CHARACTERIZATION OF PUTATIVE EXTENDED-SPECTRUM β-LACTAMASES (ESBL) PRODUCING *ESCHERICHIA COLI* ISOLATED FROM FEEDLOT CATTLE IN SOUTHERN ALBERTA

PAMELA LUSSIER

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ABSTRACT

This thesis describes the detection, and characterization of putative extended-spectrum β-lactamases (ESBLs) producing *Escherichia coli* isolated from feedlot cattle in southern Alberta. Cattle either received no antimicrobials or were administered subtherapeutic antimicrobials in feed. In total, 7,184 *E. coli* isolates were collected, and screened for resistance to either ceftazidime (2µg mL^{-1}) or cefpodoxime (2µg mL^{-1}), and from these results 237 *E. coli* isolates were considered presumptive ESBL producers. Antimicrobial resistant bacteria were isolated throughout the experiment; however, ESBL-producing *E. coli* were not prevalent throughout the study. In total, only three isolates (B221B1, C152C1, C98A1) exhibited the ESBL phenotype. Molecular subtyping of these isolates revealed no clonality between these strains. Molecular characterization of the 237 isolates investigated in this study revealed bla_{TEM} to be the most prevalent AMR determinant among the ampicillin-resistant isolates with resistance to ceftazidime (2µg mL^{-1}) or cefpodoxime (2µg mL^{-1}). These data suggest that ESBLs are not frequent among Canadian feedlot cattle and MDR resistance (55 of 237) was observed but is not prominent among both the subgroup and total isolates collected. It was determined that isolate B221B1 was ESBL-producing, and harboured the bla_{TEM-1} gene. The genes responsible for ESBL production in isolates C98A1 and C152C1 were not characterized. In order to characterize the antimicrobial resistance (AMR) genes coding for ESBL-production in these 2 isolates, cloning and conjugation experiments were attempted. However, I was unable to resolve the mechanism responsible for ESBL phenotype in these two isolates. The results of this study imply that the development of

ESBL-producing *E. coli* is complex, and is probably affected by both the administration of antimicrobials and numerous other presently undefined environmental factors.

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DEDICATION

To

My Family –

Thank you for all of your love and support

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LIST OF ABBREVIATIONS

ABBREVATION	MEANING
°C	Degree Celsius
µg	Microgram
µL	Microlitre
mL	Millilitre
AGP	Antimicrobial Growth Promoters
AMCL	Amoxicillin/Clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial Resistance
bla	β-lactamase Gene
bp	Base Pair
CFOX	Cefoxitin
CIP	Ciprofloxacin
CLOR	Chloramphenicol
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
CTZD	Ceftazidime
CZCL	Ceftazidime/Clavulanic acid
CXCL	Cefotaxime/Clavulanic acid
CTIO	Ceftiofur
d	Day
DNA	Deoxyribonucleic Acid
ENRO	Enrofloxacin
ESBL	Extended-spectrum β-lactamase
GD	Grain Diet
GENT	Gentamycin
h	Hour
HGT	Horizontal Gene Transfer
IMPN	Imipenem
Kg	Kilogram
LB	Luria Butania (microbiological medium)
MAC	MacConkey (microbiological medium)
MDR	Multi-Drug Resistance
Mg	Miligrams
MIC	Minimum Inhibitory Concentration
min	Minute
mRNA	Messenger Ribonucleic Acid

MRSA	Methicillin-Resistant *Staphylococcus aureus*
NAG	N-acetyl Glucosamine
NAM	N-acetyl Muramic Acid
NCCLS	National Committee for Clinical Laboratory Standards
NMYN	Neomycin
OMP	Outer Membrane Protein
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction
s	Second
SD	Silage Diet
STEP	Streptomycin
SULF	Sulfisoxazole
TAE	Tris-Acetate-EDTA
TD	Transition diet
TET	Tetracycline
TMSZ	Trimethoprim/Sulfamethoxazole
USA	United States of America
UTI	Urinary Tract Infection
V	Volt
v/v	Volume (of solute) per volume (of solvent)
w/v	Weight (of solute) per volume (of solvent)

1.1 IMPLICATIONS OF EXTENDED-SPECTRUM B-LACTAMASES (ESBL) IN HUMAN HEALTH

Aerobic Gram-negative bacilli infections are common in hospitalized patients, and can cause serious infections. They are commonly responsible for the majority of bacteremia and nosocomial pneumonia (Table 1-1) (Mulvey et al., 2004). These infections are associated with high mortality rates; sepsis being one of the most common causes of death in intensive-care-unit patients (Poyart et al., 1998). Providing health care to patients infected by antimicrobial-resistant organisms has contributed to the continued increase in healthcare expenditures (Larson et al., 2007). Clinicians have limited therapeutic options for the treatment of organisms producing extended-spectrum β-lactamases (ESBLs) (Pitout et al., 2005). Risk factors associated with ESBLs include increased length of hospital stay, greater severity of illness, more time in intensive care units and infections associated with urinary or arterial catheterization (Bradford, 2001).

Commensal microbial flora, such as *E. coli*, may harbor ESBL genes and through horizontal transfer, micro-biota may transmit resistant genes to pathogenic organisms such as *Klebsiella* (Levy and Marshall, 2004).

Table 1-1. Definition of medical conditions associated with ESBLs.

Term	Definition	Reference
Nosocomial pneumonia	Pneumonia that occurs more than 48 hours after admission but that was not incubating at the time of admission. Nosocomial pneumonia is the second-most-common nosocomial infection and is usually of bacterial origin	Fagon et al., 1996
Bacteremia	Presence of viable bacteria in circulating blood	McCabe et al, 1962
Sepsis	Condition where the body is fighting a severe infection that has spread via the bloodstream. If a patient becomes "septic," they will likely be in a state of low blood pressure termed "shock." This condition can develop either as a result of the body's own defence system or from toxins produced by infectious agents such as a bacteria, viruses, or fungi.	Lever and Mackenzie, 2007

A case control study of bloodstream infection comparing ESBL and non-ESBL strains of *E. coli* and *Klebsiella* showed similar infection rates. Mortalities associated with ESBL-mediated *Klebsiella* infection were considerably lower in patients treated primarily with carbapenems in comparison with patients treated with inappropriate antibiotics (Schiappa et al., 1996). In an international multicentre study of bacteremias caused by ESBL producing *Klebsiella,* mortality was found to be significantly reduced by use of a carbapenem during the first 5 days of treatment (Paterson et al., 1998). Another case control study in a New York hospital demonstrated that individuals who were infected or colonized with ESBL-producing *Klebsiella* had a greater probability of sepsis-related mortality than control patients (Qavi et al., 1999). These studies clearly demonstrated the importance of ESBL in nosocomal infections.

Enterobacteriaceae, especially *Klebsiella* producing ESBLs, such as TEM and SHV types, have been recognized since the 1980's as major causes of hospital-acquired infections (Paterson et al, 2005). Up until the late 1990s, all surveys of ESBLs conducted in Europe found exclusively TEM and SHV enzyme variants, specifically SHV-2 and SHV-5, which were largely found in *Klebsiella* spp. (Livermore et al., 2007). A similar trend was seen in the middle to late 1980's in the USA, where the first identified ESBL was derived from the TEM-1 β-lactamase in an outbreak strain of *K. pneumoniae*, along with the SHV-5 ESBL (Bush, 2008). Recent surveys from hospitals in the eastern USA have reported the most prominent ESBLs being the SHV-5 and SHV-12 enzymes (Bush, 2008). In Canada, a wide variety of *K. pneumoniae* ESBLs have been documented, with

multiple members of the TEM, SHV and CTX-M classes being found in surveillance isolates. SHV-type and CTX-M ESBLs have appeared in many Canadian isolates, with an outbreak of CTX-M-14 documented in Calgary, but limited TEM-derived ESBLs have been found (Bush, 2008).

Molecular methods have demonstrated that the majority of outbreaks originate in an intensive care unit and subsequently spread to other parts of the hospital by various means such as clonal dissemination of the ESBL-producing strain (Rice et al., 1996) or horizontal transmission of the plasmid encoding for the ESBL among nonrelated strains (Canton et al., 2003). Specifically, ESBL propagation in *E. coli* is due to plasmid transmission between unrelated strains (Hernandez et al., 2005), while clonal spreading is more common in other *Enterobacteriaceae*, such as *K. pneumoniae* (Lebessie et al., 2002). *Enterobacteriaceae*, specifically *E. coli* producing ESBLs, such as CTX-M producing strains have been an important cause of urinary tract infections (UTI's) in hospitals and community settings since the late 1990's (Babic et al., 2006). Community-acquired UTIs are among the most common bacterial infections in women and therapy for these infections is usually begun before results of microbiological tests are known (Gupta et al., 2001). During the last 5 years, CTX-M β-lactamases have become the most prevalent type of ESBL globally (Pitout et al., 2007).

As shown in Table 1-2, few surveys of ESBL producing bacteria have been conducted in Canada. Studies conducted in Canada have sporadically isolated ESBL

producing bacteria, representing a small percentage of the total *E.coli*, *K. pneumoniae*, and *Salmonella* isolates examined (Bush, 2008). A study in the Calgary Health Region (CHR) is the exception, where a large number of CTX-M β-lactamase *E. coli* were isolated from 2000 to 2002 (Pitout et al., 2005). During this period, 232 ESBL-producing *E. coli* strains were obtained from 168 patients with an overall frequency of 1.3% (232 of 17,846) (Pitout et al., 2005). Overall, 15% were positive for $bla_{\text{CTX-M}}$ genes from the CTX-M-I group, and 55% were positive for the $bla_{\text{CTX-M}}$ genes from the CTX-M-II group (Pitout et al., 2005). Another concern in regards to CTX-M –producing *E. coli* isolates from hospital and community sites is that they often exhibit AMR to other classes of antibiotics including trimethoprim-sulfamethoxazole, tetracycline, gentamicin, tobramycin, and ciprofloxacin (Pitout et al., 2007).

Table 1-2. Surveillance studies in Canada displaying the prevalence of extended-spectrum β-lactamases (ESBLs)

				β-lactamases in ESBL producers (n^b)				
Source of Samples	**Date**	**N^a**	**Organism**	**TEM**	**SHV**	**CTX-M**	**Multiple**	**Reference**
Non-repeat *E. coli* and *K. pneumoniae* isolates from 12 hospitals across Canada	October 1999-September 2000	29 323 5156	*E.coli* *K. pneumoniae*	TEM (90)[a] TEM-11 (1) TEM-12 (1) TEM-29 (1) TEM-52 (4) TEM-115 (2) TEM-120 (1)	SHV (79)[a] SHV-2(2) SHV-2a (12) SHV-30 (2) SHV-40 (2) SHV-41 (4) SHV-42 (1)	CTX-M (27)[a] CTX-M-13(1) CTX-M-14 (15) CTX-M-15 (11)	TEM + SHV (55) TEM + SHV + CTX-M (3) TEM + CTX-M (24)	Mulvey et al., 2004.
Isolates submitted to the National Laboratory for Enteric Pathogens	2000	30 of 1033	*Salmonella enterica* serovar Typhimurium				TEM-1 + SHV-2a (1)	Mulvey et al., 2003
E. coli from 168 patients from 20 towns and villages in and surrounding Calgary	2000-2002	232	*E.coli*			CTX-M-14 (87) CTX-M-14-like (67)	TEM+ SHV + OXA(20)	Pitout JDD et al., 2005

[a]*N* = number of ESBL producing isolates collected

[b]*n* = number of isolates containing specific set of ESBL gene determinants

1.2 USE OF ANTIBIOTICS IN LIVESTOCK PRODUCTION

1.2.1 History of Antimicrobials in Agriculture

In the late 1940s, inclusion of low levels of antibiotics in animal feed was first described as being growth-promoting when chickens fed fermentation waste from tetracycline production grew more rapidly than the controls (Stokestad et al., 1950). This discovery led to the widespread use of antibiotics in livestock production, both to prevent disease and to improve the efficiency of animal production (Phillips et al., 2004). Since then, the production enhancing properties associated with feeding subtherapeutic levels (less than 200g/ton of feed) of several antimicrobials has been documented (Barton, 2000).

In animals, antimicrobials are used mainly for three purposes: as therapeutics to treat sick animals, as prophylactics to prevent infection, and finally as growth promoters (Barton, 2000). Therapeutic treatments are meant for animals that exhibit clinical disease. In food animal production, sometimes animals are treated individually, but generally it is more efficient to treat entire groups by medicating feed or water (McEwen and Fedorka-Cray, 2002). The only feasible means for treating animals such as fish and poultry is via mass medication (McEwen and Fedorka-Cray, 2002). Antimicrobial growth promoters (AGPs) are defined as antibiotics added to feed to enhance their production performance and growth rate (Wegener et al., 1999). In order to enhance growth, it has been common practice for more than 30 years to supplement animal feed with antimicrobials, a practice that is estimated to account for more than half the total

antimicrobial use worldwide (Wegener et al., 1999).

Antimicrobials administered to animals at subtherapeutic levels in their feed have been shown to improve livestock production. It has been reported to increase daily weight gain by approximately 6%, and feed efficiency by 4% in beef steers and heifers (Mathews, 2001). Food production in modern agriculture has entered its most affordable stage ever by utilizing antimicrobials (Mathews, 2001).

1.2.2 Concerns with Antimicrobials in Livestock Production

In the 1950's, when antimicrobials were first used for treatment of common infections, it was believed that the development of antimicrobial resistance was improbable because the frequency of mutation to resistance in bacteria was too low (Davies, 1994). Specifically, it was assumed that in nature bacteria do not collect and exchange genetic information (Davies, 1994). However, we now know that this assumption was false. Resistance is the adaptation of bacteria to the extensive use and of antibiotics presence in the environment (Levy, 2001). Antibiotics can select for spontaneous resistant mutants and bacteria that have acquired resistance by transfer from other bacteria (Phillips et al., 2004). These resistant bacteria, as well as species that are inherently resistant, can become the prevailing members of a bacterial population and spread in host-animal populations (Phillips et al., 2004).

It is now well known that an efficient way to naturally select for antimicrobial resistance is to expose bacteria over an extended period of time to low doses of antimicrobials as is the case with AGP use (Phillips et al., 2004; Shea at al., 2004).

Animals are also exposed to antimicrobials that are analogues to those used for therapy in humans, with the exception of ionophores, and bambermycins (Turnidge, 2004). This creates an issue where these antimicrobials may promote antimicrobial resistance in microbiota to antimicrobials used for therapy in humans (Phillips et al., 2004). Bacteria resistance genes to antimicrobials could be spread through the environment by livestock through food products or via contamination of water or crops with animal excreta (Wegener, 2003). Bacterial resistance determinants to antibiotics and disinfectants have been detected in the environment in waste water, surface water, ground water, sediments and soils (Kummerer, 2004). Furthermore, bacterial spread from livestock to food products has occurred with *Salmonella*, *Campylobacter*, and *E. coli* (Wegener, 2003). Resistant bacteria are potentially being disseminated among livestock and food products by conditions such as transport of livestock in large numbers, rapid processing and centralized food processing and packaging (Shea et al., 2004). Because resistance genes are generally mobile, these resistance determinants within the bacterial flora could be transferred to more pathogenic bacteria. This could occur either in the livestock, or the human gastrointestinal tract (Winokur et al., 2001). This phenomenon will cause major health problems by limiting antimicrobial treatment options for infectious diseases in humans.

1.2.3 The Importance of Surveillance of Antimicrobial Resistance

Antimicrobial resistance, specifically in pathogenic bacteria, is a global problem that has emerged in the past two decades (Aarestrup, 2000). Agricultural animals

harboring antibiotic resistant bacteria have increased concern among both public-health professionals and veterinarians (Wegner et al., 2003). Public-health concern arises from the possibility that antimicrobial-resistance genes could be incorporated into the genomes of human pathogens, such as *Salmonella*, *Campylobacter or E. coli* and be transmitted through food-borne routes to humans (Wegner et al., 2003). Furthermore, there is concern that non-pathogenic bacteria could acquire resistance genes and transmit these genes to humans where they would be acquired by sensitive pathogenic bacteria in the human gastrointestinal tract (Wegner et al., 2003). If animals and humans comprise overlapping reservoirs of resistance, the use of antimicrobials in animals could have a direct impact on human health (Wegener, 2003).

Limited information is available regarding antimicrobial use and resistance, particularly in agriculture. It is not required for users and manufacturers of antimicrobials to report data on production or use for human or food-animal applications (Shea et al., 2004). As a result, detailed estimates of antimicrobial use in agriculture are unavailable in Canada (Conly, 2002). However, reports from the United States have estimated that nontherapeutic use in livestock is 78% of the total annual use of antimicrobial agents (Mellon et al., 2001). Drug usage in livestock is implicated in antimicrobial resistance in humans because numerous antimicrobial drugs used for agriculture are the same or members of the same antimicrobial family as those used for humans (Mathews Jr. et al., 2001). Examples of these antimicrobials include: bacitracin, tetracyclines, sulfonamides, lincosamides, penicillin and aminoglycosides (Conly, 2002).

Use of antibiotics can select for resistant bacteria in food animals, and these

bacteria can contaminate animal-derived food (Phillips et al., 2004). Bacteria from animals spread to food products during slaughter and processing (Wegener, 2003). However, these resistant bacteria can be destroyed by cooking (Phillips et al., 2004). Systems to monitor antimicrobial resistance in pathogenic and commensal bacteria have been established to a limited degree (Wegener et al., 1999). However, these systems do not monitor resistance towards antimicrobial drugs used in both humans and animals along the entire food production and consumption chain (Wegener et al., 1999).

Currently, there are only a few organizations that consistently monitor AMR in commensal and pathogenic enteric bacteria from seemingly healthy animals. These organizations include the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS; Government of Canada, 2002), the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP, 2006) and the Swedish Veterinary Antimicrobial Resistance Monitoring Program (National Veterinary Institute, 2002). The majority of surveillance studies have been conducted at the abattoir or retail level, but it is important to identify patterns of change in the occurrence of AMR throughout the different stages of animal-production to identify risk factors for resistance development. Unfortunately, only a few studies have been conducted at the farm level to investigate resistance in *E. coli* in Canada (Read, 2005; Checkley et al., 2003; Van Donkersgoed et al., 2005; Gow et al., 2005).

1.2.4 The Importance of the Surveillance of Antimicrobial Resistance in Escherichia coli

The occurrence of antimicrobial resistance in generic *E. coli* can serve as an indicator of the pool of resistance genes possibly available for transfer to pathogenic

organisms (Carson et al., 2008). Transfer of resistance genes to human pathogens is the primary concern regarding human health with an additional fear that transfer of resistance genes to animal pathogens could also reduce the therapeutic options available for veterinary medicine (Carson et al., 2008). Generic *E. coli* are commensal bacteria that are regularly found in the gastrointestinal tract of humans and animals, and are utilized as indicators to monitor changes in prevalence and patterns of resistance (Schroeder et al., 2002). Furthermore, the recovery of *E. coli* is easy and cost effective (Wegener, 1999).

As mentioned above, enteric bacteria such as *E. coli* potentially serve as a reservoir for antimicrobial resistance and may promote the dissemination of antimicrobial resistance through the food chain in a manner that imposes a health risk to humans. These problems include: increasing morbidity, mortality, cost of treatment, and limiting antimicrobial treatment options (Jones, 2001). These problems could be a direct result of *E. coli* acquiring and disseminating genes that confer resistance by a number of mobile genetic elements including plasmids, transposons and phages (Winokur et al., 2001).

1.2.5 Extended-Spectrum β-lactamases (ESBLs) in Animals

The primary reservoir of *Salmonella*, *E. coli*, and *Campylobacter* that cause enteric infection in humans is considered to be food-producing animals, and the detection of ESBL producing *E. coli* and *Salmonella* strains has increased in recent years. ESBLs are frequently reported in human medical facilities, but are not identified as regularly in the bacterial population circulating in animals (Carattoli, 2008). In human medicine, ESBLs have been studied in great detail in terms of prevalence and type in clinical

settings, but there are few reports of ESBLs in bacterial isolates of animal origin (Machado et al., 2008). Earlier studies in veterinary medicine often report just qualitative β-lactamase production. Newer studies have included phenotypic and genotypic characterization of β-lactam resistant bacteria (Li et al., 2007).

The first detection of an ESBL in an animal was reported in 1988, from a laboratory dog in Japan infected by an FEC-1 producing *E. coli* strain (Matsumoto et al., 1988). Since then, broad-spectrum derivatives of TEM-1, SHV-1 and OXA β-lactamases have been described in a few *Salmonella* and *E. coli* from animals and food of animal origin in Germany, UK, Spain, and the United States with the most common variant of these isolates being TEM-1 (Batchelor et al., 2005). However, it is only in the last few years that ESBLs relevant to human medicine have been detected in livestock.

1.3 MECHANISMS OF ANTIMICROBIAL RESISTANCE

Antimicrobials are naturally-occurring, semi-synthetic or synthetic compounds that either kill or prevent the growth of microorganisms. Typically, antimicrobials interfere with a process or structure that is essential for microbial growth and survival, leaving the host unharmed (Walsh, 2000). Resistance to an antimicrobial agent is defined as the ability of a micro-organism to withstand the effects of an antimicrobial (Kummerer, 2004). The different mechanisms of action and the family of antimicrobials they are associated with are described in Table 1-4.

Antimicrobial resistance has three components: the antimicrobial agent, the resistance gene, and the host of the microorganism (Levy and Marshall, 2004).

Antimicrobial resistance can be either inherent or acquired. Movement of genetic material between bacteria other than by descent (vertical transfer), is known as horizontal genetic transfer (HGT) (Maiden, 1998). Some bacterial species demonstrate an intrinsic resistance to specific antimicrobials, whereas others obtain resistance through mutations in their genome, or through HGT of resistance genes on mobile DNA elements (Normark and Normark, 2002). Genetic mobility entails a variety of mechanisms that have evolved to transfer DNA from one cell to another and from one DNA molecule to another. These mobile elements include plasmids, transposons, prophages, integrons, and resistance islands (Table 1-3; Khachatourians, 1998, Normark and Normark, 2002).

Bacteria can acquire resistance genes from other bacterial cells by one of three gene transfer mechanisms: conjugation, transduction and transformation (Levy, 1998). Resistance genes are commonly found on plasmids. Plasmids carrying one or more resistance gene can be transferred to recipient bacteria from a donor cell. This form of conjugation was originally believed to be restricted to within a species (Davies, 1994) but there is now evidence that this mechanism of transfer permits genetic exchange between many different bacterial genera in nature (Davies, 1994). Secondly, a virus can pick up a resistance gene from a donor bacterium and incorporate it into another bacterial cell in a process known as transduction. Finally, bacteria can take up naked DNA from lysed bacterial cells through a process known as transformation. However, genes received by a bacterium through viruses or dead cells will only be expressed if they are incorporated into the recipient's cell genome or carried on an extra-chromosomal element capable of replicating in the recipient cell such as a plasmid (Levy, 1998).

Several forms of antibiotic resistance mechanisms can confer resistance to multiple antimicrobials within one bacterium. Essentially, there are four broad mechanisms whereby antimicrobial resistance may arise including: (i) changes in bacterial cell membrane permeability, (ii) energy-dependant removal of antimicrobials by membrane-bound efflux pumps, (iii) alteration of the site of antimicrobial drug action, and (iv) inactivation or destruction of the antimicrobial (Barbosa and Levy, 2000).

Table 1-3. Definition of mobile elements responsible for transfer of DNA.

Term	Definition	Reference
Plasmid	Double stranded DNA molecule that can exist and replicate independently of the chromosome or may be integrated within it. A plasmid is not required for the host cell's growth and reproduction	Wiilley et al., 2008
Transposon	A mobile piece of DNA that is flanked by terminal repeat sequences and typically bears genes coding for transposition functions	Wiilley et al., 2008
Prophage	The latent form of a temperate phage that remains within the lysogen, usually integrated into he host chromosome	Wiilley et al., 2008
Integron	Genetic unit that contains a site-specific recombination system capable of capturing and mobilizing genes contained in mobile gene cassettes	Wiilley et al., 2008
Resistance Island	A group of resistance genes clustered close together on a chromosome	Wiilley et al., 2008

Table 1-4. Major classes of antimicrobials and their mechanism of action.

Family	Example	Mechanism of action
Aminoglycosides	Gentamicin Streptomycin	Aminoglycosides bind to the 30S subunit of the bacterial ribosome and interfere with the formation of the initiation complex causing misreading of mRNA, thereby inhibiting protein synthesis.
β-lactam	Ampicillin Penicillin Cephalosporins	Inhibit transpeptidation enzymes involved in cross-linking the polysaccharide chains of the bacterial cell wall peptidoglycan.
Macrolides	Erythromycin	Macrolides inhibit protein synthesis by binding to the 50S subunit of the bacterial ribosome.
Phenicol	Chloramphenicol	Inhibit protein synthesis by binding to the 50S ribosomal subunit. Chloramphenicol reduces the catalytic activity of peptidyltransferase preventing translation of bacterial mRNA.
Quinolones	Nalidixic acid	Quinolones block the action of bacterial topoisomerases required for DNA replication, repair or transcription.
Streptogramins	Virginiamycin	Inhibits protein synthesis by preventing substrate attachment to the A and P sites, inhibiting peptide chain elongation.
Sulfonamides	Sulfamethoxazole	Sulfonamides act as a para amino benzoic acid (pABA) analog preventing the synthesis of folic acid.
Tetracyclines	Chlortetracycline Oxytetracycline Tetracycline	Tetracyclines bind to the 30S ribosomal subunit and inhibit bacterial protein synthesis by blocking the attachment of the aminoacyl-transfer RNA to the ribosome.

1.3.1 Integrons and Multi-Drug Resistance (MDR)

The increasing incidence of microorganisms resistant to multiple antimicrobial agents has led to tremendous interest in the genetics and mechanisms of resistance in bacteria (Ahmed et al., 2006). Many antibiotic resistance genes are located on mobile genetic elements known as integrons. An integron consists of an attachment site (attI), where additional DNA gene cassettes coding for antibiotic resistance can be integrated by site-specific recombination, as well as a region that encodes for an integrase (IntI) that mediates site-specific recombination (Bennet, 1999; Carattoli, 2001; Rowe-Magnus et al., 2002). The integrase catalyzes the excision and integration of DNA fragments by performing two consecutive strand breakage and rejoining steps (Carattoli, 2001). The integrated gene cassettes are genetic elements that can exist as free, circular, non-replicating DNA molecules when relocating from one genetic site to another (Collis and Hall, 1992) or more often are linear sequences which comprise part of a larger DNA molecule such as a plasmid or bacterial chromosome (Bennet, 1999). Generally, gene cassettes harbor a single gene and an additional short sequence referred to as a 59 base element (*attI*), which functions as a specific recombination site (Hall et al., 1991).

In total, four classes of integrons have been defined based on the homology of the integrase proteins (Carattoli, 2001; Rowe-Magnus et al., 2002). Class 1 is the most predominant integron found among clinical isolates (Figure 1-1; Bennet, 1999). This class is characterized by two conserved segments: the 5'-conserved segment (5'-CS) which contains the *intI* gene, the *attI* site and the promoter, and the 3'-conserved segment (3'-CS), which contains the *sul1* gene (conferring resistance to sulphonamides), and the

qacEΔ1 gene (conferring resistance to quaternary ammonium compounds) and ofr5 (of unknown function) (Figure 1-1) (Carattoli, 2001). Overall, more than 60 resistance-gene cassettes have been identified, and up to seven different gene cassettes within a class 1 integron have been described (Mazel and Davies, 1998).

The most significant gene cassettes identified within integrons are those conferring resistance to antibiotics (Table 1-5; Ahmed et al., 2006). Of the different classes of integrons identified, class 1 and class 2 are the most prevalent in Gram-negative bacteria (White et al., 2001). Only classes 1, 2, and 3 have been recovered from antibiotic resistant clinical isolates.

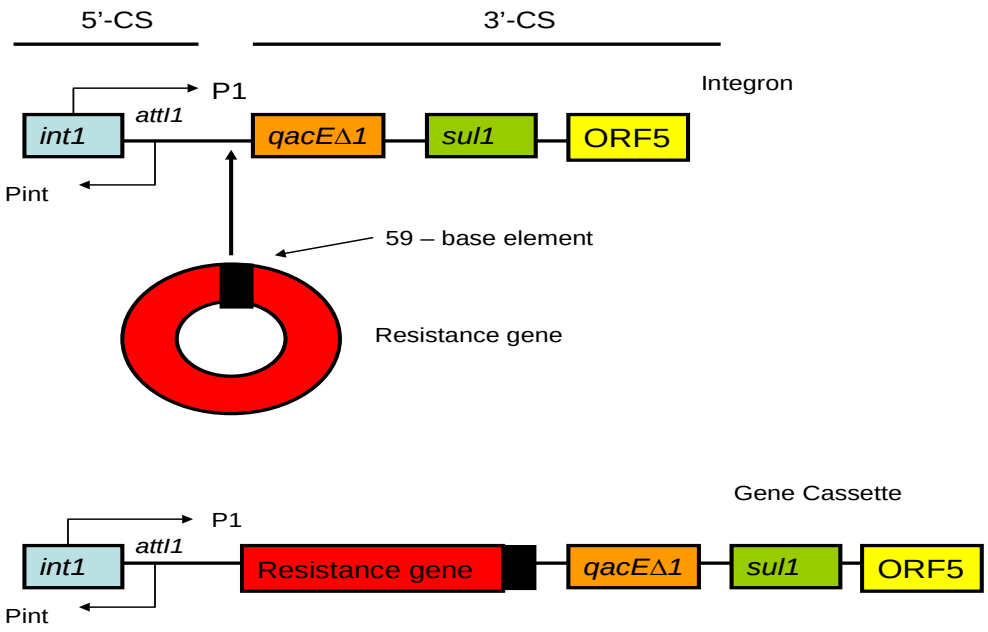

Figure 1-1. Class 1 integron structure and model for acquiring a gene cassette. A circularized gene cassette (resistance gene) is inserted at the position of *attI*. The open reading frames and genes located in the 5'-CS and 3'-CS regions are designated in boxes. The *sul1* and *qacEΔ1* genes confer resistance to sulfonamides and quaternary ammonium compounds. The 59-base element is the site of recombination. P1 is an integron associated promoter, and Pint is the integrase gene (*int1*) promoter.

Table 1-5. Antimicrobial resistance found to be associated with the four different classes of integrons*.

Integron Class	Resistance associated with Integron	Reference
1	ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfafurazole, tetracycline, trimethoprim, quaternary ammonium componds used as disinfectants	(Carattoli, 2001 White et al., 2001)
2	ampicillin, chloramphenicol, streptomycin, sulfafurazole, tetracycline, trimethoprim	(White et al., 2001)
3	bla_{IMP} gene cassette confering resistance to broad spectrum β-lactams	(Arakawa et al., 1995)
4	This integron constitutes a large pathogenicity island in the *Vibrio cholerae* genome	(Carattoli, 2001)

*For a more complete listing consult the ARDB database at http://ardb.cbcb.umd.edu/search.shtml

The integron system allows bacteria to scavenge foreign genes that may ultimately provide an adaptive advantage (Rowe-Magnus et al., 2002). Multidrug resistance (MDR) encoded by linked resistance genes has been shown to occur on class 1, 2, and 3 integrons making it possible for multiple resistance genes to be transferred among bacteria in a single transfer event (Zandbergen et al., 2007). The capture and spread of antibiotic resistance determinants by integrons underlies the rapid evolution of MDR among diverse Gram-negative isolates (Rowe-Magnus et al., 2002).

ESBL genes are normally located on conjugative plasmids (bla_{TEM} or bla_{SHV}), but several of the new emerging ESBL genes are present within integron-like structures (bla_{CTX-M}, bla_{GES}, or bla_{VEB-1}) (Bonnet, 2004; Bradford, 2001; Jacoby and Sutton; 1991). Furthermore, ESBL-producing isolates are usually associated with MDR to antimicrobials such as aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulfonamides, or quinolones (Machado et al., 2005). The presence of ESBL genes within integrons likely contributes to the propagation of these genetic elements (Bonnet, 2004).

1.3.2 β-lactam antibiotics

β-lactams have become one of the most clinically important antimicrobials in human and veterinary medicine due to their efficacy, low cost, ease of delivery, and minimal side effects (Wilke et al., 2005; Li et al, 2007). The β-lactam antibiotic class includes: amino-, carboxy-, idanyl-, and uredio-penicillins; first- to fifth-generation cephalosporins; monobactams; and carbapenems (Babic et al., 2006). In hospitals worldwide oxyimino-cephalosporins, specifically cefotaxime, ceftriaxone, ceftazidime and cefepime, are standard therapies for pneumonias, intra-abdominal infections, and

urinary tract infections (Livermore, 2008).

β-lactam antibiotics are bactericidal compounds, possessing a highly reactive four-membered ring that ultimately inhibits cell wall synthesis. In order to maintain cell wall shape and rigidity, all bacterial species rely on a heavily cross-linked peptidoglycan layer (Wilke et al., 2005). The peptidoglycan layer consists of a basic repeating glycan unit, which is an alternating disaccharide of N-acetyl glucosamine (NAG), and N-acetyl muramic acid (NAM) (Wilke et al; 2005, Babic et al, 2006). The peptidoglycan layer is formed by the formation of cross linkages between adjacent glycan strands (Babic et al., 2006). N-actetyl muramic acid becomes modified by a pentapetide, always terminating in D-Ala-D-Ala residues in both Gram- negative and Gram-positive bacteria. The final cross-linking reaction of the peptidoglycan layer occurs outside the cytoplasmic membrane, and is catalyzed by membrane-anchored cell-wall transpeptidases also known as penicillin-binding proteins, PBPs (Wilke et al., 2005). The β-lactam antibiotics mimic the D-Ala-D-Ala attached to NAM resulting in the transpeptidase (PBP) being acetylated as a result of interaction with the β-lactam. The acetylated transpeptidase can no longer hydrolyze β-lactam and cell wall synthesis is inhibited (Babic et al., 2006; Goffin and Ghysen, 1998). Consequently, the cross-linked peptidoglycan layer is weakened, causing the bacteria to be vulnerable to cell lysis and death (Wilke et al., 2005).

There are three main ways bacteria can avoid the effect of β-lactam antibiotics. The first way involves the production of β-lactamases. These are bacterial enzymes that hydrolyze the β-lactam ring and cause the antibiotic to be inactive before it reaches the transpeptidase (PBP) target (Babic et al., 2006). The second way is altered PBPs that

exhibit low affinity for β-lactam antibiotics (e.g. PBP 2X of *Streptococcus pneumoniae* and PBP 2p of *Staphylcoccus aureus*). These PBPs are resistant to inactivation by penicillins and are able to assume the functions of other PBPs (Chambers, 1997). The final way is lack of expression of outer membrane proteins (OMPs) in Gram-negative bacteria. Loss of OMPs impede the entry of β-lactams into the periplasmic space of Gram-negative bacteria, and therefore access to PBPs on the inner membrane. Resistance in *Pseudomonas aeruginosa* and *K. pneumoniae* to imipenem, which is an intravenous β-lactam antibiotic, can arise from the loss of OMP D2 and of OMP K36 (Jacoby et al., 2004; Livermore and Brown, 2001).

The hydrolytic inactivation of β-lactam antibiotics by β-lactamases is the most important resistance mechanism in Gram negative bacteria (Babic et al., 2006)

1.3.3 The emergence and development of β-lactamases

Production of β-lactamases acts as a defense mechanism to guard transpeptidases against the lethal effects of β-lactam antimicrobials (Goffin and Ghysen, 1998). The source of bacterial resistance to β-lactam antimicrobials today is most frequently due to production of β-lactamases, which hydrolyze the amide bond of β-lactam antimicrobials (Figure 1-2; Bush 1997; Livermore, 1995).

β-lactamases occur only in bacteria, coinciding with the hypothesis that their function is to protect the organism against β-lactams (Ambler, 1980). These enzymes are distributed in both Gram-negative and Gram-positive bacteria (Bush, 1997; Ambler, 1980). In Gram-positive bacteria, β-lactamases are secreted extracellularly, whereas in

Gram-negative bacteria they remain in the periplasmic space (Samaha-Kfoury and Araj, 2003).

The β-lactamase gene (*bla*) can be located on the bacterial chromosome, on plasmids, or transposons (Bush et al., 1995; Livermore, 1995). Two different mechanisms, depending on the structure of the β-lactamase, are used for catalytic process of β-lactam hydrolysis. The active site of a β-lactamase contains either a serine residue or a n^{+2} metal ion (Bush, 1997). Only a few β-lactamases utilize zinc ions to disrupt the β-lactam ring. The majority operate using the serine ester mechanism (Figure 1-3).

There are two primary ways to overcome β-lactamases, either through inhibitors (or inactivators), or by finding a new β-lactam antibiotic that has a greater affinity for the target PBP and is resistant to β-lactamases. There are currently three inhibitors used in this manner in combination with β-lactamase antimicrobials; clavulanic acid, sulbactam and tazobactam (Babic et al., 2006). All three of these compounds resemble penicillin structurally, exhibit high affinity (nM-μM) for PBP's and are poorly hydrolyzed by β-lactamases (Helfand et al., 2003).

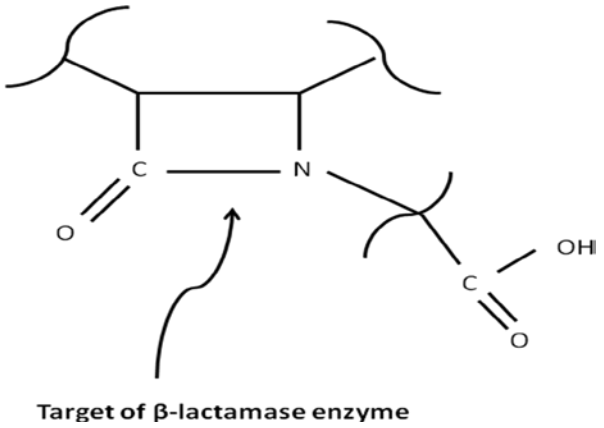

Figure 1-2. Site of action of β-lactamases on β-lactam antimicrobials. Arrow depicts the amide bond that is cleaved by the β-lactamase.

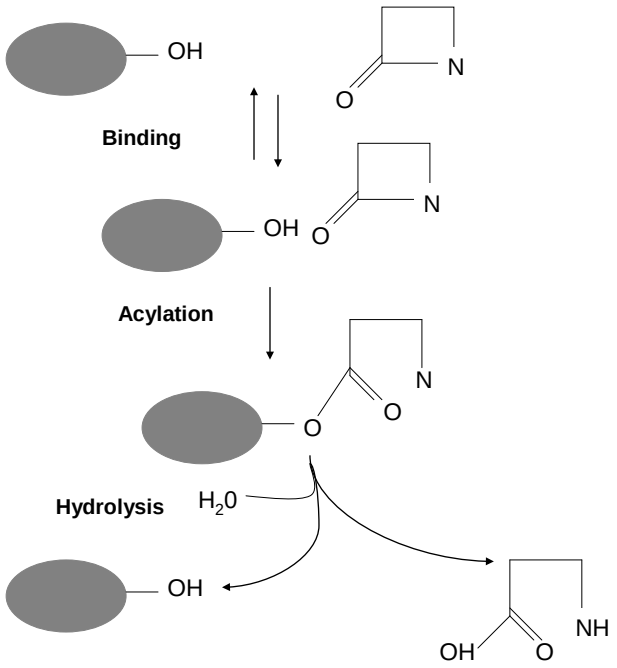

Figure 1-3. Schematic of the hydrolysis of β-lactam by a serine β-lactamase. First, a non-covalent complex forms between the antibiotic and the enzyme. The β-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue located within the active site, forming a covalent acyl ester. Finally, hydrolysis of the ester occurs, inactivating the drug (Waley, 1992).

1.3.3.1 β-lactamase Classification

β-lactamases have received plenty of attention because of their clinical importance and their ecological and evolutionary interest (Ambler, 1980). Two schemes exist to classify the 530 or more known β-lactamases (Babic et al., 2006). The Bush scheme correlates substrate and inhibitory properties of the enzyme with its molecular structure (Bush-Jacoby-Medeiros Groups 1 through 4; Bush et al., 1995; Bush, 1989). The Ambler classification divides the β-lactamases into four groups based on amino acid sequences (Figure 1-4. Ambler classes A through D; Ambler 1980).

Ambler classes A, C, and D comprises evolutionarily distinct groups of 'serine β-lactamases,' and class B contains 'the metallo-β-lactamases' (Livermore, 1995; Bush, 1997). Ambler class A is the largest class of β-lactamases, which includes those that compise part of the Bush Group 2. Several of the most commonly found β-lactamases belong to Group 2 including the staphylococcal penicillinases (Bush, 1997). This group is also associated with ESBLs. This group includes penicillinases and cephalosporinases which confer resistance to almost all penicillins, cephalosporins (excluding cephamycins) and monobactams, but bacteria possessing these determinants remain susceptible to β-lactamase inhibitors such as clavulanic acid (Bush, 1997; Gniadkowski, 2001). Ambler class D also includes ESBLs that are a part of Bush Group 2. An important distinction of this group is that they are also capable of hydrolyzing cloxacillin (Gniadkowski, 2001). Ambler class C (Bush Group 1) β-lactamases are the second largest class of β-lactamases. Class C β-lactamases hydrolyze cephamycins, as well as first, second and third generation cephalosporins (Philippon et al., 2002). Furthermore, these enzymes are not

inhibited by β-lactamase inhibitors such as clavulanic acid. The final class of β-lactamases is Ambler class B (Bush Group 3). The genes encoding these enzymes are plasmid-borne, and are present in several species of *Enterobacteriaceae,* but not *E. coli* (Mushtaq et al., 2004). These enzymes include the metalloenzymes, which unlike other classes of β-lactamases possess zinc linked to histidine or cysteine within their active site and are not susceptible to clavulanic acid (Samaha-Kfoury and Araj, 2003).

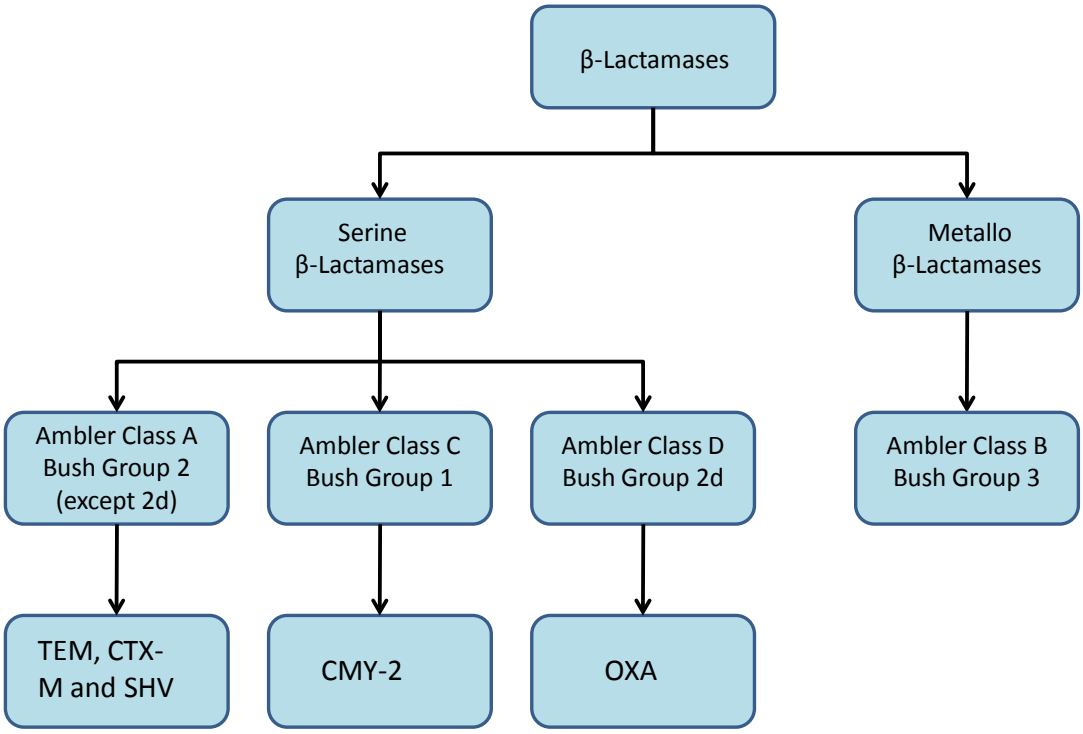

Figure 1-4. Molecular and functional classification schemes of β-lactamases (Ambler, 1980).

*1.3.3.2 Extended-spectrum β-lactamases (ESBL***)**

In order to address the challenge posed by the production of β-lactamases which confer resistance to β-lactam antimicrobials, newer extended spectrum β-lactams with greater resistance to β-lactamase activity, including cephalosporins, carbapenems, and monobactams, were introduced in the 1980's (Philippon et al., 2002). In 1983, the first clinical isolates expressing acquired ESBLs were identified in Germany (Kliebe et al., 1985).

All ESBLs contain a serine in their active site (Chaudary and Aggarwal, 2004; Gniadkowski M., 2001). These enzymes are most often found in *Klebsiella* species and *E. coli*, and confer resistance to cefotaxime, ceftazidime, ceftriaxone, aztreonam, and other oxyimino-β-lactams (Jacoby et al., 2004). The most prevalent ESBLs have evolved through point mutations resulting in key amino acid substitutions in parent TEM (Table 1-6) and SHV enzymes (Table 1-7; Al-Jasser, 2006). These substitutions include: glutamine to lysine, arginine to either serine or histidine, glycine to serine, and glutamate to lysine (Bradford, 2001). Amino acid substitutions responsible for the ESBL phenotype alter the configuration of the active site of the enzyme, allowing access to oxyimino-β-lactams and increase the spectrum of β-lactam antimicrobials that ESBLs can hydrolyze (Jacoby et al., 2005). Consequently, modification of the active site to β-lactam substrates also increases the susceptibility of isolates to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Jacoby et al., 2005).

1.3.3.3 Classification of Extended-Spectrum β-lactamases (ESBLs)

1.3.3.3a TEM-Type ESBLs (Ambler class A, Bush class 2be)

E. coli exhibiting the TEM-1 β-lactamase resistance was first isolated from the blood of a Greek patient named Temoniera (Sturenburg and Mack, 2003), and is the most common β-lactamase identified in Gram-negative bacteria (Bradford, 2001). Production of TEM-1 is responsible for up to 90% of ampicillin resistance in *E. coli* (Livermore, 1995). More than 152 TEM enzymes are currently recognized, and their diversity provides a useful way to track the spread of individual resistance genes (Jacoby and Bush, 2008). The first derivative of TEM-1 was TEM-2, which resulted from a single amino acid substitution from the original β-lactamase (Bradford 2001). Originally reported in 1989, TEM-3 was the first TEM-type β-lactamse to exhibit the ESBL phenotype (Sougakoff et al., 1988). TEM-type ESBLs are most often found in *E. coli* and *K. pneumoniae*, but have also been found in other species of Gram-negative bacteria (Bradford, 2001).

1.3.3.3b SHV-Type ESBLs (Ambler class A, Bush class 2be)

The SHV β-lactamase, named after the "sulfhydryl variable," is most prevalent in *K. pneumoniae* and is responsible for up to 20% of plasmid mediated ampicillin resistance (Bradford, 2001). To date, more than 114 varieties of SHV are currently recognized (Jacoby and Bush, 2008). SHV-type ESBLs are found in clinical isolates more frequently than any other types of ESBL (Al-Jasser, 2006).

Table 1-6. Amino acid substitutions in ESBL derivatives of the TEM-1 β-lactamase (Bradford, 2001). A complete listing can be found at http://www.lahey.org/studies/webt.htm.

Amino Acid	Position	Substitution
Leu	21	Phe
Gln	39	Lys
Ala	42	Val
Leu	51	Pro
Gly	92	Asp
Glu	104	Lys
His	153	Arg
Arg	164	Ser
Met	182	Thr
Gly	218	Glu
Ala	237	Gly or Thr
Gly	238	Ser
Glu	240	Ser or Lys
Arg	244	Leu or Ser
Thr	265	Met
Ser	268	Gly

Table 1-7. Amino acid substitutions in SHV ESBL derivatives of the SHV-1 β-lactamase.(Bradford, 2001). A complete listing can be found at http://www.lahey.org/studies/webt.htm.

Amino Acid	Position	Substitution
Ile	8	Phe
Leu	35	Gln
Arg	43	Ser
Leu	51	Pro
Leu	122	Phe
Met	129	Val
Ser	130	Gly
Asn	158	Lys
Leu	173	Phe
Asp	179	Ala or Asn or Gly
Ala	187	Thr
Arg	205	Key
Gly	238	Ala or Ser
Glu	240	Lys

1.3.3.3c CTX-M-Type ESBLs (Ambler class A, Bush class 2be)

The most common group of ESBLs not belonging to the TEM-type or SHV-type classification is termed CTX-M (Jacoby and Munoz-Price, 2005). Unlike TEM and SHV ESBLs which have arisen from point mutations, the CTX-M class of ESBLs is believed to have developed as a result of incorporation of pre-existing chromosomal ESBL genes from *Kluyvera* species onto a mobile plasmid (Bonnet, 2004). This classification of β-lactamases can be divided into five groups based on their amino acid identities: the CTX-M-1 group, the CTX-M-2 group, the CTX-M-8 group, the CTX-M-9 group, and the CTX-M-25 group (Pitout et al., 2007). CTX-M β-lactamases are more susceptible to tazobactam as opposed to clavulanic acid and sulbactam, and demonstrate greater activity against cefotaxime than against ceftazidime (Babic et al., 2006, Jacoby and Munoz-Price, 2005). These enzymes are not very closely related to TEM or SHV β-lactamases (Bradford, 2001). CTX-M enzymes have been found primarily in *Salmonella enterica* and *E. coli*, but have also been described in other *Enterobacteriaceae* (Bradford, 2001),

1.3.3.3d OXA-Type ESBLs (Ambler class D, Bush class 2d)

A total of eleven ESBLs derived from OXA-10, OXA-1 or OXA-2 by amino acid substitutions are currently known (Jacoby and Bush, 2008). OXA-type ESBLs are normally found in clinical isolates of *P. aeruginosa* (Babic et al., 2006). These β-lactamases differ from TEM- and SHV-types in that they belong to Ambler class D and functional group 2d (Bush et al., 1995). The majority of OXA-type ESBLs are relatively resistant to clavulanic acid, and are efficient at hydrolyzing oxacillin (Bradford, 2001; Thomson and Moland, 2000). The structural change or amino acid substitutions that

have led to the emergence of this phenotype have yet to be described (Babic et al., 2006). Many of the *P. aeruginosa* and *Acinetobacter baumanni* that harbor OXA β-lactamases have been isolated in Turkey and France (Babic et al., 2006).

1.3.3.3e Other ESBLs

The majority of ESBLs are derived from TEM or SHV β-lactamases, and others can be categorized into the two newer families, CTX-M or OXA. However, a few ESBLs have been reported that are not closely related to any established family (Jacoby, 1994). These β-lactamases include: BES-1, FEC-1, GES-1, CME-1, PER-1, PER-2, SFO-1, TLA-1, and VEB-1 (Bradford, 2001).

1.3.3.4 The Ambler class C β-lactamases

Ambler class C (AmpC) β-lactamases are the second largest class of β-lactamases (Bulychev and Mobashery, 1999). Like Ambler class A and D, class C also possesses a serine residue within their active site (Jacoby and Munoz-Price, 2005). These enzymes hydrolyze first, second and third generation cephalosporins, and cephamycins, but are not inhibited by β-lactamase inhibitors (Philippon et al., 2002). *E. coli* producing Ambler class C β-lactamases are resistant to all β-lactams except carbapenems and fourth generation cephalosporins (Perez-Perez and Hanson, 2002).

1.3.3.4.1 Chromosomally-Encoded Ambler Class C β-lactamases

Ambler class C β-lactamases were initially coded on the chromosomes of many

enteric and non enteric Gram-negative bacteria (Tenover et al, 2003). Expression of the chromosomal *ampC* gene is normally low, but is inducible in response to the presence of β-lactams (Hanson and Sanders, 1999).

Wild-type *E. coli* produce constitutively low levels of chromosomally encoded β-lactamase (Jaurin et al., 1982). The chromosomal *ampC* gene is not inducible due to an overlap in the fumurate reductase (*frd*) and the *ampC* operons; therefore, there is no regulatory gene (*ampR*) resulting in constitutive expression (Jaurin and Normark, 1983). However, in *E. coli* the *ampC* gene is regulated by a weak promoter and a transcriptional attenuator, resulting in low levels of AmpC β-lactamase (Fernandez-Cuenca et al., 2005). This low-level expression of the AmpC β-lactamase does not contribute to a clinically relevant level of resistance to β-lactams (Siu et al., 2003; Fernandez-Cuenca et al., 2005). *E. coli* isolates have been previously described as being resistant to various β-lactams as a result of over production of β-lactamase through gene amplification within the chromosome, or by mutations in the promoter region leading to over expression (Sui et al., 2003; Caroff et al., 1999; Fernandez-Cuenca et al., 2005; Tracz et al., 2005). The *ampC* promoter contains a -35 region (TTGTCA) and a -10 region (TACAAT) consensus sequence, separated by 16 bp (Jaurin et al., 1982). The *ampC* promoter consensus sequence has a 5/6 bp homology with the conserved promoter consensus sequence in *E. coli* recognized by RNA polymerase; specifically the -35 region (TTGACA) and a -10 region (TATAAT), or Pribnow box, separated by 17 bp spacer region (Siebenlist and Gilbert, 1980). Over-expression of the *ampC* β-lactamase can occur as a result of a number of mutations, including base substitutions in the -35 and -10 regions making them

more similar to the consensus sequence, insertions between the -35 and -10 regions that optimize the distance for transcription of 17 bp, or the creation of a new -10 and -35 region that exhibits heightened expression (Jaurin et al., 1982, Sui et al., 2003; Mulvey et al., 2005). Mutations may also occur in the attenuator, and are thought to destabilize the hairpin structure allowing for greater read-through (Tracz et al., 2005). The phenotype of cells over-expressing AmpC β-lactamases is similar to ESBLs. However, unlike ESBLs, these isolates are poorly inhibited by β-lactam-β-lactamase inhibitor combinations and hydrolytic cephamycins (Nelson and Elisha, 1999).

1.3.3.4.2 Plasmid-Encoded Ambler Class C β-lactamases

AmpC β-lactamase genes have recently been found on plasmids that transfer noninducible cephalosporin resistance. The chromosomal genes coding for these enzymes are believed to have originated from *Citrobacter, Enterobacter* or *Pseudomonas* spp. (Coudron et al., 2000). Plasmids harboring genes encoding AmpC β-lactamases frequently carry resistance genes for other classes of antimicrobials, such as aminogylcosides, chloramphenicol, sulfonamide, tetracycline, trimethoprim, or mercuric ion (Philippon et al., 2002). Clinical isolates regularly produce an AmpC β-lactamase in addition to another β-lactamase harbored either on the same plasmid, or on a different plasmid (Philippon et al., 2002). Phenotypic detection of plasmid-encoded AmpC β-lactamases is difficult, and frequently these β-lactamases can be misidentified as ESBLs (Hanson, 2003).

Plasmid-borne Ambler Class C β-lactamases can be classified into five families, namely C1 to C5, based on amino acid sequence homologies (Bauernfeind et al., 1999).

The most commonly and widely distributed of these enzymes is CMY-2 β-lactamase.

1.4 Experimental Hypothesis

It is hypothesized that the use of subtherapeutic levels of antimicrobials in feedlots as growth promoters or for prophylaxis in cattle promotes the selection and emergence of antimicrobial resistant organisms, including ESBLs in commensal microbial flora. The density of bacteria within the digestive tract may also promote the dissemination of ESBL determinants among members of the microbial community, an event that would be promoted by the fact that genes coding for ESBL are harbored on plasmids.

1.4.1 Specific Objectives

In order to investigate this hypothesis, a previous study (Alexander et al., 2008a) was designed to investigate the effect of administering subtherapeutic levels of antimicrobials on ESBL-producing *E. coli* within feedlot cattle. The specific objectives of my study are outlined below:

i) Characterization of suspect ESBL-producing *E. coli* isolated from feedlot cattle fed different subtherapeutic levels of antimicrobials. Isolates will be characterized phenotypically using antimicrobial susceptibility testing (disc diffusion). Molecular characterization of common ESBL genes found in Gram negative organisms will be determined on all suspected ESBL-producers using both multiplex and conventional PCR. Genotypic characterization will be performed on all ESBL-producers using pulsed-field gel electrophoresis (PFGE). Integron classes 1 to 3 will be screened

for in ESBL-producing isolates using PCR.

ii) Ceftazidime-resistant isolates shown not to produce an ESBL will be further characterized by examining the ampC promoter for mutations by DNA sequencing. Transcript levels of chromosomally encoded β-lactamase will be detemined by a Real-Time PCR (RT-PCR) assay.

CHAPTER 2

CHARACTERIZATION OF PUTATIVE EXTENDED-SPECTRUM B-LACTAMASES (ESBL) PRODUCING *ESCHERICHIA COLI* ISOLATED FROM FEEDLOT CATTLE IN SOUTHERN ALBERTA

2.1 INTRODUCTION

The use of antimicrobials in food-producing animals and the potential relationship it has to the emergence of resistant bacteria in the food chain is a growing issue (World Health Organization, 1997, 1998). Antimicrobial resistant bacteria originating from livestock may be directly infectious to humans or transfer resistance determinants to bacteria that negatively impact human health (Aarestrup et al., 2006; Phillips et al., 2004). Resistance to β-lactams in bacteria originating from food-producing and companion animals has increased, and these animals could serve as a reservoir for β-lactamase-producing bacteria that could be transferred to humans (Carattoli, 2008).

β-Lactams are among the critically important antimicrobials in veterinary and human medicine. In 1983, the first Extended-Spectrum β-lactamase (ESBL) was reported in Germany, and since then over 200 variants of this enzyme have been identified worldwide (Babic et al., 2006). ESBL confer resistance to penicillins, cephalosporins (including extended-spectrum cephalosporins) and aztreonam, but are sensitive to β-lactamase inhibitors such as clavulanic acid (Livermore and Woodford, 2006). ESBL's do not confer resistance to all cephamycins or carbapenems. Most commonly, ESBL-phenotypes are the result of point mutations in the bla_{TEM}, bla_{SVH}, or bla_{CTX} genes, resulting in variations of the primary amino acid sequence of the enzyme

(Bush, 2001). Genes responsible for the ESBL phenotype are generally carried on plasmids (Bradford, 2001), and are often associated with other determinants conferring resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfonamides (Nathisuwan et al., 2001). These genes are frequently associated with transposon/integrons (Li et al., 2007), increasing the potential for development of multidrug resistant (MDR) bacteria, and the propagation of the resistance determinants among bacterial species.

Identifying ESBL-producers are typically focused on clinical isolates from humans (Brinas et al., 2003; Costa et al., 2006; Duan et al., 2006). Limited studies have investigated the incidence of fecal shedding of ESBL-producers by livestock, and to date investigations on the prevalence of ESBL-producing *E. coli* in livestock in Canada have not been conducted. A study in the Calgary Health Region conducted a survey on clinical ESBL -producing *E. coli* isolates, and reported the majority of these isolates harbored $bla_{CTX\text{-}M}$ (Pitout et al., 2004). ESBL-producing *E. coli* have been isolated in a long-term care facility in Toronto, Canada where a major outbreak involving a MDR *E. coli* occurred, but the origin of this outbreak was not identified (Muller et al., 2002). It was determined that a plasmid harboring $bla_{CTX\text{-}M\text{-}15}$ was responsible for the ESBL-phenotype, and MDR (Boyd et al., 2004a).

The cephaloporinase gene (*ampC*) is constitutively expressed at low levels in wild-type *E. coli* (Jaurin et al., 1982), due to the absence of the *ampR* regulatory gene (Jaurin and Normark, 1983). As a result, the level of transcription of *ampC* in *E. coli* depends on the nature of the promoter region (Jaurin et al., 1982). The *E. coli ampC*

promoter includes an attenuator region from the fumarate reductase gene, that results in low level constitutive expression. Low-level expression of the AmpC does not result in a clinically relevant level of resistance to β-lactams (Siu et al., 2003). *E. coli* isolates that exhibit hyper-expression of AmpC have been shown to be resistant to various β-lactam antibiotics including ampicillin, cefoxitin, and expanded-spectrum cephalosporins. This hyper-production is thought to arise either through increase of ampC transcription levels either by mutations in the promoter or attenuator regions (Sui et al., 2003; Caroff et al., 1999; Fernandez-Cuenca et al., 2005; Tracz et al., 2005, 2007). In most clinical β-lactam resistant *E. coli* isolates, it appears that the high level of β-lactamase production is as a result of mutations in the promoter region of *ampC* (Caroff et al, 1999, 2000; Corvec et al, 2002).

Several different mutations can occur in the *ampC* promoter that increases the expression of the β-lactamase gene. Alterations include insertions in the consensus sigma 70 promoter sequence that increase the number of bp between the -35 box (TTGACA) and -10 box (TATAAT) regions from 16 bp to 17 bp resulting in a closer resemblance to the *E. coli* consensus boxes (Jaurin et al., 1982; Caroff et al., 1999, 2000; Corvec et al., 2002; Siu et al., 2003). Mutations creating an alternate promoter besides the *ampC* can also occur upstream of the β-lactamase gene forming a new -35 and -10 box separated by 17 bp (Caroff et al., 1999, 2000; Olsson et al, 1982).

The formation of AmpC is regulated by the attenuator component of the *ampC* promoter, which creates a hairpin structure and halts AmpC production in a growth rate-dependant manner (Jaurin and Normark, 1983). Mutations in the attenuator region are

believed to cause higher *ampC* transcription levels by destabilizing the mRNA hairpin structure which would normally decrease transcription levels and consequently core expression (Sui et al, 2003).

In this study, we characterized *E. coli* isolates recovered from 300 feedlot steers housed in the Lethbridge Research Centre Research Feedlot. The cattle received subtherapeutic levels of antimicrobials over a 314 day period and the *E. coli* isolates selected had a reduced susceptibility to either ceftazidime (MIC's of 0.5, 1, and 2 $\mu g\ mL^{-1}$) or cefpodoxime (MIC's of 0.5, 1, and 2 $\mu g\ mL^{-1}$).

2.2 MATERIALS AND METHODS

2.2.1 Feedlot location, animals, and treatment

Isolates used in this work originated from a study conducted at the Lethbridge Research Centre feedlot, using 300 crossbred steers (198 ± 20 kg initial body weight) that were blocked by weight and assigned to 30 pens. Steers were deliberately selected on the bases that they never received antimicrobials and were all purchased from the same ranch (Deseret Ranches, Raymond, AB, Canada). Upon purchase, calves were transported directly to the feedlot at the Lethbridge Research Centre. In total, five adjacent pens, with 10 steers per pen were assigned to each of six treatments: 1) Control, no antibiotics; 2) chlortetracycline and sulfamethazine (each at 350 mg $(head \cdot d)^{-1}$; fed as Aureo S®-700 G, Alpharma Inc., NJ USA; treatment denoted TET-SUL); 3) chlortetracycline (11 ppm; fed as Aureomycin®-100 G, Alpharma; treatment denoted TET); 4) monensin (25 ppm, fed as Rumensin®, Elanco Animal Health, AB Canada; treatment denoted MON); 5)

tylosin phosphate (11 ppm, fed as Tylan®, Elanco Animal Health; treatment denoted TYL); and 6) virginiamycin (250 mg (head·d)$^{-1}$, fed as V-Max®, Pfizer Animal Health, NY USA; treatment denoted VIR). The steers in this study were fed barley-based diets typical of those used in western Canadian feedlots. The feeding period included growing and fattening phases, separated by a 21-day transition between diets. For the first 115 days in the feedlot, steers were fed a silage-based, backgrounding diet consisting of 70% barley silage, 25% barley grain and a 5% mineral and vitamin supplement (dry matter basis). During the transition period, steers were gradually adapted to a grain-based finishing diet consisting of 85% barley, 10% barley silage and 5% supplement (dry matter basis). The steers were then maintained on this diet for an additional 179 d (Figure 2-1). Throughout this study, all steers were cared for in accordance to the guidelines of the Canadian Council on Animal Care (Olfert et al. 1993).

2.2.2 Sample collection and selection of isolates

Fecal samples were collected from each steer on 11 occasions during the feeding period (Figure 2-1). For each steer, two clinical swabs (Starswap®, Starplex Scientific, ON Canada) were inserted approximately 5 cm into the rectum and rotated until covered with a uniform amount of feces. All swabs were placed individually in sterile, capped test tubes, on ice and transported immediately to the laboratory. All samples were processed within 4 h of collection. Each swab was aseptically transferred and mixed with 750 μL brain heart infusion broth (BHI; Difco, Becton Dickinson, Sparks, MD USA) amended with glycerol (20%, v/v). The tubes containing swabs and medium were

vortexed and the resulting slurries from both tubes for each steer were combined.

Fecal slurry (10 μL) from each steer was plated onto MacConkey agar (Difco, Becton Dickinson) containing no antibiotics (MAC) or onto MAC amended with ampicillin (50 μg mL^{-1}; MAC+AMP), tetracycline hydrochloride (4 μg mL^{-1}; MAC+TET), gentamicin (2 μg mL^{-1}; MAC+GEN), or ciprofloxacin (2 μg mL^{-1}; MAC+CIP).

In total, 7,184 *E. coli* isolated from MAC (3,522), MAC+TET (2,212), MAC+AMP (637), and MAC+GEN (813) plates were tested using the MIC agar dilution method for ceftazidime (0.5, 1, and 2 μg mL^{-1}) and cefpodoxime (0.5, 1, and 2 μg mL^{-1}) according to NCCLS (NCCLS, 2002) to determine if presumptive ESBL-producing strains of *E. coli* were present.

E. coli isolates resistant to ceftazidime (MIC $\geq$ 2 μg mL^{-1}) or cefpodoxime (MIC $\geq$ 2 μg mL^{-1}) were designated as suspect ESBL producers as defined by the NCCLS (NCCLS, 2002). This resulted in a total of 237 *E. coli* isolated being collected. In 2005, NCCLS became CLSI and the concentrations of ceftazidime and cefpodoxime required for isolates to be designated as suspect ESBL's was increased to 8 μg mL^{-1}. These isolates were collected prior to my study.

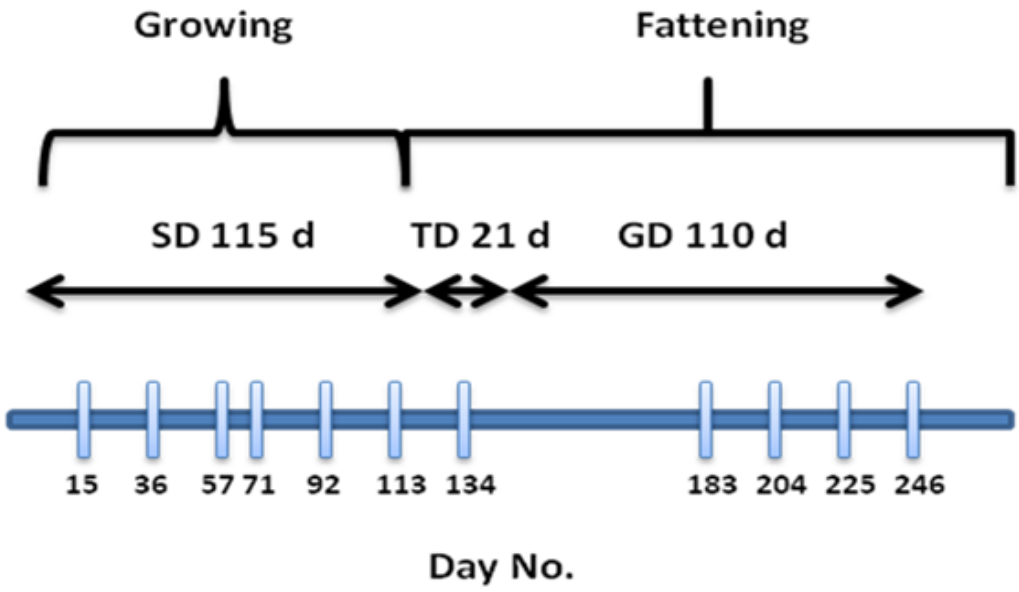

Figure 2-1. Diagram presenting the timeline for sample collection. The number of days (d) on feed are shown. Light blue rectangles represent the 11 days where samples were collected from each steer. The steers were fed a silage-based diet (SD) for the initial 115 days, a series of 3 transition diets (TD) over 21 days, and a grain-based finishing diet (GD) for 110 days.

2.2.3 Antimicrobial susceptibility testing

Potential ESBL isolates were confirmed by the disk diffusion method described by the current CLSI guidelines (CLSI, 2006) by using disks containing ceftazidime (30µg), ceftazidime/clavulanic acid (30/10 µg), cefotaxime (30µg), and cefotaxime/clavulanic acid (30/10 µg) (Becton Dickinson and Company, Mississauga , ON, Canada). A subset of 34 isolates from the original 7184 exhibited suspect ESBL-phenotype (Figure 2-2), and were re-tested in triplicate using the double disk diffusion assay. This study followed the protocols outlined by CLSI. Control strains used in this study included *K. pneumoniae* ATCC 700603, *E. coli* ATCC 52922, and *E. coli* ATCC 35218.

Susceptibilities to other classes of antimicrobials were performed on all suspect ESBL-producing isolates using the disk diffusion assay described by CLSI, 2006. The antimicrobials examined included: amoxicillin/clavulanic acid (30 µg/10 µg), ceftiofur (30 µg), cefoxitin (30 µg), chloroamphenicol (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), neomycin (30 µg), tetracycline (30 µg), streptomycin (10 µg), sulfisoxazole (0.25 µg), trimethoprim/ sulfamethoxazole (1.25 µg/23.75 µg) (BD).

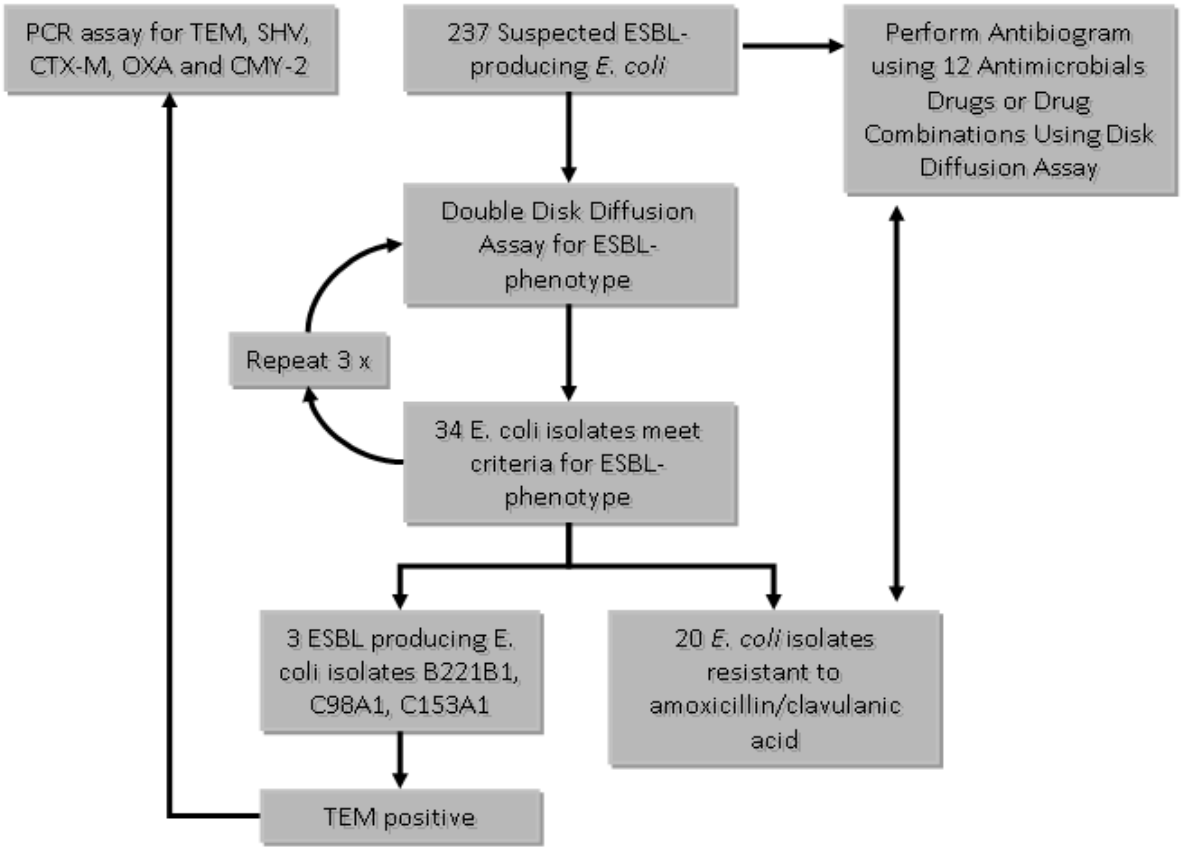

Figure 2-2. A flow diagram depicting how isolates were selected from the initial 237 putative ESBL-producing *E. coli* to 34 for further investigation. All of the 237 *E. coli* isolates were subjected to PCR analaysis, disk diffusion assay for ESBL phenotype, and the disk diffusion assay using 12 antimicrobial drugs and drug combinations.

2.2.4 Molecular characterization of study strains

The 237 putative ESBL-producing isolates were screened by both conventional and multiplex PCR assays for *bla* genes of CTX-M, TEM, SHV (Rodriguez-Villalobos et al., 2006), OXA (Ahmed et al., 2006), and CMY-2 (Susky E., 2005). DNA template was isolated from a single bacterial colony, lysed by incubating in 40 µL of TE buffer at 98°C for 5 min. The lysate was centrifuged at 13,000 x *g* for 5 min, after which 2 µL of supernatant was added to the PCR reaction as DNA template (Alexander et al. 2008).

Plasmid DNA was extracted on all confirmed ESBL producers by using the commercial QIAprep© Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada). Isolates were first grown overnight in LB amended with 50 µg mL^{-1} of ampicillin at 37°C in a 200 RPM shaking incubator and processed as per the manufacturer's guidelines. PCR assays described above was performed on all extracted plasmid DNA (Table 2-1).

PCR containing a single primer pair specific to integrons class 1 (Ahmed et al., 2006) and 2 (Machado et al., 2005) (Table 2-1) were performed on extracted DNA from a subset of 34 isolates (Figure 2-2). All PCR were performed with a DNA Dyad (M.J. Research Inc., Watertown, MA, USA). Products (15 µL) were resolved on a 2% agarose gel and stained with ethidium bromide.

Table 2-1. Primers used for ESBL detection and sequencing, *ampC* promoter, and integron detection

	Primer	Nucleotide Sequence	Amplicon size (bp)	Reference of source
ESBL detection	TEM 5'	5'-GTG CGG TAT TAT CCC GTG TT -3'		
	TEM 3'	5'-AAC TTT ATC CGC CTC CAT CC -3'	416	Rodriguez-Villalobos et al., (2006)
	SHV 5'	5'-GGA AAC GGA ACT GAA TGA GG -3'		
	SHV 3'	5'-ATC CCG CAG ATA AAT CAC CA -3'	301	
	CTX-M 5'	5'-CGY TTT SCN ATG TGC AGY AC -3'		
	CTX-M-3'	5'-TCN CCT CTG CCG CTY TTA TC -3'	510	
	OXA 5'	5'-TCA ACT TTC AAG ATC GCA -3'		Ahmed et al. (2006)
	OXA 3'	5'-GTG TGT TTA GAA TGG TGA -3'	591	
	CMY-2 5'	5'-ACA CTG ATT GCG TCT GAC G -3'		Susky E. (2005)
	CMY-2 3'	5'-AAT ATC CTG GGC CTC ATC G -3'	1244	
Integron detection	5'CS	5'-GGC ATC CAA GCA AG-3'		Ahmed et al. (2006)
	3'CS	5'-AGG CAG ACT TGA CCT GA-3'	136	
	IntI2-F	5'-CAC GGA TAT GCG ACA AAA AGG T-3'		Machado et al. (2005)
	IntI2-R	5'-GTA GCA AAC GAG TGA CGA AAT G-3'	788	
ESBL sequencing	TEM-FL-F	5'-ATG AGT ATT CAA CAT TT(CT) CGT G-3'		Rodriguez-Villalobos et al., (2006)
	TEM-FL-R	5'-TTA CCA ATG CTT AAT CAG TGA GG -3'	n/a	
ampC sequencing	AB1	5'-GAT CGT TCT GCC GCT GTG -3'		Corvec et al. (2002)
	AmpC2	5'-GGG CAG CAA ATG TGG AGC AA -3'	271	
RT-PCR	GapA1	5'-ATC AAC GGT TTG GCC GTA T -3'		
	GapA2	5'-GTT GAT AAC TTT AGC CAG CGG -3'	100	Tracz et al. (2005)
	NC3	5'-GTG AAG CCC GTC TGG TTT GAG T-3'		
	NC4	5'-TTA TTG TCA CTG CCG TTA AT -3'	57	

2.2.5 Molecular subtyping by Pulsed Field Gel Electrophoresis

Isolates (34 total, Figure 2-2) were subtyped by PFGE separation of *Xba*I-digested genomic DNA according to the Centers for Disease Control one-day standardized laboratory protocol for molecular subtyping of *E. coli* (Centers for Disease Control, 1996). Prior to PFGE, isolates from storage were inoculated onto Luria-Bertani agar (Daylin, Calgary, AB, Canada) and incubated at 37ºC for 18 h. Using a sterile swab, a single colony was harvested and suspended in cell suspension buffer (100 mM EDTA in 100 mM Tris) to an optical density (OD_{610}) of 1.3 to 1.4. Proteinase K (Sigma, Oakville, ON, Canada) was added to the cell suspension (final concentration 0.5 mg mL^{-1})and combined with 1% SeaKem Gold:1% SDS agarose (BioWhittaker Molecular, Rockland, ME USA) prepared using Tris-EDTA (TE) buffer [1 mM EDTA in 10 mM Tris, pH 8.0] amended with 1% (w/v) SDS. The mixture was immediately placed in disposable plug molds (Bio-Rad Laboratories, Mississauga, ON Canada). Plugs were left for 15 min at room temperature to allow for solidification, transferred to cell lysis buffer (0.1 mg mL^{-1} proteinase K and 1% sarcosyl in 50 mM Tris:50 mM EDTA, pH 8.0) and incubated at 54 ºC for 2 h in a shaking water bath with vigorous mixing. Following lysis, plugs were washed twice with pre-heated (50 ºC) distilled water for 15 min, then 4 times with pre-heated (50 ºC) TE buffer for 30 min. All washes were conducted in a 50ºC water bath with shaking. Plugs were cooled to room temperature and stored in TE buffer at 4ºC until PFGE was conducted. *E. coli* ATCC 25922 was included on each PFGE gel as a control. All plugs were digested with *Xba*I overnight at 37 ºC.

DNA fragments were resolved by electrophoresis in 1% SeaKem Gold agarose gel

(BioWhittaker Molecular; Rockland, Maine) in a CHEF DRII (Bio-Rad Laboratories Inc.; Mississauga, Ontario), using 0.5 Tris–borate–EDTA (45 mmol/L Tris, 45 mmol/L borate, 10 mmol/L EDTA; pH 8.3) as the buffer. Running conditions for PFGE included switching time ramped linearly from 2.2 to 54.2 s, and a voltage of 6.0 V/cm for 17.5 h. The gels were stained with ethidium bromide (0.5 mg/mL in distilled water) and visualized using a software-based image capturing system (AlphaImager; AlphaInnotech, San Leandro, California). Gels were scanned and analyzed with Bionumerics version V5.1 software (Applied Maths, Austin, TX, USA). Dendrogram analysis used UPGMA clustering of Dice coefficient values with 1.0 % optimization and 1.5 % position tolerance settings.

2.2.6 DNA sequencing

A primer pair (Table 2-1) was used to amplify a 271 bp fragment containing the -35, -10 and attenuator region of the *ampC* promoter (Corvec et al., 2002) on 20 isolates demonstrating resistance to amoxicillin/clavulanic acid (Figure 2-2). Reaction conditions for PCR included an initial denaturation (15 min at 95 ºC), followed by 35 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 57 ºC), polymerization (1 min at 72ºC), and a final extension step (7 min at 72°C). Product (10 µL) was visualized as described above prior to sequencing (Macrogen, Rockville, MD, USA).

2.2.7 RNA isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

A subset of 20 *E. coli* isolates that demonstrated resistance to amoxicillin/clavulanic acid were grown to the mid-log phase in Luria-Bertani broth

amended with 50 µg mL^{-1} ampicillin; RNA was extracted using the Qiagen RNeasy mini commercial kit. A QuantiTect Reverse Transcription Two-step Quantitative RT-PCR kit (Qiagen) was used to assay the transcript production of glyceraldehydes-3-phosphate dehydrogenase (*gapA*) and *ampC* using SYBR green PCR Master Mix (Applied Biosystems, Foster city, CA, USA). Each reaction was performed in triplicate, using the primer pairs GapA1 and GapA2, and NC3 and NC4 (Table 2-1; Corvec et al., 2003). The *gap* gene is constitutively expressed and encodes D-glyceraldehyde-3-phosphate dehydrogenase that is highly conserved in bacteria and eukaryotes (Nelson et al., 1991). The *gap* gene was used as an external control in the RT-PCR assay in a manner similar to that employed by other researchers (Corvec et al., 2003; Tracz et al., 2005). Real-Time PCR was performed in 96 well plates in an AB 7500 Fast Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). Controls included *E. coli* ATCC 255922 extracted RNA as a positive control. This strain contains a wild type promoter that produces constitutively low levels of AmpC β-lactamase (Tracz et al., 2005). A no-template negative control was also used. The reaction time conditions were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 10 s denaturation at 95 °C , and a 30 s annealing/extension time at 60 °C. Relative quantification was carried out by using the delta-delta C^T method ($2^{-\Delta\Delta CT}$). The calibrator gene used in this assay was *E. coli* ATCC 25922 *ampC*, and was assigned a $2^{-\Delta\Delta CT}$ of 1.00. The values for *ampC* were normalized to those of *gapA.* SYBR Green was used because it has been demonstrated previously to be sensitive enough to detect mRNA resistance genes, and characterize gene expression transcripts in several *E. coli* strains with different resistance phenotypes (Corvec et al., 2003). Melting curve analysis was performed at the end of the RT-PCR

assay to ensure that non-specific binding of the dye was not occurring and producing false signals. A single peak occurred during the melt curve analysis in all samples. In order to eliminate residual DNA, extracted mRNA was treated with DNase. To confirm the elimination of residual DNA, conventional PCR was conducted on DNase treated RNA. No amplification was observed, verifying no contamination due to genomic DNA (Corvec et al., 2003; Sheridan et al., 1998)

2.3 RESULTS

2.3.1 The prevalence of ESBL producing E. coli

Putative ESBL-producing *E. coli* were isolated from MAC+AMP (n=237) plates and tested for both ceftazidime (2 μg mL^{-1}) and cefpodoxime (2 μg mL^{-1}) growth according to NCCLS breakpoints to identify supect ESBL isolates. Since the collection of isolates, CLSI had altered the criteria for determining ESBL phenotype by increasing the concentration of ceftazidime and cefpodixime to 8 μg mL^{-1} as the cut-off for the ESBL phenotype. In total, 237 *E. coli* isolates were considered suspect ESBL-producers and were investigated further according NCCLS guidelines. After conducting the double disk diffusion assay according to the current CLSI guidelines, 34 isolates demonstrated resistance profiles of ceftazidime (30μg) ≤ 22 mm with an increase in zone diameter of 4 or 5 mm with ceftazidime/clavulanic acid (30/10 μg) disks, or cefotaxime (30μg) ≤ 27 mm with an increase in zone diameter of 4 or 5 mm with cefotaxime/clavulanic acid (30/10 μg) disks (Figure 2-2). After repeating this assay in triplicate, 1.27% (3/237) *E. coli* isolates of the suspect ESBL isolates demonstrated the ESBL phenotype according to CLSI standards, representing 0.0418% (3/7184) of the overall population of *E. coli*

isolated from feedlot steers. The other 31 isolates did not consistently show the ESBL-phenotype upon repeating the disk diffusion assay in triplicate, did not harbour any ESBL gene determinants screened for, and as a result were not considered to be true ESBL producers.

2.3.2 Antimicrobial susceptibility

The 237 suspected ESBL *E. coli* isolates were tested for resistance to 12 antimicrobials or combinations of antimicrobials (Table 2-2). No resistance to cefoxitin, gentamycin, imipenem and trimethoprim/sulfamethoxazole was detected in any of the suspected ESBL-producing isolates. The most prevalent antimicrobial resistance profile (14.8%; 35/237 isolates) was resistance to amoxicillin/clavulanic acid. Further, 20 of the isolates resistant to amoxicillin/clavulanic acid were part of the subset of 34 (Figure 2-2) that exhibited resistance to ceftazidime (8 μg mL^{-1}). These isolates were further characterized to investigate the genetic mechanism responsible for their resistance to β-lactam antimicrobials.

Multidrug resistance was observed in 23.2% (55/237) of these suspect ESBL-producing *E. coli* isolates, with the most common being (8.0%, 19/237) resistance to streptomycin and tetracycline. Of the 3 isolates demonstrating ESBL phenotype (B221B1, C98A1 and C152C1), 2 were susceptible to all the antimicrobials tested. However, the other confirmed ESBL isolate, B221B1, exhibited multi-drug resistance to enrofloxacin, tetracycline and sulfisoxazole with intermediate suceptibility to streptomycin.

Table 2-2 Antibiograms of presumptive-ESBL producing *Escherichia coli* (n=237) isolated from feedlot cattle[a]

Profile	Phenotype[b]	No of Isolates (n=237)	Percent (%)
A1	Amp	126	53.2
A2	Amp Step*	7	3.0
A3	Amp Amcl*	4	1.7
A4	Amp Amcl	35	14.8
A5	Amp Sulf Step Tet	2	0.8
A6	Amp Step* Tet	2	0.8
A7	Amp Tet	4	1.7
A8	Amp Amcl* Step Tet	18	7.6
A9	Amp Amcl* Clor* Step Tet	2	0.8
A10	Amp Amcl* Step	1	0.4
A11	Amp Nmyn* Step Tet	2	0.8
A12	Amp Nmyn* Step	1	0.4
A13	Amp Amcl Ctio*	1	0.4
A14	Amp Step Tet	19	8.0
A15	Amp Enro Tet	1	0.4
A16	Amp Step	3	1.3
A17	Amp Clor Sulf Step Tet	3	1.3
A18	Amp Sulf Nymn Tet	2	0.8
A19	Amp Enro Sulf	2	0.8
A20	Amp Amcl Cfox	1	0.4
A21	Amp Amcl Ctio	1	0.4

[a] *E. coli* were isolated from cattle at the Lethbridge Research Centre as described previously (Alexander et al., 2008)

[b] Suceptibilities were conducted by disc diffusion assay according to CLSI guidelines. MCL = amoxicillin-clavulanic acid; CFOX = cefoxitin; CLOR = cloramphenicol; CTIO = ceftiofur; ENRO = enrofloxacin; GENT = gentamycin; IMPN = imipenem; NMYN = neomycin; STEP = streptomycin; SULF = sulfisoxazole; TET = tetracycline; TMSZ = trimethoprim/ sulfamethoxazole

*Represents Intermediate Resistance

2.3.3 β-lactamase type

PCR screening revealed that TEM was the only category of β-lactamase observed among the 237 suspected ESBL-producing *E. coli*. No other category of β-lactamase was detected in this study. In total, 37.1% (88/237) isolates tested positive for bla_{TEM} , but only one isolate that harboured the bla_{TEM} gene exhibited an ESBL phenotype. The majority of the suspect ESBL-producing isolates harbouring the bla_{TEM} gene 16.9% (40/88) were isolated from fecal swaps collected on day 113 of the original study. The bla_{TEM} gene from this isolate was sequenced, and identified as a TEM-1 type β-lactamase and to be harboured on a 3530 bp plasmid. However, it is unlikely that this gene is responsible for ESBL-phenotype. The other two isolates that were confirmed as ESBL did not possess the common determinants (TEM, SHV, CTX-M, OXA or CMY-2) responsible for the ESBL phenotype. Plasmid DNA was isolated from these two strains; however, attempts to transform the DNA into competent cells resulted in no transformants on plates containing ceftazidime (8 μg mL^{-1}, 4 μg mL^{-1}, 2 μg mL^{-1}) suggesting that the gene responsible for ESBL phenotype was not carried on a plasmid. In total, 37.1% (88/237) putative ESBL-producing *E. coli* harboured the bla_{TEM} gene. A subset of the isolates containing the bla_{TEM} gene , namely 16.8% (40/88), were isolated from fecal swaps collected on day 113 of the original study.

2.3.4 Pulsed Field Gel Electrophoresis (PFGE)

The 34 isolates that were selected from the initial 237 suspected ESBL (Figure 2-2) were examined for clonal relationships (Figure 2-3). PFGE revealed 4 clusters based on ≥ 90% similarity, consisting of 3, 2, 2 and 2 isolates. None of the isolates within a

cluster contained an identical restriction pattern, suggesting that none of the isolates are clones of one another. The remaining 27 suspect ESBL isolates did not cluster with these closely related groups. None of the confirmed ESBL isolates clustered together, indicating that all three isolates were unique. However C152C1, one of the ESBL-producers was grouped in with cluster 1 (Figure 2-3). Strains within the 4 clusters all exhibited the same antibiogram and were resistant to amoxicillin/clavulanic acid (30/10 µg), but were susceptible to all other antimicrobials.

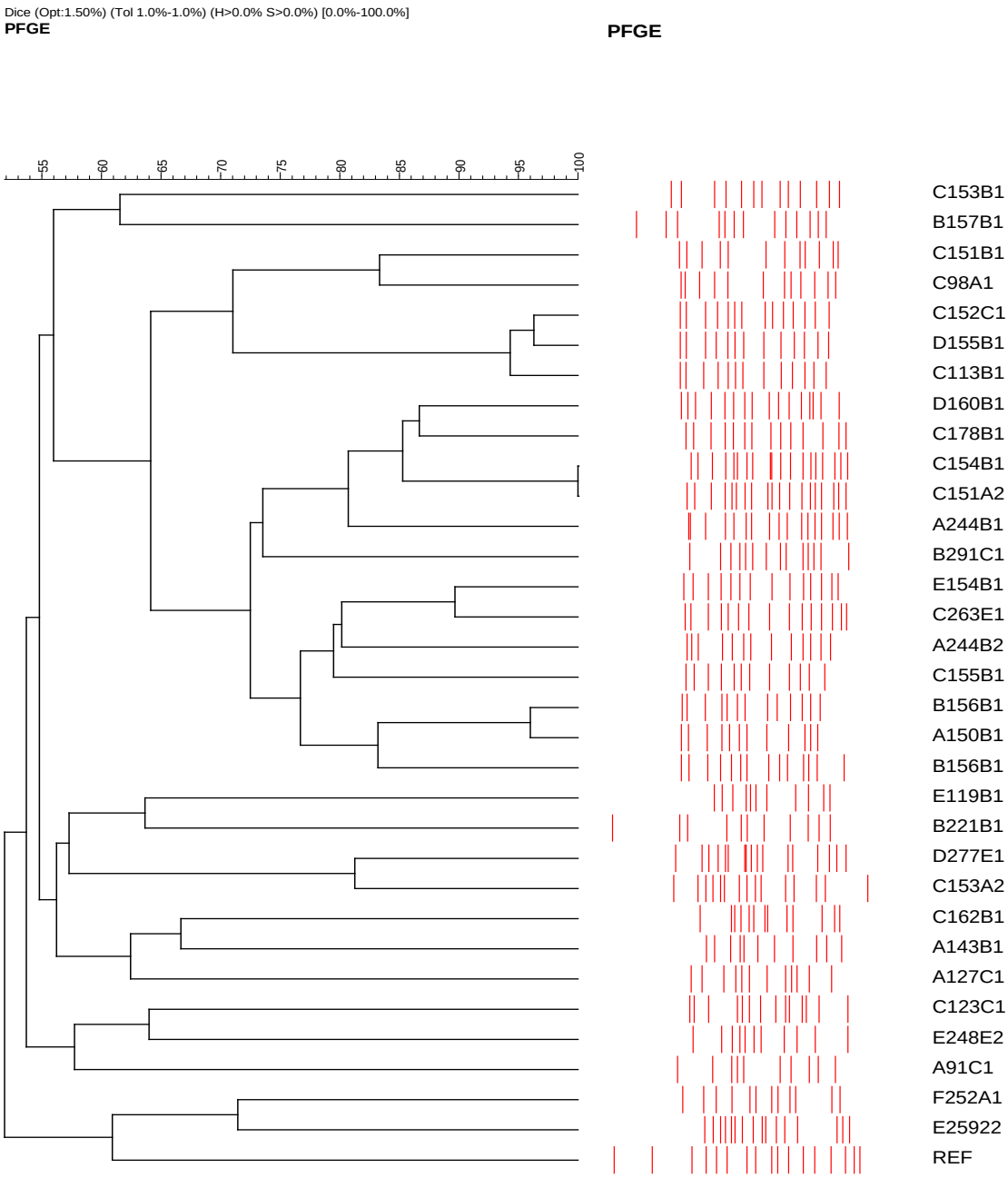

Figure 2-3. PFGE patterns and dendrogram showing the genetic relatedness of suspected *E. coli* ESBL-producers isolated from feedlot cattle. Each colour coded box represents the different clusters characterized by 90% similairity. Cluster 1 is represented by green boxes; cluster 2 is represented by red boxes; cluster 3 is represented by blue boxes; and cluster 4 is represented by yellow boxes. REF refers to the reference strain.

2.3.5 Integron Detection

A subset of 34 isolates (Figure 2-2) was examined for the presence of class 1, 2, and 3 integrons by PCR. The primer pair used for detection of the class 1 integron targeted the flanking region of the gene cassette, resulting in variable amplicon sizes. In total, 64.7% (22/34) tested positive for containing a class 1integron. In contrast, none of the isolates tested positive for class 2 integrons. All 3 confirmed ESBL producers contained the class 1 integron. A class 1 integron was not detected in plasmid DNA isolated from the three ESBL isolates; a result that suggests that the presumptive integron is located on the bacterial chromosome. Isolate B221B1 demonstrated an amplicon size of approximately 2500 bp for the class 1 integron, suggesting this isolate contained a gene cassette.

2.3.6 ampC transcript levels in promoter mutants of ceftazidime-resistant E. coli

A subset of 20 (8.43%) isolates were excluded as being ESBL producers, but still demonstrated reduced susceptibility to ceftazidime. These isolates were found to be negative for TEM-, SHV-, CTX-M-, OXA-, and CMY-2-type β-lactamase genes by PCR. Furthermore, attempts to isolate plasmid DNA were unsuccessful. DNA sequencing of the *ampC* promoter/attenuator of these 20 isolates was conducted and the results were compared against the wild type *ampC* promoter/attenuator for strain *E. coli* ATCC 25922. Subsequently, RT-PCR was used to determine if variations of the *ampC* promoter region and transcript levels could possibly account for ceftazidime resistance in these isolates.

Sequence analysis of the *ampC* promoter region revealed 5 different categories

(Figure 2-4, Table 2-3). The known functional elements include: the -35 and -10 boxes and spacer region, and the attenuator region. One isolate (A143B1) contained a transversion mutation (C→T) in the wild-type -10 box (position -11), creating a -10 box identical to the *E. coli* consensus TA**T**AAT (Figure 2-4, Table 2-3; Hawley & McClure, 1983). This isolate also had a +1 insertion in the spacer region between the wild-type -35 box and the -10 box, causing these elements to be 17 bp apart. This insertion resulted in an optimal spacing distance for efficient gene expression from sigma70 promoters (Jaurin et al., 1982). RT-PCR analysis revealed that *ampC* transcript levels were approximately 10921–fold higher in *E. coli* A143B1 relative to *E. coli* 25922. Another isolate, C153B1, also contained a transversion mutation (T→C) occurring at the +63 position, as well as a +1 bp insertion in the spacer region between the -35 and -10 consensus boxes. RT-PCR analysis demonstrated *ampC* transcript levels were approximately 88-fold higher in *E. coli* isolate C153B1 relative to *E. coli* ATCC 25922. C154B1 contained a +1 insertion in the spacer region resulting in a 17 bp spacing between the -35 box and -10 box. Corresponding 2-fold increase in the transcript levels occurred in this strain. The final class of mutation observed in this study resulted from a deletion (position -10) in the wild-type -10 box, causing it to change from TACAAT to TACA**TC.** This was observed in 5 strains, but transcript levels were measured at around wild-type levels (0-0.47-fold). Thus, it appears that this particular mutation did not result in over expression of *ampC*. The remaining strains investigated in this study contained no mutation within the *ampC* promoter. This was reflected in the RT-PCR data, where the strains demonstrated transcript levels comparable to that of the wild-type strain (0.0-2.0). The mechanism of resistance to ceftazidime in these strains remains unknown, as both *ampC* promoter

mutations and acquired β-lactamase genes were not detected.

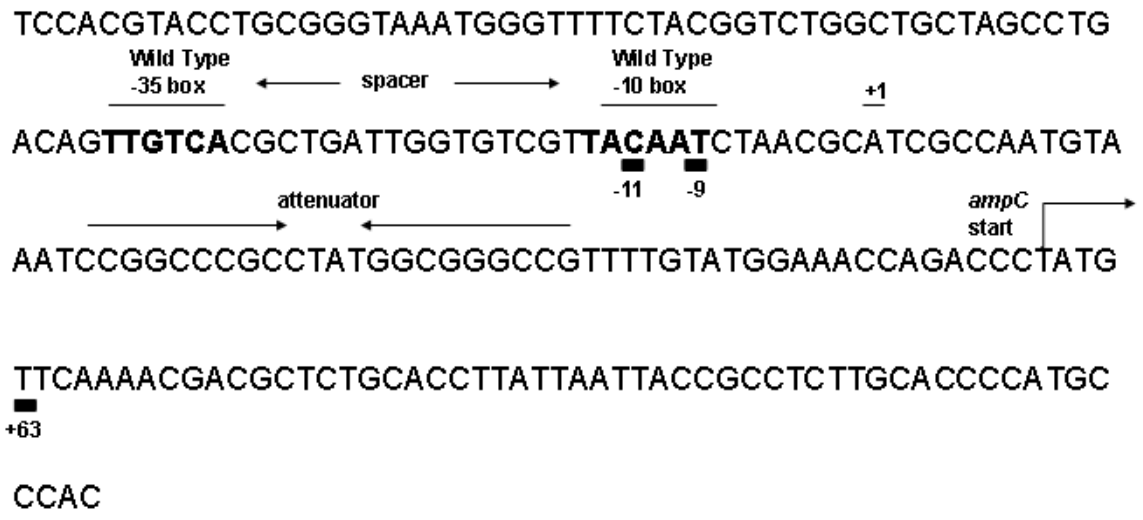

Figure 2-4. *Echerichia coli* ATCC 25922 *ampC* promoter region. Numbering is according to Jaurin et al., 1981. The boxes at position -11, -9 and +63 indicate regions were mutations have occurred creating a new *ampC* promoter in isolates investigated in this study.

Table 2-3 *ampC* promoter mutations in *E. coli* strains examined in this study. The fold change in *ampC* transcript of sample isolates is presented compared with *E. coli* ATCC 25922 as analyzed by the $2^{-\Delta\Delta CT}$ method

Location on AmpC promoter	Mutation[a]	Isolate	ΔΔCT [b]	$2^{-\Delta\Delta CT}$
spacer and wild type -10 box	+1 nt insertion in spacer region -11C->T	A143B1	-13.415	10921.31
spacer and +63 region	+1 nt insertion in spacer region +63 T-> C	C153B1	-10.888	87.69
spacer	+1 nt insertion in spacer region	C154B1	-7.7329	2.13
wild type -10 box	TACATC	D155B1	1.0825	0.4722
		C123C1	9.4896	0.0014
		C113B1	0	0
		B156B1	0	0
		E119B1	1.1224	0.4522
No mutation		A127C1	-1.019	2.0265
		A150B1	0.9399	0.5213
		A244B1	1.8327	0.2807
		A244B2	8.0497	0.2807
		C153A1	1.2054	0.4337
		C151B1	1.1063	0.4645
		D160B1	6.215	0.0135
		E154B1	5.2528	0.0262
		C178B1	0	0
		E156B1	0	0
		F252A1	0	0
		C155B1	0	0

a Defined by the location of mutation in specific promoter elements as indicated on Fig 2-4.

[b] $\Delta CT = ampC\ C_T - gapA\ C_T$

$\Delta\Delta CT$ = mean ΔCT - ATCC25922 ΔCT

Mean was calculated from triplicate reactions

2.4 DISCUSSION

ESBL-producing *Enterobacteriaceae* have mainly been associated with nosocomial infections (Pitout et al., 1997), or residents in nursing homes (Boyd et al., 2004b; Bradford et al., 1995; Wiener et al., 1999). Few reports have investigated the nature of ESBL producing bacteria associated with livestock (Allen and Poppe, 2002; Poppe et al., 2001). In this study, we describe the detection and prevalence of ESBL-producing commensal *E. coli* in feedlot steers over a 314 d collection period. The feedlot steers used in our study had no exposure to antimicrobials prior to their arrival at the Lethbridge Research Centre. Further, none of the antimicrobials administered subtherapeutically were β-lactamase antimicrobials. These isolates investigated originated from a study where it was reported that the prevalence and number of fecal *E. coli* demonstrating MDR increased in cattle over the duration of the feeding period (Alexander et al., 2008a). However, this did not seem to occur in ESBL-producing *E. coli* isolated from healthy feedlot steers. In total, 3 isolates out of 7,184 (0.0418%) were confirmed to be true ESBL-producers. Our results suggest that the subtherapeutic antimicrobials administered to steers in the described feedlot study did not directly influence the appearance of ESBL producing strains over a 314-d feeding period due to the low prevalence of ESBL-producing *E. coli*.

TEM-1 was the only category of β-lactamase identified in this study. Contrastingly, the dominant category of ESBL from food production animal isolates belongs to the CTX-M family (Li et al., 2007). Narrow-spectrum TEM-1 or derivatives have been reported in ampicillin-resistant *E. coli* and *Salmonella* spp. obtained from

food-producing animals such as cattle, turkeys, chickens and pigs (Chen et al., 2004; Maidhof et al., 2002; Olesen et al., 2004). In France, a recent study reported 52 ESBL-producing isolates (41 *E. coli*, 7 A*cinetobacter* spp., 2 *P. aeruginosa*, 1 *Citrobacter freudii*, 1 *Hafnia alvei*) from cattle exhibiting clinical symptoms belonged to the CTX-M group, an observation that coincided with the determinant group responsible for the majority of ESBL-related infections (Madec et al., 2008). In southern Alberta, (Pitout et al., 2005, 2007), most ESBL *E. coli* infections in humans are associated with the CTX-M group. This suggests cattle are unlikely the source of ESBL producing *E. coli* in infected humans. However, the isolates used in this study were collected from only a single group of cattle. Perhaps sampling from multiple groups of cattle in different locations, and screening *E. coli* for ESBL production would add further insight into whether cattle are a reservoir for ESBL producing *E. coli* associated with human infection. Another study conducted in Japan (Kojima et al., 2005) also investigated ESBL-producing *E. coli* strains from various farm animals, and reported CTX-M producing and CMY-2 producing isolates. This finding also differed from the epidemiology reports in human clinical isolates in Japan, where TOHO are the most prevalent determinants identified (Bradford et al., 2001).

MDR is common among *Enterobacteriaceae* and has been reported in cattle (Hoyle et al., 2005). Of the *E. coli* isolates in this study, 23.2% (55/237) demonstrated MDR, The most frequently observed MDR phenotype was resistance to streptomycin and tetracycline 8.02% (19/237). The prevalence of isolates resistant to tetracycline could reflect the widespread subtherapeutic use of tetracycline in the beef cattle industry a

treatment that was employed in the study of Alexander et al. (2008). A previous investigation examined antimicrobial resistance patterns in commensal *E. coli* isolated from beef carcasses in Canada, and reported tetracycline and streptomycin-tetracycline-sulfisoxazole were the most prevalent antibiograms observed (Van Donkersgoed et al., 2003). Tetracycline and sulfa drugs are frequently administered together in cattle, which may lead to the development of resistance in *E. coli* to both of these antimicrobials. Mobile elements such as plasmids harbouring resistance genes to tetracycline, streptomycin, sulfonamides, and other aminoglycosides may be responsible for the transfer of these resistances among Enterobacteriacea (Poppe et al., 2001).

Isolate B221B1, an ESBL-producer, demonstrated resistance to enrofloxacin, sulfisoxazole, tetracycline, and intermediate resistance to streptomycin. It was also determined that the bla_{TEM} gene carried by this strain was carried on a plasmid but that the integron appeared to be associated with the chromosome. It has been previously shown that ESBL can be encoded on large plasmids that also carry genes for resistance to other antimicrobials such as aminoglycosides, trimethoprim, sulfonamides, tetracyclines and chloramphenicol (Paterson, 2000). However, the plasmid associated with B221B1 was approximately 3,530 bp making it unlikely that all of the determinants needed to confer resistance were present on the same plasmid (Machado et al., 2005).

Investigators have used molecular methods such as PFGE to examine the molecular epidemiology of ESBL-producing strains involved in outbreaks of nosocomial infections (Bradford et al., 2001). All of the isolates categorized in the four different clusters were collected from different animals, at different points during the feeding

experiment. Thus, it is unlikely that any of the putative ESBL *E. coli* isolates collected from this study were clonally spread throughout the same cattle during the course of this trial. Further, it is unlikely that ceftazidime (8 μg mL^{-1})-resistant *E. coli* were propagated throughout the pens and was passed among the feedlot population. All 3 ESBL-producers demonstrated unique patterns, suggesting there is no epidemiological link between the strains. Further, all 3 ESBL- producers were collected from different animals, and during different periods. These finding have been reported in other clinical studies in humans that have surveyed ESBL populations (Machado et al., 2005; Mulvey et al., 2004).

In this investigation, we did not sequence the entire *ampC* gene but rather elected to look at alterations in the promoter region. Thus, variant AmpC proteins with amino acid alterations leading to ceftazidime resistance cannot be excluded as the source of resistance in these *E. coli* isolates. It has been reported that strains with modified AmpC proteins demonstrate altered β-lactam resistance profiles (Morosini et al., 1998; Mammeri et al., 2004). Previously, it has been suggested that alterations at +63, +70 and +81 result in amino acid substitutions in the leader peptide region and this could have an effect on transfer of the enzyme precursor into the periplasmic space (Mulvey et al., 2005). In addition, it has been suggested that altered porin expression patterns can contribute to β-lactam resistance (Martinez-Martinez et al., 2000; Anathan & Subha, 2005). It has been demonstrated that in the event there is a lack of an acquired β-lactamase gene, ceftazidime-resistant *E. coli* can have *ampC* promoter mutations that contribute to β-lactam resistance profiles.

An RT-PCR method was used to analyze *ampC* gene expression in *ampC* promoter variants in select *E. coli* strains. Previous investigations have described nucleotide insertions that create greater separation between the -35 and -10 boxes of the *ampC* promoter, and produce higher promoter efficiency (Corvec et al., 2003; Tracz et al., 2007). One study reported mutations causing a + 1 bp insertion in the spacer region, combined with or without mutations within the attenuator element. It was concluded that the main contributor to promoter strength in this case is the increased spacer length (Tracz et al., 2007). Isolate C154B1 from the present study contained a +1 insertion in the spacer region resulting in the wild-type -35 box and -10 box being 17 bp apart. The results indicated a 40-fold increase in the *ampC* transcript of this strain, which is within the same range as seen in previous investigations with the same class of mutation (Tracz et al., 2007). We also found 5 isolates containing a mutation in the wild-type -10 box from TACAAT to TACA**TC,** but this mutation had a negligible effect on *ampC* promoter strength. This mutation appears to be unique as other studies examining mutations in the *ampC* promoter region have not previously reported it.

The isolates from this study originated from an experiment that focused on intensive monitoring of a single feedlot of cattle with 11 samples being collected over the feeding period for each cow. Alexander et al. 2008a reported that ampicillin resistance in *E. coli* is lower in samples collected from the Lethbridge Research Centre versus a commercial feedlot. The ampicillin resistant *E. coli* and the suspect producing *E. coli* isolates were from the same location and study. Our results detected very low levels of ESBL-producing *E. coli*, and perhaps this is because our samples came from a single

group of cattle that originated from the same ranch. Further, in the research feedlot study there was no administration of therapeutic antimicrobials to the cattle. In a commercial feedlot setting therapeutic administration would occur, and this could have a significant impact on the development of resistance. However, the objective of my investigation was to observe the emergence of ESBL-producing E. coli on isolated origionating from cattle fed subtherapeutic levels of antimicrobials.

In this study, we characterized suspected ESBL-producing *E. coli* isolated from feedlot steers using selective plating techniques. We only detected 3 ESBL-producers, indicating the ESBL-producing levels in the total *E. coli* population in the feedlot were low. Even though at present there is a low level of isolation in food-producing animals, such as cattle, it is necessary to monitor the spread of ESBL-producing bacteria and further research including animals and humans and the environment. Acquisition of ESBL-producing bacteria from food-producing animals could transfer to humans resulting in clinical complications. Previously, it was clearly determined that a ceftriaxone-resistant *Salmonella* isolate from cattle was acquired by a child (Fey et al., 2000). Furthermore, Shiraki et al., 2004, suggested that $bla_{CTX\text{-}M}$ could have originated from cattle and subsequently been transmitted to the food chain by contamination of cattle carcasses. Although our data does not demonstrate a need for surveillance when cattle are subtherapeutically administered the investigated antimicrobials, other studies (Madec et al., 2008, Meunier et al., 2006) emphasizes the need for a long-term survey for ESBL emerging phenotypes in parallel in human and veterinary fields. Correct assessment of the factors responsible for increasing the risk of emergence of ESBL

bacteria would be an important component of any prudent antimibrobial use program.

2.4.1 Chapter Summary

- In total, 3 *E. coli* isolates of the 237 presumptive ESBL-producers demonstrated the ESBL phenotype. One of these isolates, B221B1 harboured the bla_{TEM} gene on a plasmid.
- Molecular subtyping of the 3 isolates demonstrated that the ESBL phenotype revealed no epidemiological link between these strains.
- MDR was observed, but was not predominant among the presumptive ESBL-producers.
- Three isolates contained mutations within the *ampC* promoter region that was demonstrated to be linked with increased transcript levels of the chromosomal *ampC* β-lactamase gene.
- Feeding antimicrobials subtherapeuticially to feedlot cattle does not appear to aid in the development of ESBL-producing commensal *E. coli*.

CHAPTER 3

ATTEMPTS TO DETERMINE THE GENE DETERMINANT RESPONSIBLE FOR EXTENDED-SPECTRUM ß-LACTAMASES (ESBL) IN TWO *ESCHERICHIA COLI* STRAINS ISOLATED FROM FEEDLOT CATTLE IN SOUTHERN ALBERTA

INTRODUCTION

Plasmid-mediated extended spectrum β-lactamases (ESBL) are typically TEM, SHV or CTX-M β-lactamases (Bradford, 2001) with one or more amino acid substitutions surrounding their active site (Merdeiros and Crellin, 1997). Currently, 167, 98 and 92 forms of TEM, SHV, and CTX-M β-lactamases, respectively have been reported (Jacoby and Bush, 2008). Members of these groups are all class A β-lactamases, and generally are inhibited by clavulanic acid. In addition to these classical ESBL categories, alternate ESBL-derivatives have been reported in clinical isolates and in some instances have been associate with a specific geographical location; TOHO-1, TOHO-2, and SFO-1 in Japan (Ishii et al., 1995; Ma et al., 1998; Matsumoto et al., 1999), PER-1 mostly in Turkey (Nordman et al., 1998), and VEB-1 in Thailand and Vietnam (Naas et al, 1999; Poriel et al, 1999). Further, recent spread of VEB-1 and PER-1 has occurred in *A. baumannii* in France and Belguim (Potriel et al., 2003; Naas et al., 2006), and *P. aeruginosa* in northern Italy (Pagani et al., 2004). These derivatives are inhibited by clavulanic acid and belong to the Bush functional group 2be (Bush et al., 1995), and Ambler molecular class A (Ambler, 1980) with the genes conferring resistance usually

being plasmid mediated (Merdeiros and Crellin, 1997).

In this investigation, we examined 7,184 *Escherichia coli* isolated from 300 feedlot steers in southern Alberta receiving subtherapeutic levels of antimicrobials over a 314-day period. A subset of 237 isolates were suspected of being ESBL-producers based on elevated MIC's for either ceftazidime (2 μg mL^{-1}) or cefodoxime (2 μg mL^{-1}) according to NCCLS standards. In total three isolates (0.0418%) exhibited the ESBL phenotype, but when trying to resolve the genetic determinants responsible, only one isolate was found to contain bla_{TEM-1}. The objective of this study was to identify and characterize the gene determinants responsible for the ESBL phenotype in the two remaining *E. coli* strains (C152C1 and C98A1). Isolates were subjected to conjugation, plasmid extraction and transformation, and cloning techniques in an effort to isolate the resistance genes.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3.1.

Conjugation experiments

In an effort to determine if the genes responsible for the ESBL phenotype in strains C98A1 and C152C1 were harbored on a transferable plasmid, mating experiments were undertaken. Conjugation experiments were performed between both isolate strains and nalidixic acid resistant *E. coli* recipient K12 JM109 (NAL^R) (New England Biolabs, Pickering, ON, Canada). Both the donor and recipient strain were grown for 18 hr at 37

°C in a total volume of 5 mL of LB, diluted 20 times in LB and grown for an additional 12 h at 37 °C. Conjugation experiments were performed using various donor: recipient ratios of 1:1, 1:2, and 1:10 in a 5 mL total reaction volume of LB. Liquid mating was performed in LB broth and incubated for either 30 min or overnight at 37 °C (Gray et al., 2006). Transconjugants were selected on LB agar plates containing both nalidixic acid (32 μg mL^{-1}) and ampicillin (50 μg mL^{-1}) or nalidixic acid (32 μg mL^{-1}) and ceftazidime (2 μg mL^{-1}).

Transformation experiments

Plasmid DNA from *E. coli* C152C1 and C98A1 was extracted with the commercial QIAprep© Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada) from 5 mL of overnight culture, and concentrated using a Savant DNA 120 SpeedVac Concentrator (Thermo electron corporation, Gormley, ON, Canada). Ten microliters of plasmid DNA (between 5 and 10 kb) was resolved on a 1.0% (w/v) agarose gel and stained with ethidium bromide, using a lambda DNA marker cut with *Hin*dIII and *Eco*R1 (Fermetas, Burlington, ON, Canada) to estimate the size of the plasmids . Plasmid DNA (20 ng/μL) was subsequently transformed into One Shot®TOP10 chemically competent cells (Invitrogen, Burlington, ON, Canada). Transformants were selected on LB agar plates containing ceftazidime (2, 4, 8 μg mL^{-1}) or ampicillin (50 μg mL^{-1}).

Genomic DNA extraction and Partial Restriction

Genomic DNA from isolates C98A1 and C152C1 was extracted using a DNA extraction method previously described (Puhler and Timmins, 1984). DNA was resolved

by electrophoresis on a 0.8 % (w/v) agarose gel, followed by staining with ethidium bromide and viewing with a transilluminator. The DNA was then subjected to a time-course digest (0, 5, 10, 15, 20, 30 min) using the restriction enzyme *Sau*3AI (New England Biolabs). Five µL of each digest was analyzed on a 1.0% (w/v) agarose gel, following electrophoresis and visualized by staining with ethidium bromide. A lambda *Hin*dIII marker was included on the gel. The quality of genomic DNA was examined by agarose gel electrophoresis and subsequently the partial digest was repeated again (0 min, 6 min, 6 min 15 sec, 6 min 30 sec, 6 min 45 sec, 7 min) with time points selected on the basis of those that contained greatest amount of DNA fragments ranging from 4000-6000 bp. The time point 6 min 15 sec was selected for further digests because it was associated with the greatest amount of DNA consisting of 4000-6000 bp. DNA was excised from the gel and extracted using a Qiaquick Gel Extraction Kit (Qiagen) and quantified using a Flurometer (Biorad, Mississauga, ON, Canada). The DNA was concentrated using a Savant DNA 120 SpeedVac Concentrator (Thermo electron corporation, Gormley, ON, Canada), requantified and diluted to a concentration of 50 ng/µL.

Cloning Experiments

A Zero Background^{TM} Cloning Kit with selection on Kanamycin (Invitrogen) was used in subsequent cloning experiments. The pZErO-2^{TM} vector was digested with the restriction enzyme *Bam*HI (New England Biolabs) for 20 minutes and purified using a DNeasy Blood and Tissue Kit (Qiagen) to eliminate the restriction enzyme. The cut vector was then dephosphorylated using Antarctic phosphatase (New England Biolabs)

according to manufacturer's specifications. The DNA (50 ng) digested with *Sau*3AI (New England Biolabs) was then ligated into the cleaved pZErO^{TM} -2 (100 ng DNA) with either a 2:1 or a 1:2 vector:insert ratio using T4 DNA ligase (Invitrogen) (4U/µL) and ligation conditions as shown in Table 3-2. Ligations were transformed into One Shot® TOP10 chemically competent cells (Invitrogen) according to manufacturers specifications. Transformants were selectively plated on LB agar amended with either ceftazidime (2, 4, 8 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) or ampicillin (50 µg mL^{-1}) and kanamycin (50 µg mL^{-1}).

Table 3-1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or phenotype	Source of reference
Strains		
E. coli K12 JM109	*F´ traD36 proA^{+}B^{+} lacI^{q} Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14^{-} gyrA96 recA1 relA1 endA1 thi hsdR17*	New England Biolabs
E. coli C152C1	Amp^{r} Caz^{r}	This Study
E. coli C98A1	Amp^{r} Caz^{r}	This Study
E. coli One Shot® TOP10	*F-mcr AΔ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15ΔlacX74 recA1araD139 Δ(ara-leu)7697 galU galK rpsL endA nupG*	Invitrogen
Plasmids		
pZErO™ -2	Kan^{r}	Invitrogen
Plasmid isolated from C152C1	Unknown	This Study
Plasmid isolated from C98A1	Unknown	This Study

Table 3-2. The various ligation times and temperature conditions used in cloning attempts for gene determinants responsible for ESBL phenotype observed in *E. coli* strains C152C1 and C98A1.

Ligation	Time	Temperature
pZErO-2™ + Digested DNA (1:2)	1 hr	16°C
	18 hr	16°C
	18 hr	4°C
	24 hr	4°C
pZErO-2™ + Digested DNA (2:1)	1 hr	16°C
	18 hr	16°C
	18 hr	4°C
	24 hr	4°C

3.3 RESULTS

Molecular Characterization, Conjugation and Transformation Analysis

Preliminary PCR experiments using primers to amplify internal fragments of ESBL genes (TEM, SHV, CTX-M, CMY-2 and OXA derivatives) failed to detect any of these determinants in *E. coli* strains C98A1 and C152C1. Mating experiments performed in liquid medium between *E. coli* K12 JM109 with both *E. coli* C152C1 or *E. coli* C98A1 failed to generate transconjugants. Plasmid DNA extracted from both isolates C152C and C98A1 (Figure 3.1) were transformed into chemically competent *E. coli* One Shot® TOP10. No colonies were observed after selective plating onto LB amended with either ceftazidime (2, 4, 8 µg mL^{-1}) and or ampicillin (50 µg mL^{-1}).

3.3.2 Partial Digest and Cloning Experiments

Partial Digest and Cloning Experiments

To investigate if the gene determinants responsible for the ESBL phenotype were located on the chromosome of isolates C152C1 and C98A1, genomic DNA was extracted and subsequently partially digested with *Sau*3AI over a 30-minute period.. The digested DNA was ligated into the pZErO-2™ using the various reaction conditions described in Table 3.1. and then transformed into competent cells and selectively plated on LB. No colonies were observed after incubation for 18 hours at 37 ºC.

DISCUSSIONS AND CONCLUSIONS

Attempts to determine the origin of the ESBL phenotype in *E. coli* strain C152C1 and C98A1 was unsuccessful. Others have also been unable to clone antibiotic resistance determinants from ESBL producing *E. coli* exhibiting an ESBL phenotype (Kojima et al., 2005; Mulvey et al., 2004). Our results indicate that both isolates C152C1 and C98A1 harbor plasmids, which can be problematic in transformation reactions because as the size of a plasmid increases, the transformation efficiency decreases. In order to address this issue, conjugation experiments were conducted to determine if the ESBL resistance determinant was located on a plasmid. However, conjugation experiments failed to produce recipient strains resistant to ceftazidime.

Alternative approaches could be explored to determine the cause for ESBL phenotype in both these isolates. It would be worthwhile to test using PCR for other less common gene determinants responsible for ESBL-production, such as bla_{PER}, bla_{VEB}, bla_{GES}, bla_{CME}, bla_{SFO}, bla_{TOHO}, and bla_{TLA}. Further, it could be beneficial to make gene libraries using different resistriction enzymes, and attempt cloning techniques using the DNA fragments created from restriction digestion.

There are other mechanisms responsible for resistance to β-lactam antimicrobials besides production of β-lactamases. Outer membrane impermeability is a source of resistance to β-lactams in Gram-negative bacteria (Livermore, 1991). In *P.aeruginosa,* broad and narrow spectrum resistances can arise through mutation in OMP's,

specifically, resistance has been shown to result form loss of the D2 outer membrane protein, providing resistance to carbapenems but not other β-lactams (Livermore, 1991). Broad spectrum-resistance has resulted from unknown mechanisms that simultaneously provided resistance to many β-lactams, but not imipenem (Livermore, 1991). PBP insensitivity also can cause resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) (Livermore, 1991). Unlike the mechanisms described above, an insensitive PBP is active against all β-lactams, including carbapenems as well as penicillins and cephalosporins (Livermore, 1991). Perhaps one of the mechanisms mentioned above has occurred in isolates C152C1 and C98A1.

CHAPTER SUMMARY

- Two *E. coli* strains, (C152C1 and C98A1) isolated from feedlot cattle demonstrated a ESBL phenotype, but no detectible gene determinants could be identified using PCR.
- I used conjugation, plasmid extraction and subsequent transformation, and a direct selection cloning method to try and identify a novel gene, but all methods failed to obtain the determinants responsible for ESBL phenotype.

CHAPTER 4

CONCLUSIONS AND PROSPECTS

The potential connection between the use of antimicrobials in food-producing animals and the emergence of resistant bacteria in the food chain has become of great concern (World Health Organization 1997; 1998). The European Union has banned the use of many AGPs. In contrast, antimicrobials (e.g., penicillin, tetracycline, the macrolides and sulfonomides) are routinely used in North America for livestock production either subtherapeutically to prevent disease, increase growth and improve feed efficiency, or therapeutically to treat disease (Blake et al., 2003; Wegener, 2003). To date, few studies have investigated the influence of subtherapeutic administration of antimicrobials to feedlot cattle and its relationship to the development of AMR, specifically in the development of ESBL-producing commensal *E. coli*. The lack of available information creates difficulties in forming regulatory guidelines regarding the use of in-feed antimicrobials in beef production. A concern about livestock production is that the constant exposure of bacteria to antimicrobials in the animal's intestine could create an environment that is conducive for the development of AMR. These resistant microbes could be transferred through the food chain to humans and be problematic to treat with antimicrobials in the event that they cause disease.

In Canada, ESBL-producing *Enterobacteriaceae* are important in nosocomial infections. Baudry et al., 2008 reported that CTX-M ESBLs were the most prevalent type of ESBL in clinical isolates of *E. coli* in Canada. Investigations involving ESBL-producers are typically derived from clinical isolates from humans (Brinas et al., 2005;

Costa et al., 2006; Duan et al., 2006; Shiraki et al., 2004). Few studies have examined the incidence of fecal carriage of ESBL producers in livestock, and to date no investigations on the prevalence of ESBL-producing *E. coli* in feedlot cattle have been reported in Canada.

The purpose of this study was to characterize a subset of putative ESBL-producing *E. coli* isolated for a group of feedlot cattle housed at the Lethbridge Research Centre Feedlot in southern Alberta. To my knowledge, this is the first experimental study to investigate the nature of possible ESBL *E. coli* from feedlot cattle. My isolates originated from an experiment that was designed to maximize the detection of ceftazidime and cefpodoxime resistant *E. coli* (and therefore presumptive ESBL-producers) throughout the feeding period both with and without the administration of subtherapeutic levels of antimicrobials by first plating on ampicillin, and then screening for ceftazidime (2 μg mL^{-1}) and cefpodoxime (2 μg mL^{-1}). Antimicrobial treatment was selected based on prevalence of use in the feedlot industry and included: chlortetracycline + sulfamethazine (44ppm; fed as Aureo® S -700G); chlortetracycline (11ppm; fed as Aureomycin® -100 G); virginiamycin (44 ppm, fed as V-Max®); monensin (25 ppm, fed as Rumensin®); tylosin (11 ppm, fed as Tylan®), or no antibiotic supplementation (control). In total, 7184 *E. coli* isolates were tested for ampicillin resistance using MAC amended with ampicillin plates (50 μg mL^{-1}), and then examined ceftazidime and cefpodoxime of varying concentrations (0.5 μg mL^{-1}, 1.0 μg mL^{-1}, 2 μg mL^{-1}). Isolates that were resistant to either ceftazidime (2 μg mL^{-1}) or cefpodoxime (2 μg mL^{-1}) were categorized as presumptive ESBL producers based on NCCLS guidelines at the time of

isolate collection. Using antimicrobial resistance profiling, pulse field gel electrophoresis (PFGE) and molecular characterization I investigated the nature of suspect ESBL *E. coli* isolates collected. My findings offer insight into the development of ESBL-producing commensal *E. coli* in feedlot cattle. The results suggest that feeding subtherapeutic levels of antimicrobials to cattle does not result in the presence of high numbers of ESBL-producing *E. coli* in feedlot cattle. None of the antimicrobials fed to the cattle included β-lactam as these antibiotics are not approved for subtherapeutic use in feedlot cattle in Canada. Another important component to consider is that the cattle in this study originated from a single herd and were transported directly from the ranch to the feedlot environment. Results may have varied if sampling occurred at multiple feedlots containing animals originating from multiple herds across western Canada. If approval could be obtained, a similar study feeding cattle β-lactams subtherapeutically over a larger time frame to cattle located in multiple feedlots would provide additional insight into the impact that this practice would have on the emergence of ESBL-producing *E. coli*. However, as such a practice does not take place, commercially the relevance of such a study to Canadian feedlot cattle production conditions could be questioned even though it is likely that the frequency of ESBL producing isolates would increase. Finally since the time of isolate collection in this study, the CLSI guidelines that define ESBL-producers have been altered to increase the concentration of ceftazidime or cefpodoxime required to confirm the ESBL phenotype. This could explain why initially, 237 *E. coli* isolates were presumptive ESBL-producers based on the old criteria, but after further analysis and using the new criteria only 34 of the original isolates would have been considered presumptive ESBL-producers. This demonstrates that resistance of *E. coli* to

extended-spectrum β-lactams occurs as a continuum of sensitivity to these antibiotics and although we establish a cut-off point for resistance, a range of resistance actually occurs within the *E. coli* population.

Attempts to use conjugation, transformation, and gene cloning proved to be unsuccessful at resolving the genetic determinants responsible for the phenotype observed in isolates C152C1 and C98A1. Further analysis should be conducted to resolve the mechanisms of resistance in these two isolates that exhibit the ESBL-phenotype. Perhaps further optimization of steps used to construct the gene library (i.e. preparation of genomic DNA inserts with a different restriction enzyme, ligation) would aid in achieving this goal.

Although 88 isolates were confirmed to harbour the bla_{TEM} gene by PCR analysis, it has yet to be determined what genetic mechanism is responsible for resistance to ceftazdime (2 μg mL^{-1}) in the other isolates that were non ESBL producing. Furthermore, we do not know if the bla_{TEM} gene is located on a plasmid or if it is chromosomal. This could be another area to investigate, because although these isolates are not ESBL-producers, they still show reduced suceptibility to a third generation cephalosporins. Identifying the genetic mechanism responsible in the other presumptive ESBL-producers, and also determining if the bla_{TEM} genes responsible for ceftazidime (2 μg mL^{-1}) resistance are clones or have been propagated through the feedlot would be another worthwhile area to investigate. This could provide further insight on the impact of AMR in food animals on human health, and help understand the consequences of the use of antimicrobial agents on the occurrence and spread of resistant clones and genes

that impact AMR in bacterial reservoirs. In turn, this could aid in implementing guidelines and regulations on the usage of antimicrobial agents in feedlots and other livestock production systems.

This study offers insight into the nature of ESBL-producing *E. coli* in feedlot cattle. This investigation was the largest study seen in Canada in cattle concerning ESBL-production, and was conducted under natural conditions. In turn, this approach offers a more realistic outlook on the prevalence and emergence of ESBL-producing *E. coli* within a feedlot. The results of this study suggest that current production practices in feedlot cattle are unlikely to promote the emergence of high levels of ESBL's in feedlot cattle. Overall, my findings make an important contribution to establishing a knowledge base regarding the development and emergence of ESBL *E. coli* in feedlot cattle.

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