance (87), was detected in four *E. coli* isolates, including in two pandemic clones, ST10 and ST69. Detecting a gene associated with colistin resistance is a public health concern as colistin is considered a last-resort antibiotic for treating infections caused by ESBL- and carbapenemase-producing *Enterobacteriaceae* (88, 89). The current study did not test for phenotypic colistin resistance because the panel of antibiotics used did not include colistin. Thus, the colistin gene's impact on colistin resistance needs further study, but its presence still raises significant health risks. Furthermore, detecting colistin resistance genes in ST10 and ST69, epidemic strains, highlights the high risk associated with consuming raw milk. The plasmid-mediated colistin resistance gene, *mcr-1*, was previously detected in *E. coli* at a very low level (0.1- 0.02%) in the U.S. (90, 91) but no previous report on *Enterobacteriaceae* with any colistin resistance gene from BTM. The MDR ESBL-producing strains are already challenging to treat, and the additional presence of colistin-resistance genes increases the risk of treatment failure (92). Detection of colistin resistance determinant in BTM was unexpected as colistin has never been marketed for use in the U.S. dairy farms (<https://www.center4research.org/8094-2/>).

All ESBL-*E. coli* isolates in this study carried several virulence genes exhibiting pathogenic properties (adhesion, invasion, and toxin production) in their mammalian host. The most frequently detected virulence genes in ESBL-*E. coli* were *fimH* (type1 fimbriae), *csgA* (Curli fimbriae gene), *yehA-D* (various fimbriae cluster), and *fdeC* (intimin-like adhesin) for adhesion; *iss* (increased serum survival protein encoding gene), *aslA* (arylsulfatase-like gene), *terC* (Tellurium ion resistance protein encoding gene), *gad* (glutamate decarboxylase encoding gene), and *traT* (outer membrane protein responsible for complement resistance), and *sitA* (iron transport protein) for bacterial replication and invasion; and *hlyF* (hemolysin) for toxin secretion (93-97). The virulence genes belong to various pathotypes of *E. coli*, but most are related to extra-intestinal pathogenic *E. coli* (ExPEC) (94, 95, 97). Among the *E. coli* strains, the ST69 has the most abundant and diverse virulence genes, including three toxin genes, *hlyE* (hemolysin), *astA* (heat-stable enterotoxin EAST-1), *espP* (serine protease autotransporter), suggesting the public health hazard associated with this strain. The ST69 *E. coli* is reported as the second most common cause of extraintestinal infection across the globe (82).

In some isolates, virulence genes and ARGs were co-located on the same plasmids or associated with other MGEs. For example, in ST69, *traT* (a virulence gene that helps the bacteria to survive the host serum bactericidal effect of the complement system) is located on IncFII (F2:A: B) plasmids together with *tet(A)* and *bla*_{TEM-1}. Hayer *et al.* (86). also reported an association between the *traT* virulence gene and IncFI plasmids from *E. coli* isolates obtained from pigs in the U.S. The virulence gene, *traT*, was indicated to be frequently associated with sepsis and urinary tract infections in humans (94, 98). Thus, co-location favors the clonal and horizontal spread of the virulence and resistance genes and may cause a significant clinical impact on people consuming raw milk. In addition to detecting the virulence gene in each strain, in silico assessment of the pathogenicity of the bacteria to the human host predicted that all the ESBL-*E. coli* are highly likely (probability > 0.92) to be a human pathogen (56). *E. coli* is one of the most important milk borne human pathogens (99). *E. coli* causes a wide range of severe infections in humans (100) and is also among the frequent cause of environmental mastitis in dairy cattle (101), along with colibacillosis in calves [6]. Thus, the presence of multiple ARGs, combined with the presence of virulence factors, make ESBL-*E. coli*, a greater threat to human health. Furthermore, the detection of potential pathogenic and MDR ESBL-*E. coli* isolates in this study support the argument about the possible convergence between ESBL-production and virulence (102).

The ESBL-*E. coli* isolate that showed resistance to nine antibiotics was found to have the same resistant pattern as most *E. coli* isolates retrieved from fecal samples from the same farm (Farm A), suggesting possible fecal contamination of the bulk tank milk from the cow. Similarly, WGS analysis of this isolate identified eleven different ARGs, including *mph(A)* (macrolide phosphotransferases), *aadA1*(aminoglycoside nucleotidyltransferase), *dfrA*(dihydrofolate reductase), and *sul1*(dihydropteroate synthase) which was detected only in this isolate. Multiple *E. coli* isolates with the same serotype (045:H25) and sequence types (ST2325) were isolated from a fecal sample obtained from the same farm. Detection of the same serotypes and sequence of *E. coli* isolates from fecal samples strongly indicates fecal contamination of the BTM. This highlights the importance of implementing hygiene and sanitation measures to prevent

contamination and reduce the risk of transmission of pathogenic or commensal MDR bacteria to humans.

Unlike ESBL-*E. coli*, the two *K. pneumoniae* isolates did not exhibit acquired multi-drug resistance phenotypes. However, the detected resistance genotypes of the *K. pneumoniae* isolates were consistent with their expressed phenotypes. Even without MDR phenotypes, the presence of ESBL-producing *K. pneumoniae* is still a concern, as it can contribute to the spread of antibiotic resistance (103). Both *Klebsiella* strains harbored three beta-lactamase encoding genes, two ESBL genotypes, and one non-ESBL genotype. The detection of non-ESBL or narrow spectrum beta-lactamase, *bla*_{TEM-1B} in one and *bla*_{SHV-2} in the other strain is consistent with the established literature as *Klebsiella* spp. are inherently resistant to penicillin (104). Detection of multiple resistance genes to beta-lactam antibiotics suggests that these isolates have accumulated multiple mechanisms for resistance to beta-lactam antibiotics. The observed ESBL phenotypes might be mediated by *bla*_{CTX-M-27} and *bla*_{SHV-62} in ST867 and *bla*_{CTX-M-1} and *bla*_{SHV-40} in the novel strain (105).

Sequence typing assigned one of the *Klebsiella* isolates, BM-MK, to ST867, but its serotype was new and could not be assigned. The other isolate, DM-MK, was also not assigned to a particular sequence type or serotype, i.e., its genetic makeup or antigenic characteristics did not match any known *K. pneumoniae* subtypes based on its DNA sequence. This *K. pneumoniae* isolate was identified as a novel serotype and sequence type; it was not previously reported in the scientific literature. This finding highlights the importance of continuous monitoring and surveillance for the emergence of new strains of ESBL-producing bacteria to detect them early before their spread and inform public health efforts to control them.

In the novel strain, *K. pneumoniae*, the ESBL genotypes co-existed with resistance genotypes that mediate resistance to three CIAs, including aminoglycosides (*aph*(6)-Id), fluoroquinolone (*qnrB19*), a macrolide (*mph*(A)). However, the corresponding resistance phenotypes were not observed against these antibiotics during AST, suggesting a lack of proper expression of the respective genes. The resistance to sulfisoxazole observed in the ST867 *Klebsiella* strain may be due to the *oqxAB* operon, a plasmid-borne multi-drug efflux pump (106). The absence of known

genes related to folate antagonists does not rule out the possibility of efflux pump-mediated resistance. Detection of multiple resistance genes in *K. pneumoniae* is consistent with the previous studies and reviews that identified *K. pneumoniae* as a "key trafficker" of multi-drug resistance genes (103, 107, 108).

The two *K. pneumoniae* strains predominately harbored the IncF plasmid families. The predominance of epidemic resistance plasmids (formerly known as narrow host plasmids) in these two strains of *K. pneumoniae* is consistent with the previous findings that reported a frequent association between ESBL-producing *Klebsiella* spp. and IncF plasmid families (84). The IncFII(K) and IncFIB(K) plasmids identified in the novel strain are highly similar to (96 - 99% nucleotide identity) those plasmids identified from MDR carbapenemase-producing ST258 *K. pneumoniae* isolate from a human in Italy (109). This suggests closely related epidemic-resistance plasmids are circulating in both animals and humans across the globe. The col440I plasmids that carried *qnrB19* in the novel strain were similar (91% nucleotide identity) to the plasmid identified from *K. pneumoniae* isolates from human blood samples in the U.S. (Accession: CP023920.1; the data not published).

The ESBL- *K. pneumoniae* were also enriched with virulence determinants (*fimH*, *ccI*, *clpK1*, *traT*, *Irp2*, *fyuA*, *iutA*, *mrkA*) that may enhance their pathogenic potential. The siderophore receptor gene (*fyuA*), the Ferric aerobactin receptor gene (*iutA*), and the iron regulatory protein (*Irp2*) encode proteins associated with iron uptake and metabolism. Type 1 fimbriae (*FimH*) help the bacteria adhere to the mammalian host cell. The genes *ccI* (encodes for cloacin), *clpK1* (encodes heat shock survival AAA family ATPase), and *traT* (encodes outer membrane protein responsible for complement resistance) help evade the host defense mechanism and contribute to the pathogenicity (66, 110, 111). In addition, further analysis of the *Klebsiella* genome predicted both strains as possible human pathogens with a high probability (probability > 0.87). The presence of ESBL and multiple virulence genes in *K. pneumoniae*, a frequent cause of severe infections in humans and mastitis in dairy cattle, highlights the significant public health and veterinary risk posed by this bacterium (28, 29). Monitoring and controlling the ESBL-

producing Enterobacteriaceae, particularly those with increased virulence, is crucial to reduce infection risk and to improve public and animal health.

Conclusion

The results of this study revealed a high prevalence of multidrug resistant ESBL-*E. coli* and presence of ESBL-*K. pneumoniae* in bulk tank milk in East Tennessee dairy farms. These bacteria carry several resistance and virulence genes often linked to mobile genetic elements, making it easy for them to spread to other bacteria. Half of ESBL-*E. coli* isolates from this study are considered high-risk clones due to their ability to cause disease and acquire genetic traits that give them an advantage over other bacterial clones, including virulence factors, epidemic plasmids, and ARGs. Detection of these highly resistant and virulent bacteria in raw bulk tank milk threatens public health. The bacteria can cause clinical infection or transfer their resistance and virulence gene to a more clinically relevant strain. Feces were also found to be a potential source of ESBL-*E. coli*, which might be a source of milk contamination, suggests the need for hygienic milking practices on farms. The WGS data of *E. coli* isolates revealed a diverse population of bacteria with seven distinct serotypes, six STs, and eight different cgSTs. The discovery of a new allele type for the IncFIA (HI1) plasmid in the novel ST of *K. pneumoniae* can help to further our understanding of the diversity of strains of this bacterium and its plasmids. This study indicates that raw milk is a reservoir of potentially zoonotic MDR *E. coli* and *K. pneumoniae*, carrying readily transferable resistance and virulence genes. Thus, the consumption of raw milk and milk products has a public health risk with potentially MDR infections.

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Appendix

Table 6. 1. Antimicrobial resistance phenotypes and mobile genetic elements associated with resistance genes in ESBL-*E. coli*

Isolate		Antimicrobial Resistance								MGE* linked to resistance genes
Phonotypes		Genes								
		βRG	FQRG	ARG	MRG	PRG	FARG	TRG	PM	
AG-M	AXO, AMP, AZI, CIP, NAL, STR, CHL, STX, TET	<i>bla</i> _{CTX-M-32} ^a	<i>qnrB19</i> ^b	<i>aadA1</i>	<i>mph(A)</i> ^c	<i>floR</i> ^d	<i>sul1</i> , <i>dfrA1</i>	<i>tet(A)</i>	<i>gyrA</i> <i>parC</i>	IS630 ^a Col440I ^b IncR ^c IS6 ^d
RM-M	AXO, AMP, FIS, TET	<i>bla</i> _{CTX-M-32} ^e , <i>bla</i> _{TEM-1A} ^f	<i>qnrB19</i> ^g	<i>aph(3'')-Ib</i> ^f , <i>aph(6)-Id</i> ^f	-	<i>floR</i> ^f	<i>sul2</i> ^f	<i>tet(A)</i> ^f	<i>gyrA</i> <i>parE</i> <i>parC</i>	IS5 ^e , IS91 ^f Col440I ^g
DM-M	AXO, AMP, CIP, NAL, GEN, STR, CHL, TET	<i>bla</i> _{CTX-M-27} ^h , <i>bla</i> _{TEM-1c} ⁱ	<i>qnrB19</i> ^j	-	-	-	<i>sul2</i>	<i>tet(A)</i> ⁱ	<i>gyrA</i> , <i>gyrB</i> , <i>parC</i> , <i>pmrB</i> , <i>ampC</i> [*]	IncFII ^h , IS6 ^h Tn1 ⁱ , IncFII(pCoo) ⁱ , Col440I ^j Tn3 ^j , Col440I ^k , IncFIA(HI1) ^l
ST-M	AXO, AMP, STR, CHL, FIS, TET,	<i>bla</i> _{CTX-M-32} , <i>bla</i> _{TEM-1A} ^j	<i>qnrB19</i> ^k	<i>aph(3'')-Ib</i> ^j , <i>aph(6)-Id</i> ^j	-	<i>floR</i> ^l	<i>sul2</i> ^j	<i>tet(A)</i>	<i>parC</i> <i>pmrB</i>	Tn3 ^j , Col440I ^k , IncFIA(HI1) ^l
DA-M	AXO, AMP, TET	<i>bla</i> _{CTX-M-65} ^m	-	-	-	-	<i>sul2</i> ^m	<i>tet(A)</i>	<i>parC</i> , <i>ampC</i> [*]	IS6 ^m
RC-M	AXO, AMP, TET	<i>bla</i> _{CTX-M-32} , <i>bla</i> _{TEM-1B} ⁿ	<i>qnrB19</i> ^o	-	-	-	-	<i>tet(A)</i> ⁿ	<i>parC</i> , <i>parE</i> , <i>gyrA</i> , <i>pmrB</i>	Tn1721 ⁿ , Col440I ^o
BET-M	AXO, AMP, CIP, NAL, STR, CHL, FIS, TET	<i>bla</i> _{CTX-M-32} ^p , <i>bla</i> _{TEM-1A} ^q	<i>qnrB19</i>	<i>aph(3'')-Ib</i> ^q , <i>aph(6)-Id</i> ^q	-	<i>floR</i> ^q	<i>sul2</i> ^q	<i>tet(A)</i> ^q	<i>gyrA</i> <i>parE</i> <i>parC</i>	IS5 ^p , IS91 ^q
CD-M	-	-	-	-	-	-	-	-	<i>parC</i> , <i>ampC</i> [*] , <i>pmrB</i>	-

Table 6. 2. Continued

*MGE: mobile genetic elements. ^{a-q} Resistance genes with the same superscript letters indicate they are co-located on the same contigs together with mobile genetic elements with the same superscripts. *: Mutation is on the promoter regions of *ampC*. AXO: ceftriaxone; FOX: ceftiofur; AUG2: amoxicillin-clavulanic acid; AMP: ampicillin; MERO: meropenem; AZM: azithromycin; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; TET: tetracycline; FIS: sulfisoxazole; STX: trimethoprim-sulfamethoxazole, β RG: beta-Lactamases resistance genes; FQRG: Fluoroquinolones resistance genes; ARG: aminoglycosides resistance genes; MRG: macrolides resistance genes; PRG: phenicol resistance genes; FARG: folate antagonist resistance genes; TRG: tetracycline resistance genes; PM: point mutation

Table 6. 3 . ESB L - $E. coli$ genetic diversity and pathogenomics

Isolate	Serotype	MLST ^a	CgMLST ^b	Virulence genes and their roles			Predicted Probability of pathogenicity to human
				Colonization (adhesins)	Fitness (invasins)	Effectors (toxins)	
AG-M	O45:H25	2325	133328	<i>fimH</i> , <i>yeh</i> B, C & D, <i>nlpI</i>	<i>cea</i> , <i>sitA</i> , <i>terC</i> , <i>iss</i> , <i>hlyE</i> <i>csgA</i> , <i>gad</i>		0.935
RM-M	O101:H9	10	34072	<i>fimH</i> , <i>fdeC</i> , <i>yeh</i> A, B, C & D, <i>nlpI</i>	<i>iss</i> , <i>terC</i> , <i>csgA</i> <i>aslA</i> , <i>hha</i>	<i>hlyE</i>	0.921
DC-M	O17/O44:H18	69	33877	<i>afaA</i> , B, C, & D; <i>yeh</i> A, B, C & D; <i>fae</i> C, D, F, I & J; <i>aalF</i> <i>lpfA</i> , <i>fimH</i> , <i>fdeC</i> , <i>air(eaeX)</i> , <i>eila</i> , <i>nlpI</i>	<i>traT</i> , <i>traJ</i> , <i>sitA</i> , B, & C; <i>iss</i> , <i>terC</i> , <i>chuA</i> <i>ompT</i> , <i>csgA</i> , <i>cvaC</i> , <i>aslA</i> , <i>gad</i> , <i>kpsMIII_K98</i> , <i>kpsE</i> , <i>mchF</i> , <i>hha</i>	<i>hlyE</i> , <i>astA</i> , <i>espP</i>	0.933
ST-M	O9:H30	540	160799	<i>fdeC</i> , <i>fimH</i> , <i>lpfA</i> <i>yeh</i> A, B, C & D, <i>nlpI</i>	<i>aslA</i> , <i>terC</i> , <i>iss</i> , <i>gad</i> , <i>csgA</i> , <i>hha</i>	<i>hlyE</i>	0.935
DA-M	O36:H36	10	115817	<i>yeh</i> A, B, C & D; <i>fdeC</i> , <i>fimH</i> , <i>nlpI</i>	<i>csgA</i> , <i>terC</i> , <i>traT</i> , <i>fyuA</i> , <i>aslA</i> , <i>irp2</i>	<i>hlyE</i>	0.934
RC-M	O142:H29	5728	5861	<i>yeh</i> B, C & D; <i>fdeC</i> , <i>nlpI</i>	<i>iss</i> , <i>csgA</i> , <i>terC</i> , <i>aslA</i> , <i>gad</i> , <i>hha</i>	<i>hlyE</i>	
BET-M	O101:H9	10	34081	<i>fdeC</i> , <i>fimH</i> , <i>yeh</i> A, B, C & D, <i>nlpI</i>	<i>aslA</i> , <i>terC</i> , <i>iss</i> , and <i>csgA</i> , <i>gad</i> , <i>hha</i>	<i>hlyE</i>	0.925
CD-M	O112ab:H19	5891	197619	<i>yehA</i> , B, C, & D, <i>lpfA</i> , <i>fimH</i> , <i>fdeC</i> ,	<i>terC</i> , <i>csgA</i> , <i>gad</i>	<i>hlyE</i>	0.944

^aMLST: Multilocus Sequence Type; CgMLST^b: core genome Multilocus Sequence Type.

Table 6. 4. Virulence genes and their association with mobile genetic elements

Isolate	Virulence Genes and Their Role		
	Colonization (adhesins)	Fitness (invasins) and Effectors (toxins)	The Mobile Genetic Element Associated with the Virulence Genes
AG-M	<i>fimH</i> , <i>yeh</i> B, C &D,	<i>cea</i> , <i>sitA</i> , <i>terC</i> , <i>iss</i> , <i>csgA</i> , <i>gad</i>	None
RM-M		<i>iss</i>	(IS1, IS630)
DC-M	(<i>afaA</i> -D, & <i>terC</i>) ^a <i>ompT</i> ^b (<i>yeh</i> A, B, C &D) ^c	<i>traT</i> ^d , (<i>terC</i> , <i>kpsMIII_K98</i> , <i>kpsE</i> & <i>nlpI</i>) ^e , <i>mchF</i> ^f , <i>gad</i> ^h , <i>espP</i> ^g	IS630 ^a , IS1 ^b , IS605 ^c , (Tn1 and IncFII(pCoo) ^d , IS5 ^e , IncFIB(AP001918) ^f , (IS30 and IS1) ^g , (IS30 and IS1) ^h
ST-M	(<i>fdeC</i> & <i>iss</i>) ⁱ	<i>gad</i> ^l , (<i>nlpI</i> & <i>terC</i>) ^k	IS1 ⁱ , IS150 ^j , IS630 ^k
DA-M	<i>fdeC</i> ^l , <i>fimH</i> ^m ,	<i>terC</i> ⁿ	(IS1, IS630) ^l , IS903 ^m , (IS51&IS1) ⁿ
RC-M		<i>terC</i> ^o , <i>gad</i> ^p , (<i>hlyE</i> & <i>gad</i>) ^q , <i>aslA</i> ^r	IS630 ^o , Tn1 ^p , (IS605, IS3& IS4) ^q , IS3 ^r
BET-M	(<i>fdeC</i> & <i>iss</i>) ^s	<i>terC</i> ^t , <i>gad</i> ^u ,	(IS1 & IS630) ^s , (IncHI2A and IncHI2) ^t , (IS150 and IS1) ^u
CD-M	(<i>yehA</i> , B, C, & D) ^v , <i>terC</i> ^x <i>fdeC</i> ^w		(IS3, IS630) ^v , (IS1 and IS630) ^w , IS630 ^x

^{a-x} virulence genes with the same superscript indicate they are co-located on the same contigs together with mobile genetic elements bearing the same superscripts. Those virulence genes in the same parentheses are co-located with Mobile genetic element/s having the same superscript.

Table 6. 5. Phenotypic and genotypic resistance in ESBL-*K. pneumonia* isolates

Isolate	Resistance Phenotype	Resistance Genes by Antibiotic Class						MGE	PHP
		βRG	FQRG	ARG	MRG	MEP	FRG		
ST867	AXO	<i>bla</i> _{CTX-M-27} ^a ,	<i>qnrB19</i> ^b	-	-	<i>oqx</i> <i>B</i> ,	<i>fosA</i>	<i>IncFII</i> ^a	0.89
	AMP	<i>bla</i> _{SHV-62} ,				<i>oqx</i> <i>A</i>		<i>Col440I</i>	
	FIS	<i>bla</i> _{TEM-1B} ^a							
Novel S	AXO	<i>bla</i> _{CTX-M-1} , <i>bla</i> _{SHV-2}	<i>qnrB19</i> ^c	<i>aph</i> (6)-Id	<i>mph</i> (A)	<i>oqx</i> <i>A</i>	<i>fosA</i>	<i>Col440I</i>	0.88
	AMP	<i>bla</i> _{SHV-40}							

AXO: ceftriaxone; AMP: ampicillin; FIS: sulfisoxazole; βRG: beta-lactamases resistance genes; FQRG: Fluoroquinolones resistance genes; ARG: aminoglycosides resistance genes; MRG: macrolides resistance genes; MEP: multidrug efflux pump; FRG: Fosfomycin resistance genes; MGE: Mobile Genetic Element associated with resistance genes; PHP :probability of being a human pathogen

Chapter 7. General Conclusion

This study investigated the prevalence, antimicrobial resistance, resistance spread mechanisms, risk factors, and public health implications of ESBL-*E. coli* and -*Klebsiella* spp. isolates from diverse samples obtained from dairy farms. The samples include rectal feces, bulk tank milk, manure, feed, and water. All samples were tested for the presence of ESBL-*E. coli* and -*Klebsiella* spp. by culturing on selective chromogenic media and presumptive isolates were confirmed by MALDI TOF. The confirmed ESBL-*E. coli* and -*Klebsiella* isolates were tested for antimicrobial susceptibility and subjected to whole genome sequencing.

The prevalence of ESBL-*E. coli* was very high, where almost half of the tested animals carried the resistant bacteria in their GIT. Overall, ESBL-*E. coli* was detected in 41% of all the samples (rectal feces, manure, water, and feed). We did not find any significant difference in the prevalence of ESBL-*E. coli* among different samples suggesting that the ESBL-*E. coli* is widespread in the dairy farm. The occurrence of ESBL-*E. coli* was widespread, and at least one animal in each study farm had the ESBL-*E. coli* in their feces. Most animals in nearly half of the study farm had ESBL-*E. coli* in their feces. The study identified recent exposure to ceftiofur, the third-generation cephalosporin (3GC), higher parity number, and calve linked with increased odds of fecal carriage of ESBL-*E. coli*, which are essential factors to consider when intervention is planned.

Almost all ESBL-*E. coli* isolates were resistant to at least one of the tested antibiotics, where 94.4% exhibited resistance to three or more classes of antibiotics, indicating their multidrug resistance pattern. Almost all the tested *E. coli* isolates were concurrently resistant to ceftriaxone, 3GC, and ampicillin. All farms had a common concurrent resistance among beta-lactam antibiotics (ceftriaxone and ampicillin) and other antibiotics such as streptomycin, tetracycline, sulfisoxazole, and chloramphenicol. None of the tested ESBL-*E. coli* isolates were resistant to carbapenems. This study identified six families of beta-lactamase genes (*bla*) mediating resistance to beta-lactam antibiotics, where about 99% of ESBL-*E. coli* harbored at least one of these genes. The *bla*_{CTX-M} and *bla*_{TEM} were the most dominant *bla* genes identified in the genomes of ESBL-*E. coli* at 96% and 52.4%, respectively. In addition to *bla* genes, a chromosomal mutation in the promoter region of the *ampC* gene was detected in many isolates,

potentially contributing to the observed resistance to beta-lactam antibiotics. We identified several types and subtypes of resistance genes (ARGs) against nine classes of antibiotics co-harbored with *bla* genes. Most ESBL-*E. coli* co-harbored ARGs to at least three classes of antibiotics. Resistance genes for critically important classes of antibiotics, specifically fluoroquinolones and aminoglycosides, were commonly co-harbored with the *bla* gene, which could have significant implications for public health.

This study identified diverse genotypes of ESBL-*E. coli* circulating in dairy farms, including previously uncharacterized and novel strains indicating dairy cattle could be a source of new ESBL-*E. coli*. This study also identified several identical STs of ESBL-*E. coli* within a farm showing a possible clonal expansion of the bacteria via animal-to-animal transmission. Identical *E. coli* ST was also isolated in samples obtained from different farms suggesting the possible inter-farm spread of the bacteria. Interestingly, nearly one-third of these STs were high-risk clones, including the ST10 and ST69, members of the most frequent high-risk pandemic lineages associated with MDR extraintestinal human infection across the globe.

This study demonstrated that ESBL-*Klebsiella* species was present in 10% of all the tested samples (rectal feces, manure, feed, and water), four times less than ESBL-*E. coli*. The animal-level prevalence of ESBL-*E. coli* was 7.2%. Although ESBL-*Klebsiella* spp. was detected in six farms, the ESBL-*Klebsiella* were isolated from rectal fecal samples only in five farms. In addition, most of the isolates were obtained from a single farm, suggesting that it is less widespread than ESBL-*E. coli*. Calves and cows with higher parity were associated with an increased risk of fecal carriage of ESBL-*Klebsiella* spp. All ESBL-*Klebsiella* spp. were isolated from dairy cattle with no recent exposure to ceftiofur, indicating that they may have acquired the resistance gene from other animals or maintained the gene they had for over six months prior to sampling. We found a high prevalence of ceftriaxone resistance among ESBL-*Klebsiella* spp. isolates, with the majority being co-resistant to sulfisoxazole. Furthermore, a significant proportion of isolates were MDR, with some being resistant to at least six classes of antibiotics, suggesting a potential threat of antimicrobial resistance in dairy farms.

This study detected a diverse range of *bla* genes in ESBL-*Klebsiella* spp. isolates, with *bla*_{SHV} and *bla*_{CTX-M} being the most frequently observed type. One or more variants of *bla*_{SHV} and *bla*_{CTX-M} co-occurred in about 93% ESBL-*Klebsiella* genomes, indicating that this bacterium is a reservoir of *bla* genes. More than two dozen horizontally transferable antibiotic resistance genes detected in the ESBL-*Klebsiella* spp. This highlights the genetic complexity of these isolates and the potential for the rapid spread of resistance in dairy farm settings. This study demonstrates a high genetic diversity among *K. pneumoniae* isolates, identifying fifteen distinct STs. The study also showed that ESBL-*Klebsiella* isolates harbor a diverse range of plasmid replicon types, with some carrying up to nine replicon types. The IncF family, narrow host range (epidemic resistance) plasmids, and Col440I plasmids were the most frequently detected types. In a farm where several ESBL-*K. pneumoniae* was isolated, most of the identical and distinct ESBL-*K. pneumoniae* STs shared an indistinguishable ST of IncFII plasmid that carried the *bla*_{CTX-M-27} and *bla*_{TEM-1B} genes. This suggests that this plasmid containing these genes may be transmitted vertically within specific lineages of ESBL-*K. pneumoniae*, leading to clonal expansion of these ARGs. On the other hand, the finding of the same plasmid ST associated with the same variant of *bla* genes in different *K. pneumoniae* STs in the farm suggests possible horizontal transmission of ARGs. This indicates that this epidemic resistance plasmid may play a crucial role in spreading the *bla* genes among *Klebsiella* isolates via two mechanisms.

This study provides valuable insights into the prevalence, molecular characteristics, and potential public health risks associated with ESBL-*E. coli* and -*K. pneumoniae* in bulk tank milk (BTM) in U.S. dairy cattle farms. The findings reveal that ESBL-*E. coli* is more prevalent than ESBL-*K. pneumoniae*, detected in 24.2% and 6.1% of BTM samples, respectively.

Moreover, all ESBL-*E. coli* strains isolated from BTM were found to be MDR and carried *bla*_{CTX-M}, and other ARGs, including those associated with mobile genetic elements. In addition, the study showed that the ESBL-*E. coli* isolated from BTM had several virulence markers which potentially help the bacteria attach to host cells, survive or evade the host's defense mechanisms, and secrete toxins that can affect the host cells. Most of these virulence genes were associated with mobile genetic elements (MGE) in all eight *E. coli* isolates from BTM. The finding of multiple ARGs and virulence markers associated with MGE underscores the potential for

horizontal transfer and spread of these genes, potentially leading to emerging pathogens with increased virulence and resistance. The study also identified matching STs and resistance patterns in ESBL- *E. coli* isolates obtained from BTM and fecal samples from the same farms, suggesting feces as a possible source of BTM contamination.

Additionally, the study identified that half of the ESBL-*E. coli* isolates from BTM belong to high-risk clones (ST10 and ST69), known to cause severe infections in humans. These findings suggest that BTM can serve as a source for zoonotic ESBL-*E. coli* and -*K. pneumoniae*, carrying readily transferable resistance and virulence genes. These bacterial strains can pose a threat to public health due to the high chance of transmission to humans through the food chain, especially through unpasteurized milk consumption. The results of this study serve as a wake-up call for the dairy industry and public health officials to take proactive control measures to safeguard the public from these potentially dangerous bacteria.

This study provides comprehensive insights into the prevalence, antimicrobial resistance, risk factors, molecular epidemiology, and potential public health risk associated with ESBL-*E. coli* and -*Klebsiella* spp., in dairy farms. These findings are significant in providing the status and spread mechanism of these AMR bacteria in dairy farms which are crucial to develop evidence-based intervention measures. It also serves as baseline data for monitoring the trend of ESBL status in dairy farms. Moreover, the finding of new sequence types of ESBL-producing *E. coli* and -*K. pneumoniae*, plasmids, and other results from this study provide additional evidence-based data of great importance to the scientific community. It also improves our understanding of the status of ESBL-*E. coli* and *Klebsiella* spp. in dairy farms. Further investigation, such as the longitudinal monitoring of ESBL-*E. coli* and -*Klebsiella* spp. colonization of dairy cattle throughout the lactation cycle and possible direct transmission between cattle and farm workers who are in direct and frequent contact with animals and their feces is needed.

It is crucial to take the following limitations into account while analyzing the findings of this study. Because only farms that volunteered to participate were included, and since the individual animals on those farms were not chosen randomly, selection bias might occur. Furthermore, there is a chance that survey respondents' responses about the use of beta-lactam antibiotics and

other farm management methods will result in misclassification bias. It should also be highlighted that this study did not assess the quantity of beta-lactam antibiotics used, which influences selection pressure and antibiotic resistance. In addition, the study cannot comment on the prevalence of commensal *E. coli* resistance to other antibiotics because it focused on ESBLs-*E. coli* resistance.

Moreover, since all but one of the farms included in the study were from East Tennessee, the results may not directly apply to dairy cattle farms in other parts of the state. Furthermore, the testing method employed to identify ESBLs-producing may introduce the possibility of misclassification, which may marginally affect reported prevalence. The use of short-read sequencing technology in this work may have impacted the number of contigs scaffold lengths obtained, possibly resulting in the under-detection of resistance genes in the genome studies. Furthermore, the thresholds for percent identity and coverage set to identify the different genes may result in under-detection of the respective genes.

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Vita

Benti Gelalcha was born in Ethiopia. He obtained a DVM degree from Addis Ababa University. He received his dual MSc degree from the Free University of Berlin and Addis Ababa University. He joined the University of Tennessee, Knoxville, in 2019 to pursue his Ph.D. study under the mentorship of Dr. Kerro Dego. He is passionately engaged in exploring the field of molecular microbiology with a specific focus on Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* species. He successfully defended his Ph.D. dissertation on May 9, 2023.