Molecular Epidemiology of Extended-Spectrum β-Lactamase-, AmpC β-Lactamase-, and Carbapenemase-Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated in Canadian Hospitals from 2007 to 2012

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ABSTRACT

Members of the Enterobacteriaceae, including *Escherichia coli* and *Klebsiella pneumoniae* are among the top ranked pathogens causing bacterial disease in Canadian hospitals. A growing proportion of clinical *E. coli* and *K. pneumoniae* isolates now demonstrate resistance to oxyimino-cephalosporins, largely attributable to extended-spectrum β-lactamase (ESBL) or AmpC β-lactamase (AmpC) production. Infections caused by β-lactamase-producing organisms pose a significant threat to patient outcome; as such organisms undermine empiric therapy and are frequently multidrug resistant (MDR). In addition, ESBL- and AmpC-producers hold serious implications for both public health and infection control practices.

We hypothesized that the prevalence of oxyimino-cephalosporin resistance would increase among clinical *E. coli* and *K. pneumoniae* isolates in Canada and that this trend would driven by increased β-lactamase production within these organisms. As a result, the primary objective of this thesis was to assess the prevalence of ESBL-, AmpC-, and carbapenemase-producing *E. coli* and *K. pneumoniae* in Canadian hospitals over time. *E. coli* and *K. pneumoniae* clinical isolates were collected from 2007 to 2012, inclusive, as part of the ongoing CANWARD national surveillance study. This study was utilized in order to collect a large nationally representative cohort of *E. coli* and *K. pneumoniae* isolates. Antimicrobial susceptibility testing was performed to detect putative ESBL-, AmpC-, and carbapenemase-producers, which were then further characterized by PCR and sequencing to detect resistance genes.

ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* are firmly established in Canadian hospitals. The prevalence of ESBL-

producing *E. coli* [2007: 3.4%, 2012: 7.6% (*P*<0.001)], AmpC-producing *E. coli* [2007: 0.7%, 2012: 2.2% (*P*=NS)], and ESBL-producing *K. pneumoniae* [2007: 1.5%, 2012: 3.6% (*P*=NS)] increased during the study period. ESBL-producing *E. coli* and *K. pneumoniae* were resistant to a variety of antimicrobials, with 78.8% and 66.7%, respectively, demonstrating a MDR phenotype. The majority of ESBL-producing *E. coli* (>95%), AmpC-producing *E. coli* (>97%), and ESBL-producing *K. pneumoniae* (>90%) remained susceptible to colistin, amikacin, ertapenem, and meropenem.

Isolates were generally unrelated by PFGE (<80% similarity); however, the pandemic *E. coli* clone sequence type (ST) 131 was identified among 56.9% and 31.7% (*P*<0.001) of ESBL-producing *E. coli* and AmpC-producing *E. coli*, respectively. CTX-M-15 was the dominant ESBL produced by both *E. coli* (66.5%) and *K. pneumoniae* (48.0%), while the dominant AmpC β-lactamase produced by *E. coli* was CMY-2 (53.2%).

The prevalence of carbapenemase-producing *E. coli* and *K. pneumoniae* is currently low in Canada. From 2009-2012, 0.06% (2/3260) and 0.09% (1/1059) of *E. coli* and *K. pneumoniae*, respectively, were found to produce a carbapenemase enzyme, all of which produced KPC-3.

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%5-SBA	Trypticase soy agar with 5% sheep blood
%I	% Intermediate
%R	% Resistant
%S	% Susceptible
AMC	Amoxicillin/clavulanic acid
AmpC	AmpC β-lactamase
ATCC	American Type Culture Collection
B-J-M	Bush-Jacoby-Medeiros
Bfp	Bundle-forming pili
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAN-ICU	Canadian Intensive Care Unit Surveillance Study
CANWARD	Canadian Ward Surveillance Study
CAZ	Ceftazidime
CHEF	Contour-clamped homogeneous electric field electrophoresis chamber
CI	Confidence interval
CLA	Clavulanic acid
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeter
CNISP	Canadian Nosocomial Infection Surveillance Program
CTR	Ceftriaxone
DAEC	Diffusely adherent *E. coli*
dH_2O	Distilled H_2O
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EAST	Enteroaggregative heat-stable enterotoxin
EDTA	Ethylene diamine tetra acetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
g	Grams
HSC	Health Sciences Centre

ICU	Intensive care unit
IHMA	International Health Management Associates Inc.
Ins	Insertion
kb	Kilobase
KPC	*Klebsiella pneumoniae* carbapenemase
LT	Heat-labile enterotoxin
M	Molar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
MBL	Metallo-β-lactamase
MDR	Multidrug resistant
mg	Milligram
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
ml	Millileter
MLST	Multilocus sequence typing
mm	Millimeter
mM	Millimolar
MNEC	Meningitis-associated *E. coli*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NDM	New-Delhi metallo β-lactamase
ng	Nanogram
nm	Nanometer
NS	Not statistically significant
OR	Odds ratio
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field Gel Electrophoresis
pH	Power of hydrogen
RPM	Revolutions per minute
Sen	Shigella enterotoxin
SENTRY	SENTRY Antimicrobial Surveillance Study
SMART	Study for Monitoring Antimicrobial Resistance Trends
ST	Heat-stable enterotoxin
ST-	Sequence type

Stx	Shiga toxins
SXT	Trimethoprim-sulfamethoxazole
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TZ	Tazobactam
TZP	Piperacillin/tazobactam
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
V	Volt
VRE	Vancomycin resistant enterococci
XDR	Extremely-drug resistant
µg	Microgram

1. INTRODUCTION

1.1 Enterobacteriaceae

The Enterobacteriaceae are a large, diverse family of organisms belonging to the phylum Proteobacteria, class Gammaproteobacteria, and the order Enterobacteriales (1). This family contains a number of clinically important genera including *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, and *Yersinia*, as well as other less common but important human pathogens including *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Providencia*, and *Serratia* (2). Enterobacteriaceae are typically large, non-spore forming, Gram-negative rods measuring 2-4 μm in length (2). All members characteristically ferment glucose, reduce nitrates, are oxidase negative, and grow rapidly on simple media under both aerobic and anaerobic conditions (1, 2). Members of the Enterobacteriaceae comprise seven of the top 20 organisms isolated from Canadian hospitals, a list which includes *Escherichia coli* (#1), *Klebsiella pneumoniae* (#5), *Enterobacter cloacae* (#9), *Proteus mirabilis* (#13), *Serratia marcescens* (#14), *K. oxytoca* (#15), and *E. aerogenes* (#20) (3). Clinically, Enterobacteriaceae are responsible for approximately 40% of bloodstream infections and greater than 70% of urinary tract infections (UTIs) in Canadian hospitals, with *E. coli* alone accounting for greater than 50% and 20% of UTIs and bloodstream infections, respectively. The term "enterics" is often used to describe this family, as the majority of organisms can be found to colonize the mammalian intestinal tract.

1.2 *Escherichia coli* and *Klebsiella pneumoniae*

1.2.1 *Escherichia coli*

Escherichia is the type genus of the family Enterobacteriaceae, and *E. coli* is the type species of the genus *Escherichia* (1). This genus contains a number of other species including *Escherichia albertii*, *Escherichia blattae*, *Escherichia fergusonii*, *Escherichia hermanii*, and *Escherichia vulneris*, of which, *E. coli* is the species most frequently isolated from humans (1). *E. coli* is the etiological agent of one of three general clinical syndromes including diarrheal disease, UTI, and systemic infection (4). Such systemic infections include sepsis, meningitis (including neonatal), nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis, and infectious arthritis (4, 5). Similar to the majority of Enterobacteriaceae, *E. coli* is motile by peritrichous flagella and is able to ferment D-glucose and other sugars (1). *E. coli* is differentiated biochemically from other members of the Enterobacteriaceae by its ability to ferment lactose and other carbons sources, and the ability of most strains to produce indole from tryptophan (1). The majority of *E. coli* strains grow rapidly on a variety of media, producing smooth round colonies of approximately 2-4 mm in diameter. Growth occurs optimally at 37^{o}C in approximately 16 to 18 hours, though some strains demonstrate a decreased rate of growth. *E. coli* is generally non-hemolytic on blood agar, producing colonies with a white/grey color, while on MacConkey agar, *E. coli* colonies typically appear pink/red as a result of strong lactose fermentation.

1.2.2 Klebsiella pneumoniae

Organisms of the genus *Klebsiella*, including *K. pneumoniae*, are found to colonize mucosal surfaces of mammals such as humans, horses, and swine, as well as in

environmental habitats such as surface water, sewage, and soil (6, 7). In this respect, *Klebsiella spp.* resembles other members of the Enterobacteriaceae such as *Citrobacter spp.* and *Enterobacter spp.*, but not others such as *Shigella spp.* and *E. coli*, which are not common in the environment (6). *K. pneumoniae* is noted as being the most medically important species of this genus, and is a common cause of UTI, pneumonia, sepsis, neonatal sepsis, bacillary meningitis, endocarditis, and wound infections (6, 7). *K. pneumoniae* is a common cause of nosocomial infections, specifically those in the very young, the elderly, and those who are immunocompromised or suffer from some underlying disease (7). Similar to other members of the Enterobacteriaceae, *K. pneumoniae* is defined as Gram-negative, non-motile rods, that are usually encapsulated (6). All produce lysine decarboxylase but not ornithine decarboxylase and are typically positive in the Voges-Proskauer test (6). In the clinical laboratory, *K. pneumoniae* can be biochemically differentiated from *K. oxytoca*, another relevant human pathogen of the genus *Klebsiella*, by a lack of indole production and inulin fermentation (7).

1.2.3 Pathogenesis

E. coli contains three main surface antigens, which are important to immunogenicity and typing of various strains. These surface antigens include the O (lipopolysaccharide) antigen, K (capsular) antigen, and H (flagellar) antigen (8). Perhaps more applicable is the categorization of virulent *E. coli* into various pathotypes based on certain disease and pathogenicity characteristics. These include the intestinal pathotypes enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (4). Extraintestinal pathogenic *E. coli* (ExPEC) is

typically separated into two main pathotypes, namely uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC) (4). Virulence factors in *E. coli* include those allowing attachment and colonization, such as fimbriae or other adhesins, as well as various entero- and cytotoxins (4, 5, 9). Characteristics of the various diarrheagenic *E. coli* pathotypes are summarized in Table 1.

Pathogenesis in *K. pneumoniae* is typically attributable to the presence and characteristics of four key virulence factors, including capsular polysaccharide, pili (fimbriae), lipopolysaccharide/serum resistance, and siderophores (6). Factors responsible for virulence in *K. pneumoniae* are summarized in Table 2.

TABLE 1. The clinical characteristics and virulence factors associated with diarrheagenic *E. coli* pathotypes. Modified from (1, 4, 10).

E. coli Pathotype	Spectrum of Disease	Susceptible Population	Adhesins/Invasins	Secreted Toxins
EPEC (Enteropathogenic *E. coli*)	Non-specific gastroenteritis (infantile diarrhea); Non-bloody (watery) diarrhea, vomiting, and fever in infants	Children under 2 years of age in under-developed countries	Bfp, intimin	*E. coli*-secreted protein F
EHEC (Enterohemorrhagic *E. coli*)	Hemorrhagic colitis, non-bloody diarrhea, hemolytic uremic syndrome (HUS)	Children and the elderly in industrialized countries	Intimin	Stx 1 and 2
ETEC (Enterotoxigenic *E. coli*)	Watery diarrhea (ranging form mild/self-limiting to severe purging disease)	Children in under-developed countries; travelers to those countries	Cf antigens	LT and ST
EAEC (Enteroaggregative *E. coli*)	Persistent diarrhea	Children and adults in under-developed countries; travelers to those countries	Aggregative adherence fimbriae (AAF/I etc.)	EAST; plasmid-encoded cytotoxin
EIEC (Enteroinvasive *E. coli*)	Bacillary dysentery	All ages; more common in under-developed countries	Invasion-plasmid antigens (IpaC etc.)	Sen
DAEC (Diffusely Adherent *E. coli*)	Non-bloody diarrhea	Children, particularly those >12 months of age	Dr fimbriae	N/A

LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; Sen, *Shigella* entero-toxin; Bfp, bundle-forming pili; Stx, Shiga toxins;

EAST, enteroaggregative heat-stable enterotoxin.

TABLE 2. Virulence factors associated with pathogenic *K. pneumoniae* and their function. Modified from (6).

Virulence Factor	Virulence Characteristics		
	Description	Primary Type	Function
Capsular antigens	77 serological types; essential to *Klebsiella* virulence	Strains possessing K1 and K2 capsule are highly likely to be virulent	Prevents phagocytosis; prevents killing by bactericidal serum factors
Pili (fimbriae)	Type 1 pili, Type 3 pili, *Klebsiella* adhesins	Type 1 pili most common	Important for colonization of genital and respiratory tract
Lipopolysaccharide (LPS)	Complement protein C3b binds O-polysaccharide side chain, preventing MAC formation	Serum resistance associated with O1 subtype	Serum resistance; endotoxin
Siderophores	Enterobactin, aerobactin	Enterobactin most common	Iron scavenging; increased growth

MAC: membrane attack complex.

1.3 Treatment

Treatment of diarrheal disease caused by *E. coli* is typically supportive in nature (11). Extraintestinal infections caused by *E. coli* and *K. pneumoniae* are diverse, including UTI, sepsis, meningitis, hospital- (ventilator- and non-ventilator-associated) and community-acquired pneumonia, as well as various intra-abdominal infections such as peritonitis. Such infections are often treated empirically with a variety of antibiotics including β-lactams, aminoglycosides, fluoroquinolones (in adults), trimethoprim-sulfamethxazole, and other agents. As the incidence of multidrug resistant (MDR: resistance to ≥3 different antimicrobial classes) and extremely-drug resistant (XDR: resistance to ≥5 different antimicrobial classes) isolates has increased, treatment has also moved to last line agents such as tigecycline and colistin for those infected with these pathogens. Treatment in such cases relies on culture and the availability of antimicrobial susceptibility testing to guide patient treatment. The β-lactam antibiotics will be the focus of this thesis and will be discussed in further detail.

1.4 β-lactam Antibiotics

1.4.1 Introduction and Mechanism of Action

The modern era of antimicrobial chemotherapy began with the introduction of sulfanilamide in 1936 and was followed by the release of the first β-lactam, benzylpenicillin, which became clinically available in 1941 (12). The β-lactams are bactericidal antibiotics that target the penicillin-binding proteins (PBPs) responsible for peptidoglycan synthesis and remodeling within the bacterial cell wall (13-15). Structurally, the characteristic four-membered β-lactam ring can be found among all members of this class. Beyond this universal characteristic, this class can be further

divided into a number of groups based on structure. These groups include the penicillins, cephalosporins, carbapenems, and monobactams (Figure 1) (14). This structural diversity contributes to variable pharmacologic properties, the propensity with which resistance develops to the various groups of β-lactam antibiotics, and ultimately how the different β-lactams are utilized in a clinical setting.

As mentioned, the β-lactams exert their bactericidal effect through the inhibition of cell wall synthesis, more specifically by targeting transpeptidation during the synthesis of peptidoglycan. Peptidoglycan is an essential component of the cell wall providing protection from osmotic forces, contributing toward cell shape, as well as influencing cell growth and development (15). This heteropolymer is composed of glycan chains consisting of two alternating sugars, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), in which NAM contains a short peptide that is cross-linked to an adjacent glycan strand giving the peptidoglycan its complex mesh-like structure (12, 14). Transpeptidation is the final stage in peptidoglycan synthesis, which takes place outside the cell membrane and is carried out by the PBP (12). The peptide chain attached to NAM is composed of five residues including a terminal D-ala-D-ala. In transpeptidation, PBPs catalyze the linkage of neighboring glycan strands through this peptide chain. The linkage occurs between the third position (diaminopilemic acid) of one side-chain and the fourth position (D-ala) of the other, and consists of either a peptide cross-bridge or a direct linkage (12, 16). In the case of the β-lactams, the antibiotic is sterically similar to the D-ala-D-ala of the pentapeptide, and therefore upon localization within the periplasmic space is mistakenly used as substrate by the PBPs (16). This leads to the PBP becoming acylated, inhibiting hydrolysis of the drug as well as

transpeptidation (15). With transpeptidation inhibited, autolytic enzymes responsible for peptidoglycan turnover continue to act in the absence of new synthesis (15). As a result, the peptidoglycan becomes structurally compromised leading to the eventual lysis of the cell (15). The process of peptidoglycan synthesis is summarized in Figure 2.

FIGURE 1. Chemical structure of β-lactam antibiotics (1-4), site of β-lactamase action (5), and the chemical structure of commonly used β-lactamase inhibitors (6-8). Figure reproduced with permission from (14).

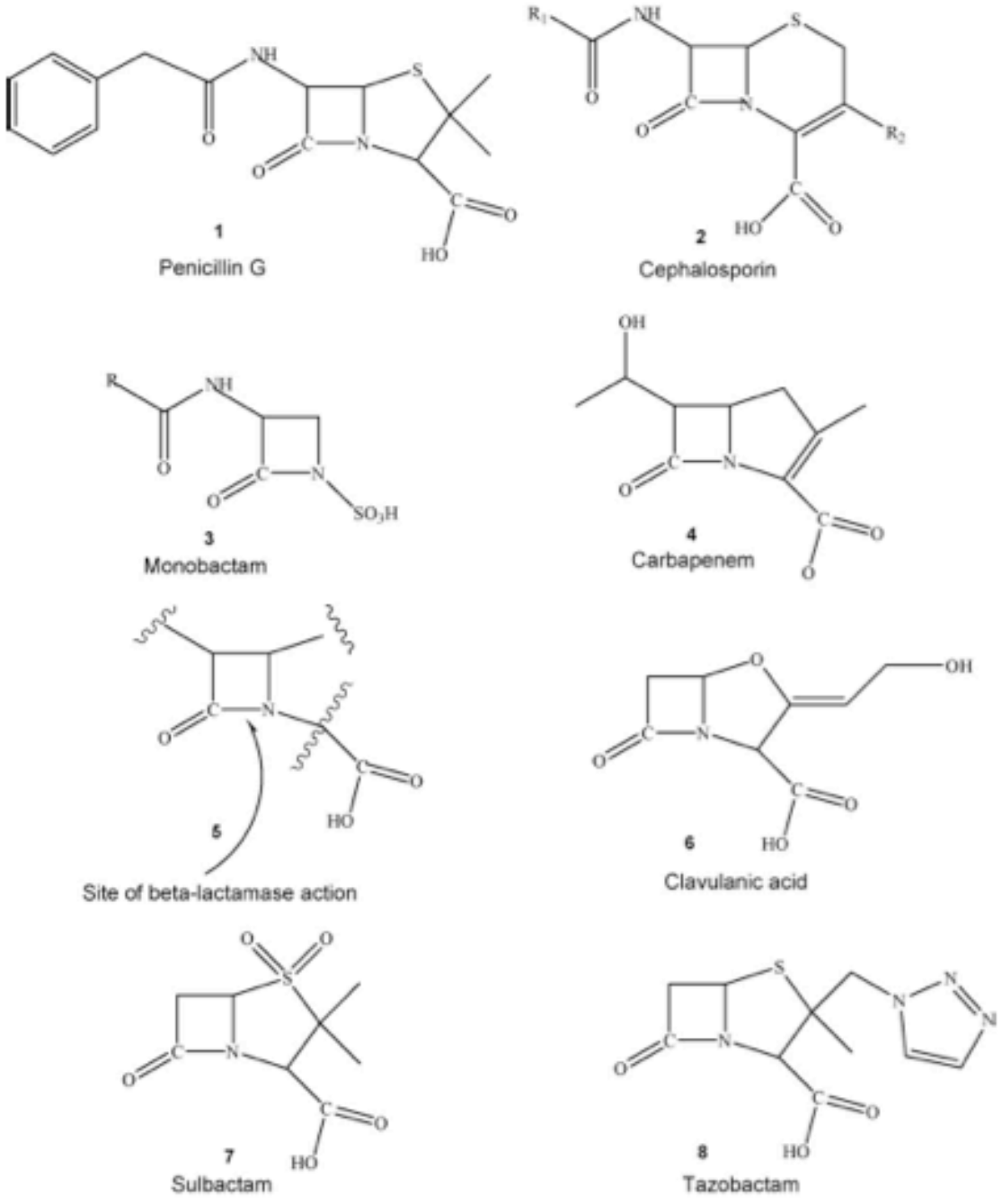
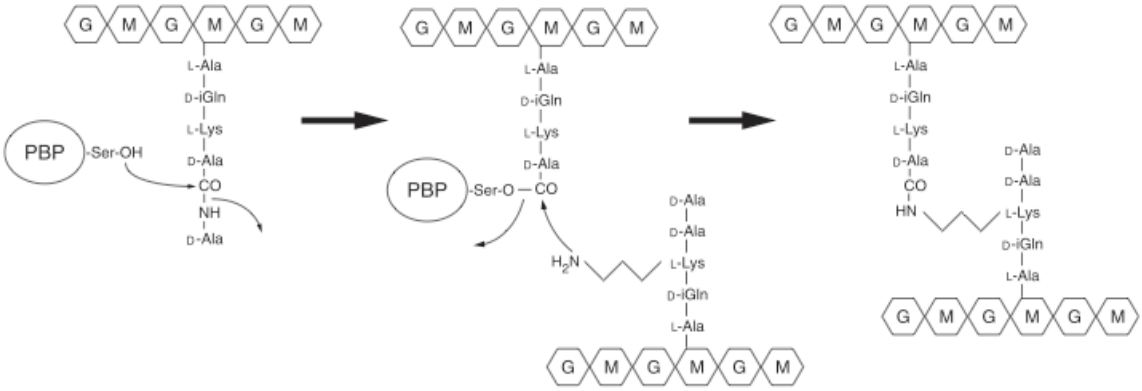


FIGURE 2. Transpeptidation during peptidoglycan synthesis. Glycan chains composed of G (N-acetylglucosamine) and M (N-acetylmuramic acid) with the pentapeptide chain extending from M illustrated; PBP attacks the fourth residue of the pentapeptide (D-ala) releasing the terminal D-ala; Amine group attacks acyl-ester of PBP releasing the enzyme and linking glycan strands. Figure reproduced with permission from (16).



1.4.2 Resistance to the β-lactams

The β-lactam antibiotics are the most widely used class of antimicrobials worldwide, representing over 65% of the global market (13). This large class of antibiotics is comprised of over 50 marketed drugs and the widespread use of these agents can be attributed to their efficacy, safety profile, and their broad spectrum of activity (12, 13). Though these agents remain an integral part of our armamentarium, their effective use in a clinical setting is currently being challenged by a perpetual cycle where the genetic plasticity of bacterial pathogens continually reduces their susceptibility to antibiotic action. Resistance to the β-lactams can arise through a number of mechanisms including drug inactivation, target-site alteration, reduced permeability, and efflux (13, 14). Target-site alteration involves the production of modified PBPs demonstrating reduced affinity for the β-lactam agent and is a common contributor of resistance in Gram-negative pathogens such as *Haemophilus influenzae* and *Neisseria spp*., as well as Gram-positive pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (13). Gram-negative organisms are capable of reducing β-lactam permeability into the cell and therefore reducing access of the β-lactam agent to their PBP target through reduced expression of outer membrane porin proteins (14). Reduced permeability is generally not capable of generating high-level resistance on its own and is often found in conjunction with the production of drug inactivating β-lactamase enzymes or other resistance determinants (13). Similarly, efflux involves a reduction in the intracellular concentration of antibiotic at the target-site through the production of proteinaceous active transporters (13). While there are five recognized families of efflux systems, members of the resistance-nodulation-division family are chromosomal and

prevalent among Gram-negative organisms, and are the primary variants associated with resistance to clinically relevant antimicrobials (13).

β-lactamases can be considered the greatest single source of β-lactam resistance, especially among Gram-negative organisms (14, 17). Functionally, β-lactamases are enzymes that cleave the amide bond of the four-membered β-lactam ring rendering the drug inactive (14). β-lactamases comprise a diverse family of enzymes with over 950 unique variants (18). The emergence of β-lactamase-mediated resistance within the clinically important Enterobacteriaceae poses a significant threat to patient outcome. Such resistance delays initiation of appropriate therapy increasing length of hospital stay and hospital cost (19). Furthermore, delays in administering appropriate therapy correlate with increased patient mortality (19).

1.5 β-lactamases

1.5.1 Overview and History

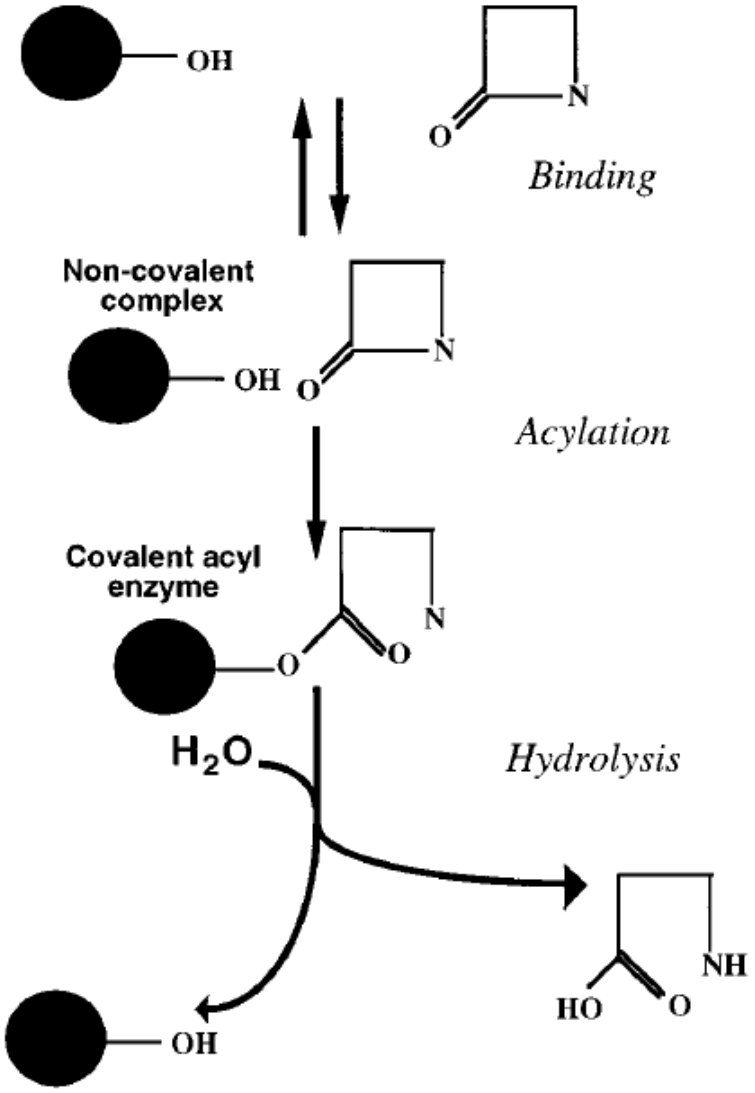
As the β-lactam class has expanded, β-lactamase co-evolution has followed closely alongside new drug development. The first β-lactamase was discovered in *E. coli* during the early 1940s prior to penicillin becoming clinically available (17). Similarly, the release of the broad-spectrum penicillins in the 1960s was followed by the identification of a number of new β-lactamases able to hydrolyze these antibiotics (17, 20). Among these newly identified β-lactamases were the TEM-1, TEM-2, and SHV-1 types, with TEM-1 spreading rapidly and increasing in prevalence among the Enterobacteriaceae where it was identified at rates of 30-50% in *E. coli* by the early 1970s (21). Further drug development, driven primarily by TEM-1, led to the extended-spectrum cephalosporins becoming clinically available by the early 1980s (17). Such

investigations also led to the synthesis of clavulanic acid and the penicillanic acid sulfones, suicide inhibitors active against TEM-1 and other class A β-lactamases, and with the subsequent release of the carbapenems it seemed that β-lactamase-mediated resistance had been overcome (17). However, selection resulting from heavy antibiotic use led to the evolution of SHV- and TEM-derived β-lactamases with "extended" activity against the oxyimino-cephalosporins (17). Furthermore, the various β-lactamases identified has continued to expand beyond SHV- and TEM-, and the current emergence of carbapenem resistance is of great concern.

1.5.2 Mechanism of Action

β-lactamases are structurally related to PBPs and it has been hypothesized that they evolved from these cell wall enzymes (22). As a result, the action of β-lactamases is mechanistically similar to what occurs during cell wall synthesis, where β-lactamases are able to bind, acylate, and hydrolyze the β-lactam ring using a water molecule (14). Similar to the PBPs, β-lactamases are located in the periplasmic space. Here, the enzyme will bind to the drug and in the case of serine β-lactamases, an active site serine residue will attack the β-lactam ring forming an acyl-ester (17). This acyl-ester intermediate is then attacked by a water molecule yielding hydrolysis of the amide bond of the β-lactam ring rendering the drug inactive and restoring the activity of the enzyme (Figure 3) (14, 17). Another group of β-lactamases, known as metallo-β-lactamases (MBLs), are distinctly different from those containing an active site serine. The mechanism of action for the MBLs is more complex and can vary depending on the active site chemistry of a given enzyme (23, 24). While the exact mechanism is beyond the scope of this thesis, the overall process of binding and subsequent hydrolysis remains the same.

FIGURE 3. Mechanism of action for serine β-lactamases. Serine residue of the β-lactamase active site attacks the β-lactam ring forming an acyl-ester; a water molecule then attacks the acyl-ester freeing the β-lactamase and the inactivated β-lactam molecule. Figure reproduced with permission from (25).



1.5.3 Clinical Relevance

In recent years, the burden of antibiotic resistance seemed to center squarely on Gram-positive pathogens, primarily methicillin-resistant *S. aureus* (MRSA) and vancomyin resistant enterococci (VRE). While infections caused by these organisms remain a serious issue, the emerging consensus seems to be that Gram-negative pathogens pose the greatest threat to patient outcome (26). Central to this current paradigm shift is the continued emergence of β-lactamase-mediated resistance among the Enterobacteriaceae. The increasing prevalence of infections caused by such organisms as well as the overall importance of β-lactam antibiotics in clinical practice creates two antagonistic problems. Simply put, resistance threatens our ability to effectively use these antibiotics, meanwhile their use selects for increasing resistance. In cases where empirical treatment is required such as with sepsis, resistance to first-line agents can have significant implications (27, 28). Furthermore, the increasing rate of multidrug resistance among these isolates, especially to the fluoroquinolones, complicates this problem (29). Additional factors contributing toward the weight of this issue are the rate at which resistance appears to be spreading both intra- and interspecies, along with the apparent insufficiency of antibacterial coverage to sustain treatment for the foreseeable future (26).

1.5.4 Classification and Diversity

Similar to the β-lactam antibiotics, β-lactamases can be broadly separated into four groups based on substrate specificities. These four groups include the penicillinases, AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), and the carbapenemases (13). The hydrolytic activity of these four groups is summarized in Table 3. Especially relevant to the Enterobacteriaceae is the current emergence of the ESBLs, able to

hydrolyze the extended-spectrum cephalosporins (e.g. cefotaxime, ceftazidime, ceftriaxone) and monobactams (e.g. aztreonam), and the carbapenemases able to hydrolyze a wide range of β-lactams including the carbapenems (e.g. imipenem, meropenem, ertapenem) (20, 30).

There are two main classification schemes utilized to categorically organize the large number of β-lactamases. The Ambler molecular classification scheme places these enzymes into four groups, namely A-D, based on their corresponding amino acid sequences rather than on phenotypic characteristics (13). In this scheme, groups A, C, and D can all be considered serine β-lactamases based on the presence of an active site serine residue. In contrast to these groups, group B contains MBLs, where the presence of one or more active site zinc (Zn^{2+}) ions is required for substrate hydrolysis (Table 4) (13, 14). Perhaps a more applicable system for clinical use is the Bush-Jacoby-Medeiros scheme as functional characteristics are used in order to group β-lactamases based on their substrate and inhibition profiles (31). This system is composed of four main groups and multiple subgroups. The first group contains cephalosporinases belonging to molecular class C while the second group is made up of serine β-lactamases of molecular class A and D (31). The third group in this system contains carbapenemases belonging to molecular class B and the fourth group is reserved for β-lactamases with unknown molecular characteristics (31). In this system groups 1-3 are divided into subgroups that indicate various characteristics such as extended hydrolysis of oxyimino-cephalosporins or resistance to various β-lactamase inhibitors (e.g. clavulanic acid) (Table 4) (31). For simplicity we will focus on the Ambler classification system in our further discussion of β-lactamases.

The Ambler class A β-lactamases are a large group featuring several notable members. This group contains penicillinases common in Gram-positive organisms such as the PC1 β-lactamase of *S. aureus* (14). More notable in Gram-negative pathogens, in particular the Enterobacteriaceae, are the other members of this class. These include the narrow spectrum β-lactamases TEM-1, TEM-2, and SHV-1, as well various ESBLs including SHV- and TEM-derived variants and the widespread CTX-Ms (13, 32). Other members of class A include the TEM-derived inhibitor resistant β-lactamases (e.g. TEM-30, TEM-51), and numerous carbapenemases including IMI-1, GES-2, and SHV-38 as well as the *Klebsiella pneumoniae* carbapenemases (KPC-) which have now spread globally and are a major source of carbapenem resistance (13, 32).

β-lactamases belonging to molecular class B can all be considered carbapenemases and are inhibited by chelating agents such as EDTA (13). The members of this class have the ability to hydrolyze most β-lactam antibiotics including the carbapenems, as well as the third and fourth generation cephalosporins (e.g. 4th generation - cefepime) (14, 30). Bacteria harboring class B enzymes are among the most resistant organisms identified clinically (14). This class includes members of the VIM-, SPM-, and IMP- β-lactamases, as well as the recently characterized NDM-1.

Ambler class C contains a number of chromosomal and plasmid-mediated extended-spectrum cephalosporinases known as AmpC β-lactamases (14). Similar to the ESBLs of class A, class C enzymes have the ability to hydrolyze oxyimino-cephalosporins and monobactams with the added ability to hydrolyze cephamycins (e.g. cefoxitin, cefotetan) and avoid inhibition by classical β-lactamase inhibitors (e.g. clavulanic acid, tazobactam, sulbactam) (19). The presence of chromosomal AmpCs in

the Enterobacteriaceae is common, as they are found among *E. coli, E. aerogenes, E. cloacae, Morganella morganii, and S. marcesens* (14). The contribution of chromosomal AmpCs toward β-lactam resistance is variable dependent on gene expression (14). Some examples of plasmid-mediated AmpCs include DHA-, ACT-, FOX, and CMY-, with CMY-2 being the predominant AmpC found in Canadian hospitals (29).

Molecular class D is composed of various members of the OXA- family, named for their ability to hydrolyze oxacillin (14). The OXA- enzymes display varying degrees of activity against the β-lactams ranging from penicillinases, to ESBLs and carbapenemases (14). This class of β-lactamases is common among the non-fermenters *Pseudmonas aeruginoas* and *Acinetobacter baumanii* (14). Notable variants relevant to the Enterobacteriaceae include the OXA-1 penicillinase often found in association with various ESBLs, and the recent emergence of the OXA-48 (and point-mutant analog OXA-181) carbapenemase.

TABLE 3. Overview of the substrate specificities associated with the various β-lactamase classes.

	~Propensity of resistance development →						
β-lactam Class:	**Peniciilins**	**Cephalosporins**			**Monobactams**	**Cephalosporins**	**Carbapenems**
		1st generation	*2nd generation*	*3rd generation*		*4th generation*	
Examples:	Amoxicillin	Cefazolin	Cefuroxime	Cefotaxime	Aztreonam	Cefepime	Imipenem
	Ampicillin	Cephalexin	Cefamandole	Ceftriaxone			Ertapenem
	Pipercillin	Cephalothin		Ceftaxidime			Meropenem
	Carbenicillin						
	Ticarcillin						
Broad-Spectrum (e.g. TEM-1/2, SHV-1, OXA-1[a])	[a]OXA-1 also has the ability to hydrolyze the penicillins methicillin and oxacillin						
Extended-Spectrum (SHV- and TEM-derived)							
Extended-Spectrum (CTX-M and AmpC[b], [c])	[b]AmpC β-lactmases have the ability to confer resistance to the cephamycins (cefoxitin and cefotetan) [c]Not influenced by the classical β-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam)						
Carbapenemase[d] (e.g. KPC-, NDM-, VIM-, OXA-48)	[d]variable activity against the monobactams						
	Spectrum of β-lactamase activty →						

TABLE 4. The molecular (Ambler) and Bush-Jacoby-Medeiros classification schemes for β-lactamase enzymes. Modified from (13, 31).

Molecular (Ambler) Class	B-J-M[a] Group	Primary Substrate	Inhibition Profile		Representative Examples
			CA/TZ[b]	EDTA	
A	2a	Penicillins	Yes	No	PC1
	2b	Penicillins, early cephalosporins	Yes	No	TEM-1, TEM-2, SHV-1
	2be	Extended-spectrum cephalosporins, monobactams	Yes	No	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
	2br	Penicillins	No	No	TEM-30, SHV-10
	2ber	Extended-spectrum cephalosporins, monobactams	No	No	TEM-50
	2c	Carbenicillin	Yes	No	PSE-1, CARB-3
	2ce	Carbenicillin, cefepime	Yes	No	RTG-4
	2e	Extended-spectrum cephalosporins	Yes	No	CepA
	2f	Carbapenems	Variable	No	KPC-2, IMI-1, SME-1
B	3a	Carbapenems	No	Yes	IMP-1, VIM-1, CcrA, IND-1, NDM-1
	3b	Carbapenems	No	Yes	CphA, Sfh-1
C	1	Cephalosporins	No	No	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
	1e	Cephalosporins	No	No	GCI, CMY-37
D	2d	Cloxacillin	Variable	No	OXA-1, OXA-10
	2de	Extended-spectrum cephalosporins	Variable	No	OXA-11, OXA-15
	2df	Carbapenems	Variable	No	OXA-23, OXA-48

[a]B-J-M: Bush-Jacoby-Medeiros, [b]CA/TZ: clavulanic acid/tazobactam.

1.6 Extended-spectrum β-lactamases

1.6.1 Introduction: Defining an ESBL

The term ESBL was first used in the mid-1980s to describe derivatives of the TEM-1 and SHV-1 β-lactamases that had gained the ability to hydrolyze the oxyimino-cephalosporins (21). These initial ESBLs came as the result of a single amino acid change, Gly238Ser within the active site of TEM-1 and SHV-1 broadening the enzymes' hydrolytic activity (14). Since these initial reports, the ESBLs have become a large, diverse family with varying characteristics, substrate specificities, and inhibition profiles. Generally, ESBLs belong to functional group 2be and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (33). They are able to confer resistance to the penicillins, oxyimino-cephalosporins, and monobactams, but not the cephamycins or carbapenems, and kinetically are able to hydrolyze oxyimino-cephalosporins or monobactams at rates of at least 10% that of benzylpenicillin (19, 21, 33). While the above provides a working definition for the properties of an ESBL, it should be noted that exceptions do occur. Such exceptions include enzymes that do not rigorously meet hydrolysis criteria, members of functional groups such as 2d, 2e, or 2f that closely resemble group 2be, and enzymes with variable inhibition characteristics (33).

1.6.2 TEM and SHV ESBLs

As mentioned, the earliest ESBL enzymes belonged to the TEM and SHV families. Since their initial appearance, these enzymes have gained a worldwide distribution (20, 34). Both of these families are classified in molecular group A and can be considered structurally similar with SHV-1 sharing 68% of its amino acid sequence

with TEM-1 (32). SHV and TEM are among the largest families of β-lactamases with over 150 and 100 variants recognized, respectively (*http://www.lahey.org/Studies*, Date accessed: June 13, 2013).

SHV and TEM ESBLs can be found most commonly in *K. pneumoniae* and to a lesser extent *E. coli*, as well as in other members of the Enterobacteriaceae (13). Historically organisms producing SHV and TEM ESBLs have been associated with outbreaks within a hospital setting, more specifically within the ICU, and members of these families represented the dominant ESBLs observed in the late 1980s and throughout the 1990s (19, 34). Generally, the diversity of SHV ESBLs arises from fewer amino acid substitutions than is observed with TEM, and currently the most common variants of SHV and TEM ESBLs include SHV-2, -5, -12, and TEM-3, -26, -51 (19, 35).

1.6.3 CTX-M ESBLs

ESBLs of the CTX-M family were first discovered in 1989 in Munich, Germany, and were named for their increased hydrolytic activity against cefotaxime as compared to ceftazidime, though it should be noted this is not always the case (32, 34). In the initial years following their discovery, CTX-M-producing organisms were rarely encountered clinically, as TEM- and SHV-ESBLs remained the dominant enzymes identified (34). Throughout the 1990s CTX-M-ESBLs were mainly associated with the occasional nosocomial outbreak, primarily caused by CTX-M-2-producing Enterobacteriaceae in South America, with the largest outbreaks occurring in Argentina (19). However, since 2000, the molecular epidemiology of ESBL-producing organisms has drastically changed to where CTX-M ESBLs, especially CTX-M-15, have become the dominant family observed throughout the world (30). Moreover, while organisms producing SHV and

TEM ESBLs have typically been confined within a hospital setting, CTX-M-producing organisms have emerged as an important cause of community-onset UTIs and bloodstream infections contributing toward significant increases in the prevalence of ESBL-producing organisms (30).

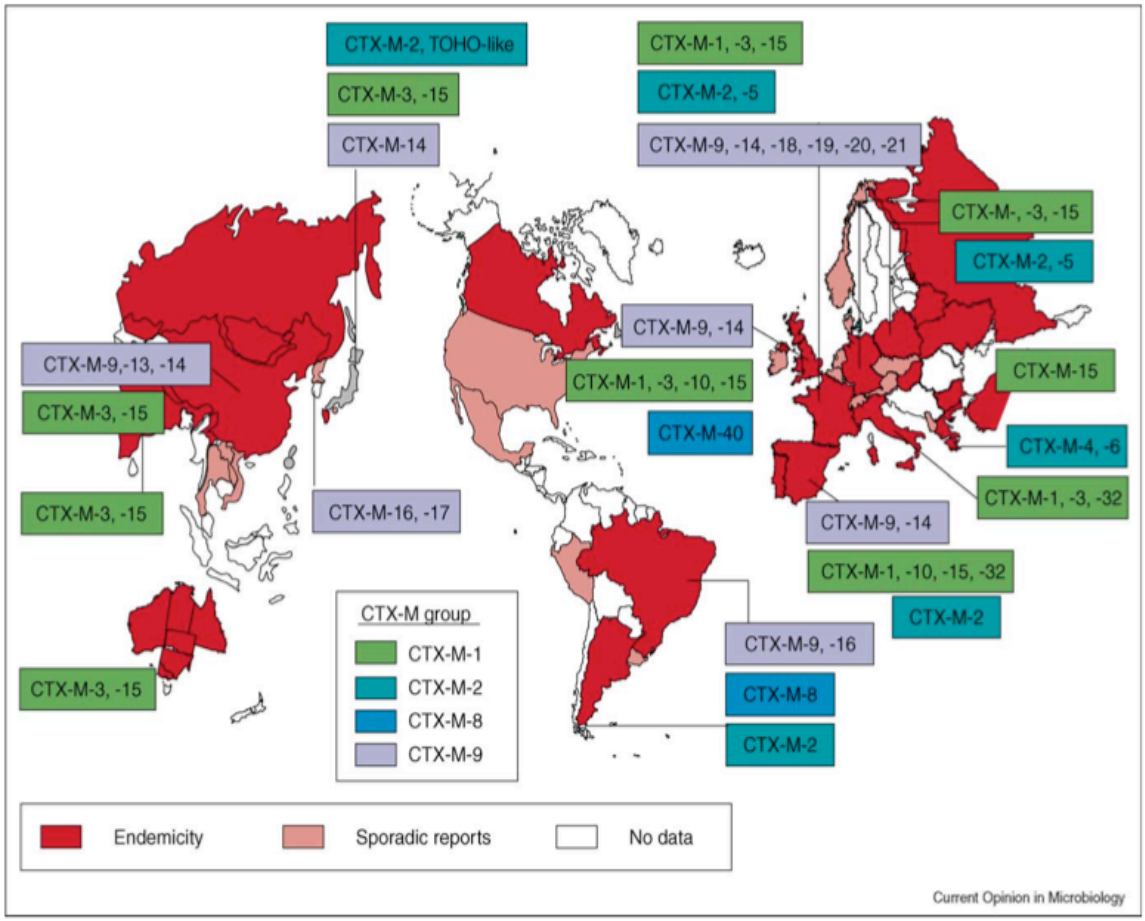
Currently, there are over 100 different CTX-M variants that can be placed into six groups based on amino acid sequence (19). These groups include CTX-M-1, -2, -8, -9, -25, and -45, with the various groups demonstrating >94% amino acid identity within each individual group and ≥90% identity between groups (Table 5) (19, 34). CTX-M β-lactamases are believed to have originated through the mobilization of chromosomal β-lactamases of the environmental organisms *Kluyvera georgiana* and *Kluyvera ascorbata,* due to the high degree of similarity between CTX-M ESBLs and the KLUG-1 and KLUA-1 β-lactamases of these organisms (34). The distribution of CTX-M ESBLs is variable dependent upon geographic region, with CTX-M-1 being common in Italy, CTX-M-2 in Israel and Argentina, CTX-M-3 in Poland, CTX-M-9 in Spain, CTX-M-14 in Spain, Canada and China, while CTX-M-15 is found worldwide (Figure 4) (30).

TABLE 5. CTX-M β-lactamases: Examples and origin. Modified from (36).

	CTX-M Group				
	CTX-M-1	CTX-M-2	CTX-M-8	CTX-M-9	CTX-M-25
Year (enzyme, country)[a]	1989 (CTX-M-1, Germany)	1986 (FEC-1, Japan)	1996 (CTX-M-8, Brazil)	1994 (CTX-M-9, Spain)	2000 (CTX-M-25, Canada)
Examples	CTX-M-1 CTX-M-3 CTX-M-10 CTX-M-11 CTX-M-12 CTX-M-15 CTX-M-22 CTX-M-23 CTX-M-29 CTX-M-30 CTX-M-32 CTX-M-33 CTX-M-28 CTX-M-36 CTX-M-54 UOE-1	CTX-M-2 CTX-M-4 CTX-M-6 CTX-M-7 CTX-M-20 CTX-M-31 CTX-M-44[b] FEC-1	CTX-M-8 CTX-M-40	CTX-M-9 CTX-M-13 CTX-M-14 CTX-M-16 CTX-M-17 CTX-M-18 CTX-M-19 CTX-M-24 CTX-M-27 CTX-M-45[c] CTX-M-46 CTX-M-47 CTX-M-48 CTX-M-49 CTX-M-50	CTX-M CTX-M-26 CTX-M-25 CTX-M-39 CTX-M-41
Origin	*K. ascorbata*	*K. ascorbata*	*K. georgiana*	*K. georgiana*	Unknown

[a]Year of isolation (first enzyme discovered, country of discovery); [b]previously TOHO-1; [c]previously TOHO-2.

FIGURE 4. The global distribution of CTX-M-producing organisms. Figure reproduced with permission from (36).



1.6.3.1 *E. coli* O25:H4- ST131

Much of the global spread and increasing prevalence of CTX-M-15-producing organisms has been attributed to the emergence of a single clone, *E. coli* O25:H4 sequence type 131 (ST-131) (19). *E. coli* ST-131 is a pandemic clone identified by multilocus sequence typing (MLST) and is predominantly associated with community-onset antimicrobial resistant infections (37). This clone represents a unique combination between virulence and antibiotic resistance as it belongs to the highly virulent phylogenetic group B2 while also carrying a MDR IncFII plasmid (19). The occurrence of other β-lactamase genes including $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$ are commonly found along with $bla_{\text{CTX-M-15}}$ on such plasmids, and rates of fluoroquinolone and trimethoprim-sulfamethoxazole resistance are typically quite high among ST-131 isolates attributable to the presence of additional resistance determinants (37).

In one study performed by Johnson *et al*., it was found that 44% of fluoroquinolone resistant *E. coli* isolates causing UTIs in Canada between 2002 and 2004 belonged to ST-131, while among this cohort, cephalosporin susceptibility remained high at 98% (38). Such results provide insight into a possible origin suggesting that ST-131 may have already been prevalent among fluoroquinolone resistant isolates prior to the acquisition of CTX-M-15 (38). An additional study by Peirano *et al*. analyzed all ESBL-producing *E. coli* isolated from blood cultures in the Calgary Health Region from 2000-2010 (39). From 2000-2006, 27% of ESBL-producing *E. coli* were found to be ST-131, while from 2007-2010, 69% of isolates were ST-131, providing further evidence to the importance of ST131 in the changing epidemiologic landscape of ESBL-producing organisms (39).

1.6.3.2 Phylogenetic Group B2 and IncFII Plasmids

Phylogenetic analysis places strains of *E. coli* into one of four main groups, namely A, B1, B2, and D (40). In addition, a link between phylogenetic grouping and virulence has been reported with extraintestinal pathogenic strains belonging primarily to group B2, and in some cases group D (40, 41). The virulence potential of group B2 strains was historically observed to occur at the expense of antibiotic resistance, where previously Johnson *et al.* have reported that B2 strains commonly infect non-immunocompromised hosts, express P fimbriae, hemolysin, and aerobactin determinants, while lacking antimicrobial resistance (42). Interestingly, the majority of ST-131 ESBL-producing *E. coli* have been reported to belong to group B2 (19, 43). It is this fact that makes the ST-131 clone unique, as it represents a combination of phylogenetic group B2 virulence and antibiotic resistance via a MDR IncFII plasmid carrying $bla_{\text{CTX-M-15}}$ (19). In addition to $bla_{\text{CTX-M-15}}$ such IncFII plasmids carry genes encoding $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, *aac(6')-Ib-cr* capable of acetylating various fluoroquinolones including ciprofloxacin, the *tet(A)* tetracycline efflux pump, and the aminoglycoside acetyltransferase *aac(3')-IIa* within a class 1 integron (19, 44). Among ST-131 ESBL-producing *E coli* isolates, IncFII plasmids were found to be closely related at sizes of 85-200 kb (19, 44).

1.6.4 Other ESBLs

While ESBLs belonging to the TEM, SHV, and CTX-M families are the most common, certain variants of the OXA family are also capable of conferring an ESBL phenotype. The contribution of OXA ESBLs toward oxyimino-cephalosporin resistance within the Enterobacteriaceae is limited as the majority of OXA variants poorly hydrolyze the extended-spectrum cephalosporins and those that do are found more

frequently in *P. aeruginosa* and other non-fermenters (33). Some examples of OXA ESBLs include OXA-10, -11, -14, -16, -28, and -31 among others (13, 33). While OXA-1 is not considered an ESBL, it has been found in frequent association with mobile plasmids harboring other ESBL genes, especially in *E. coli* (33, 35).

The enzymes discussed above can be considered the main ESBLs that are most commonly encountered, rather than an all-inclusive list. For completeness, it should be noted that other ESBL families do play a part in the generation of extended-spectrum cephalosporin resistance. These can include variants such as PER-1 and PER-2 which are occasionally found within members of the Enterobacteriaceae, as well as other enzymes including VEB-1, TLA-21, SFO-1, BES-1, and GES-1 (13).

1.7 AmpC β-lactamases

1.7.1 Introduction: Defining an AmpC

AmpC β-lactamases belong to Ambler class C and are capable of conferring resistance to multiple β-lactams similar to what is seen in the case of ESBLs (13). While ESBLs within the Enterobacteriaceae can be primarily considered extra-chromosomal, AmpC β-lactamases are commonly found on both the chromosome and in association with mobile plasmids (13, 45). The spectrum of activity of AmpC enzymes is in many ways broader than what is observed with ESBLs. AmpC β-lactamases have the ability to hydrolyze penicillins, oxyimino-cephalosporins, cephamycins, and monobactams, are not inhibited by the classical β-lactamases inhibitors, and typically maintain susceptibility to the carbapenems (45). Unlike ESBLs however, AmpC β-lactamases are typically poor at hydrolyzing the ureidopenicillins and fourth-generation cephalosporins (e.g. cefepime),

though in the case of cefepime, susceptibility decreases with increasing inoculum size (13, 45).

1.7.2 Chromosomal AmpC β-lactamases

Chromosomal AmpC β-lactamases are widespread within the Enterobacteriaceae and can be found in *E. coli, Citrobacter spp., Enterobacter spp., Serratia spp., M. morganii., Providencia stuartii* and others (13). With the exception of *E. coli*, chromosomal AmpC β-lactamases are inducible by β-lactam exposure (13). Chromosomal AmpC expression in *E. coli* is constitutive, however expression levels are low due to the presence of a weak promoter and strong attenuator (46). Normally expression is too low to allow resistance though mutations within the promoter/attenuator region can lead to hyperproduction, and therefore an AmpC phenotype (46, 47). The contribution of chromosomal AmpC β-lactamases toward overall resistance is variable and the presence of inducible gene expression can be deceptive in a clinical setting as organisms testing susceptible to various β-lactams *in vitro* may in fact be resistant *in vivo* leading to therapeutic failure (45). The induction mechanism for this process can be considered complex and depends on sufficient levels of peptidoglycan breakdown products within the cytoplasm in order to bind to AmpR, the AmpC regulatory protein required for expression (45, 46).

1.7.3 Acquired AmpC β-lactamases

In 1990, Papanicolaou *et al.* published the first report confirming the presence of a plasmid-mediated AmpC β-lactamase (48). Acquired AmpC β-lactamases came as the result of mobilization of the chromosomal AmpC of several different organisms (13). In the majority of cases, acquired AmpC β-lactamases are not inducible due to the loss of

ampR and their contribution toward resistance can normally be attributed to promoter mutations as opposed to the effects of plasmid copy number (13, 45). Multiple families of acquired AmpC β-lactamases have been reported and common examples include CMY, MOX, FOX, DHA, ACT, ACC, and MIR (45). The molecular epidemiology of acquired AmpC genes varies based on geographic region, however in Canadian hospitals, CMY-2 is by far the most frequently encountered (29, 47). In two recent studies by Simner *et al*. and Denisuik *et al.*, it was reported that ~56% of *E. coli* isolates from across Canada demonstrating an AmpC resistance phenotype were found to contain an acquired AmpC β-lactamase gene, of which ~98% were positive for $bla_{\text{CMY-2}}$ (29, 49).

1.8 Carbapenemases

1.8.1 Introduction: Defining a Carbapenemase

The carbapenems represent a critically important group of antibiotics as they are often used as last-line agents in the treatment of severely ill patients or in cases where treatment options are limited due to resistant organisms (50). Among the β-lactam class, the carbapenems demonstrate the broadest spectrum of activity and have the greatest potency against both Gram-positive and Gram-negative pathogens (50). The importance of maintaining the clinical efficacy of the carbapenems is apparent as the prevalence of infections caused by ESBL- and AmpC-producing organisms able to hydrolyze all β-lactams except the carbapenems continues to increase. The first carbapenemase-producing member of the Enterobacteriaceae was identified in 1993 and since then, resistance to the carbapenems, primarily attributed to the production of carbapenemase enzymes, has become a global problem (51). The hydrolytic activity of carbapenemases can be considered variable (Table 6). By definition, all carbapenemases display some

hydrolytic activity against the carbapenems; however this hydrolysis can vary from levels below specified susceptibility breakpoints up to high-level resistance. Such enzymes are also generally able to hydrolyze all β-lactam agents, although this is variable. It should be noted that many carbapenemases are found in association with ESBLs or acquired AmpC β-lactamases therefore enhancing resistance and further challenging treatment (13, 51, 52).

TABLE 6. The hydrolytic and inhibition profile of common carbapenemase variants. Modified from (26, 53-61).

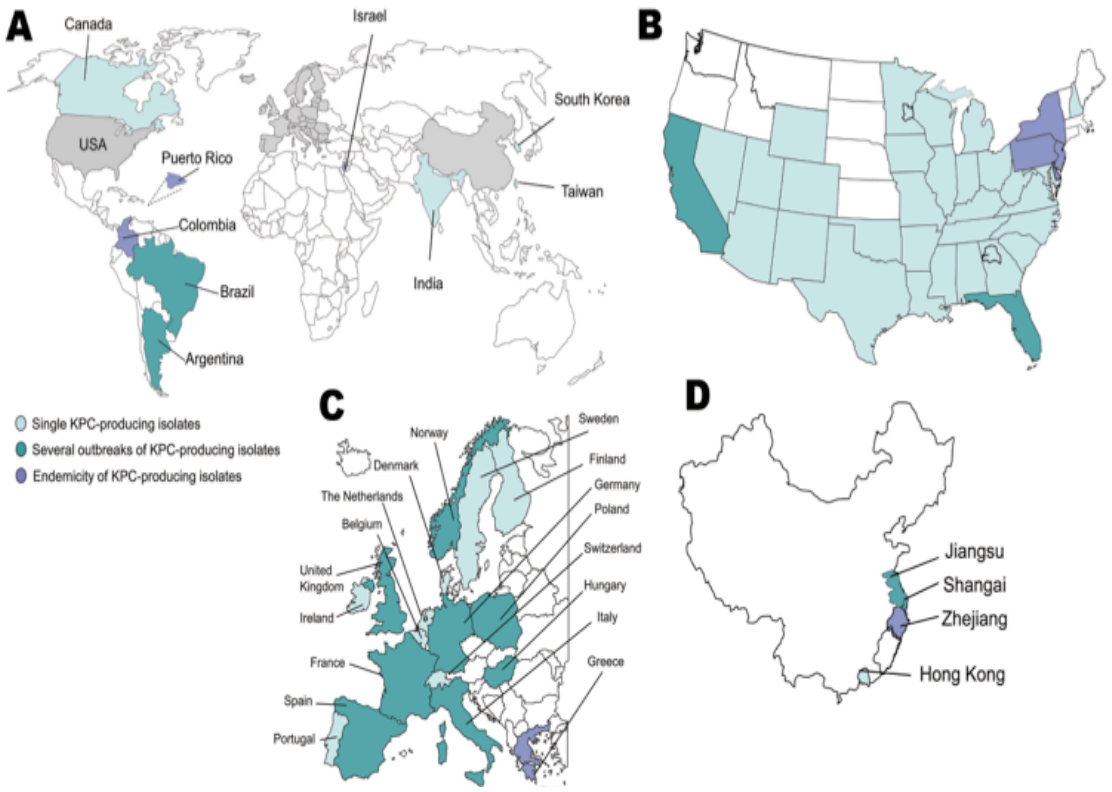
Molecular (Ambler) Class	B-J-M[a] Class	Enzyme	Hydrolytic Profile[b]					Inhibition Profile[c]	
			Penicillins	Early Cephalosporins	Extended-spectrum Cephalosporins	Aztreonam	Carbapenems	EDTA	CA
A	2f	NMC	+	+	+	+	+	-	+
		IMI	+	+	+	+	+	-	+
		SME	+	+	±	+	+	-	+
		KPC	+	+	+	+	+	-	+
		GES	+	+	+	-	±	-	+
B	3	IMP	+	+	+	-	+	+	-
		VIM	+	+	+	-	+	+	-
		GIM	+	+	+	-	+	+	-
		SPM	+	+	+	-	+	+	-
		NDM	+	+	+	-	+	+	-
D	2d	OXA	+	+	±	-	±	-	±

[a]B-J-M: Bush-Jacoby-Medeiros; [b]+:strong hydrolysis, ±: weak hydrolysis, -: no measurable hydrolysis; [c]+: strong inhibition, ±: weak inhibition, -: no measurable inhibition, CA: clavulanic acid.

1.8.2 Molecular Class A

Molecular class A contains a number of carbapenemases including chromosomal variants such as Nmc-A, Sme, IMI-1, and SFC-1, as well as variety of plasmid-mediated variants such as KPC-, IMI-2, and GES- (51). Carbapenemases of molecular class A are all at least partially inhibited by clavulanic acid. The most notable and common enzymes of this group are the KPCs (62). The first report of a KPC enzyme in a clinical setting came from North Carolina in 2001 and was subsequently followed by reports of a single amino acid variant KPC-2 from the eastern United States (63). KPCs are most frequently associated with *K. pneumoniae* but are also found to a lesser extent in *E. coli* and other members of the Enterobacteriaceae (52). KPC-producing organisms are commonly associated with nosocomial infections, though less frequent, community-acquired infections due to KPC-producers are on the rise (52). Among KPC-producing *K. pneumoniae,* a single clone known as ST-258 has been identified worldwide pointing to its possible role in the dissemination of bla_{KPC} (52, 62). As a result of decreased treatment options and delayed administration of effective therapy, mortality rates associated with KPC-producing infections are high at >50% (51). The global distribution and approximate prevalence of KPC-producing Enterobacteriaceae is summarized in Figure 5.

FIGURE 5. The global distribution of KPC-producing Enterobacteriaceae. Figure reproduced with permission from (51).



A: worldwide geographic distribution of KPC-producing *K. pneumoniae*, regions in grey are shown separately; B: distribution in the United States; C: distribution in Europe; D: distribution in China.

1.8.3 Molecular Class B

Carbapenemases belonging to molecular class B can all be considered MBLs (or metallo-carbapenemases) and are therefore inhibited by chelating agents rather than by clavulanic acid. Metallo-carbapenemases generally confer high-level resistance to all β-lactams except aztreonam; however, the frequent association of class B carbapenemases with ESBLs limits the clinical efficacy of the monobactams in this case (51). The most common class B carbapenemases are the VIM, IMP, and NDM types with MBLs of the VIM and IMP types being found endemically in Greece, Taiwan, and Japan (51).

The most notable carbapenemase of molecular class B is the recently characterized **N**ew **D**elhi **M**etallo-1 (NDM-1). NDM-1 was first characterized in 2008 from a patient in Sweden who had recently been hospitalized in New Delhi, India, with a *K. pneumoniae* UTI found to harbor a novel metallo-β-lactamase (64). Clinical isolates producing NDM-1 typically contain multiple resistance genes including the nearly ubiquitous occurrence of a 16S rRNA methylase gene conferring high-level resistance to the aminoglycosides (51). In many cases, the only remaining treatment options for such infections are tigecycline, colistin, and fosfomycin, all of which are not ideal and have limited use based on certain clinical situations (51, 65). Further concern surrounding NDM-1 stems from the apparent dissemination of the gene among multiple species as well as the potential role of the environment in dissemination (26, 51). The global distribution of NDM-producing Enterobacteriaceae is summarized in Figure 6.

FIGURE 6. The global distribution of NDM-1-producing Enterobacteriaceae. Figure reproduced with permission from (51)[a,b,c].

[a]Geographic distribution is representative of July 15, 2011; [b]star size is indicative of number of cases that have been reported; [c]red stars represent cases traced back to India, Pakistan, or Bangladesh, green stars indicate cases traced back to the Balkan states or the Middle East, and black stars indicate cases of unknown origin.

1.8.4 Molecular Class D

The number of class D β-lactamases demonstrating carbapenemase activity is limited with the majority being found in *A. baumanii* (13). The most relevant class D carbapenemase among the Enterobacteriaceae is OXA-48 and to a lesser extent the point mutant analog OXA-181 (51). Class D carbapenemases will generally yield weak hydrolysis of the carbapenems often below susceptibility breakpoints; however, the enzymes do in fact contribute toward resistance and treatment failure *in vivo* through the synergistic effect of β-lactamase action and cell wall impermeability (51). Though somewhat limited as compared to other carbapenemases, the geographic distribution of OXA-48-producing Enterobacteriaceae is currently expanding. Outbreaks associated with OXA-48 have been frequently reported from Turkey and reports from other parts of Europe, Asia, and Africa are becoming more common (51).

2. HYPOTHESES

(A) Oxyimino-cephalosporin resistance among Canadian *E. coli* and *K. pneumoniae* isolates will primarily be attributable to ESBL or AmpC production.

(B) The prevalence of ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae* will increase in Canadian hospitals.

(C) ESBL-producing organisms will frequently display a MDR phenotype.

(D) CTX-M-15 will continue to be the dominant ESBL among clinical *E. coli* and *K. pneumoniae* isolates collected from Canadian hospitals.

(E) The ST-131 clone will be prevalent among CTX-M-15-producing *E. coli* and will be an important factor in the increase of ESBL-producing *E. coli* in Canadian hospitals over time.

(F) The prevalence of AmpC-producing *E. coli* will increase in Canadian hospitals with CMY-2 representing the major contributor of acquired AmpC resistance among clinical *E. coli* isolates.

3. SCIENTIFIC RATIONALE AND OBJECTIVES

Members of the Enterobacteriaceae, including *Escherichia coli* and *Klebsiella pneumoniae* are among the top ranked pathogens causing bacterial disease in Canadian hospitals. Infections caused by β-lactamase-producing Enterobacteriaceae hold serious implications for both public health and infection control practices. Such infections are associated with delays in the administration of effective therapy, as β-lactam resistance often undermines empiric treatment regimens. As a result patients are subject to increased length of hospital stay, increased hospital cost, as well as an elevated risk of infection-related mortality. Furthermore, β-lactamase-producing Enterobacteriaceae have demonstrated an overwhelming ability to both disseminate and persist within a hospital and community setting, and their frequent association with MDR makes the study of such organisms essential to the improvement of patient care, ensuring effective implementation of antibiotic chemotherapy, and the continued expansion of our current armamentarium.

The CANWARD study is a national, ongoing multi-centre surveillance study evaluating the distribution of pathogens causing infectious disease in Canadian hospitals and the associated patterns of antibiotic resistance. The primary objective of this thesis was to assess the prevalence of ESBL-, AmpC-, and carbapenemase-producing *E. coli* and *K. pneumoniae* in Canadian hospitals. In order to address this primary objective, a nationally representative cohort of clinical *E. coli* and *K. pneumoniae* isolates was collected via the CANWARD study, which includes centres in eight of the 10 Canadian provinces (Newfoundland/Labrador and Prince Edward Island are not represented).

Secondly, we sought to characterize the patterns of antimicrobial susceptibility and molecular epidemiology of these organisms. Molecular characterization was used to evaluate the genetic composition of β-lactamase-producing *E. coli* and *K. pneumoniae* in Canada through time.

4. MATERIALS AND METHODS

4.1 Bacterial Isolates

Bacterial isolates were collected as part of the ongoing CANWARD Surveillance study from January 2007 to December 2012. The CANWARD study receives annual approval by the University of Manitoba Research Ethics Board (H2009:059).

4.1.1 CANWARD Surveillance Study

The CANWARD study is a national, ongoing multi-centre surveillance study evaluating the *in vitro* activity of commonly used and investigational antimicrobial agents against bacterial pathogens isolated from Canadian hospitals (3, 66). From January 2007 to December 2012, tertiary-care medical centres (12 in 2007, 10 in 2008, 15 in 2009, 14 in 2010, 15 in 2011, and 12 in 2012) submitted clinically relevant isolates (consecutive, one per patient per infection site) from in- and outpatients attending hospital clinics, medical and surgical wards, emergency rooms, and ICUs with blood, urine, wound, and respiratory tract infections. Each year, centres submitted 100 to 200 respiratory specimen isolates (2007: 200; 2008: 150; 2009-2012: 100), 25 to 50 wound specimen isolates (2007-2010: 50; 2011-2012: 25), 25 to 100 urine specimen isolates (2007-2008: 100; 2009-2010: 50; 2011-2012: 25), and 10/month to 30/month bloodstream infection isolates (2007: 30/month; 2008: 20/month; 2009-2010: 15/month, 2011-2012: 10/month).

Bacterial isolates submitted to the CANWARD study were deemed clinically relevant and identified by routine methods at the originating site. Isolates were shipped to the Health Sciences Centre (Winnipeg, MB) on Amies charcoal swabs. Upon receipt, all swabs were planted using the appropriate media, subcultured, and then stored in skim milk at -80^{o}C.

TABLE 7. Participating medical centres of the CANWARD Study: 2007-2012.

City, Province Centre	Investigator	CANWARD Study Year					
		'07	'08	'09	'10	'11	'12
Vancouver, BC							
Vancouver General Hospital†	Dr. D. Roscoe	✓	✓	✓	✓	✓	✓
Edmonton, AB							
University of Alberta Hospital†	Dr. J. Fuller/ Dr. R. Rennie	✓	✓	✓	✓	✓	✓
Saskatoon, SK							
Royal University Hospital†	Dr. J. Blondeau	✓	✓	✓	✓	✓	✓
Winnipeg, MB							
Health Sciences Centre†	Dr. D. Hoban/ Dr. G. Zhanel	✓	✓	✓	✓	✓	✓
Hamilton, ON							
St. Joseph's Hospital	Dr. C. Lee	✓	✗	✗	✗	✗	✗
London, ON							
London Health Sciences Centre†	Dr. Z. Hussain	✓	✓	✓	✓	✓	✓
Ottawa, ON							
The Ottawa Hospital	Dr. M. Desjardins	✗	✗	✓	✗	✓	✗
Children's Hospital of Eastern ON†	Dr. F. Chan	✓	✓	✓	✓	✓	✓
Toronto, ON							
Mount Sinai Hospital†	Dr. S. Poutanen	✓	✓	✓	✓	✓	✓
St. Michael's Hospital	Dr. L. Matukas	✗	✗	✓	✓	✓	✓
Montreal, QC							
Hôpital Maisonneuve-Rosemont†	Dr. M. Laverdière	✓	✓	✓	✓	✓	✓
Montreal General Hospital	Dr. V. Loo	✓	✓	✓	✓	✓	✗
Royal Victoria Hospital	Dr. V. Loo	✓	✗	✓	✓	✓	✗
Trois-Rivières, QC							
CHRTR Pavilion Ste. Marie	Dr. M. Goyette	✗	✗	✓	✓	✓	✓
Moncton, NB							
South East Regional Health Authority	Dr. M. Kuhn	✗	✗	✓	✓	✓	✓
Halifax, NS							
Queen Elizabeth II HSC†	Dr. R. Davidson	✓	✓	✓	✓	✓	✓

†: Centre has participated in all study years (2007-2012).

4.2 Antimicrobial Susceptibility Testing (AST)

4.2.1 Antibiotic Preparation

Antibiotic stock solutions were prepared in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines using laboratory grade powders (67). The antimicrobial activity of these solutions was confirmed by the macrodilution broth method as described by CLSI using at least two of the following quality control strains, as appropriated for the antimicrobial agent: *E. coli* ATCC® 25922, *Staphylococcus aureus* ATCC® 29213, *Enterococcus faecalis* ATCC® 29212, and *Pseudomonas aeruginosa* ATCC® 27853 (68).

4.2.2 Broth Microdilution

Following two subcultures from frozen stock on 5%-SBA, the activity of amoxicillin/clavulanic acid, cefazolin, cefoxitin, ceftriaxone, ceftazidime, cefepime, piperacillin/tazobactam, ertapenem, meropenem, ciprofloxacin, amikacin, gentamicin, tigecycline, trimethoprim-sulfamethoxazole, nitrofurantoin, and colistin was determined by the broth microdilution method as described by CLSI M07-A9 guidelines (67). Minimum inhibitory concentrations (MICs) were determined in duplicate using in-house prepared, custom designed, 96-well microtitre panels. The concentration of tazobactam was kept at a constant of 4 μg/ml, while clavulanic acid concentrations were equal to 50% that of amoxicillin (67).

4.2.3 Agar Dilution

Following two subcultures from frozen stock on 5%-SBA, the activity of fosfomycin tromethamine was tested by the agar dilution method using Mueller-Hinton

agar (MHA) plates supplemented with 25 µg/ml of glucose-6-phosphate as described by CLSI M07-A9 (67).

4.2.4 MIC Interpretive Criteria

MIC interpretive standards were defined by CLSI M100-S22 (2012) breakpoints (68). Food and Drug Administration interpretative criteria have been used for colistin (susceptible: ≤2 µg/ml, resistant: ≥4 µg/ml) and tigecycline (susceptible: ≤2 µg/ml, intermediate: 4 µg/ml, resistant: ≥8 µg/ml). MDR was defined as resistance to ≥3 different antimicrobial classes and XDR was defined as resistance to ≥5 different antimicrobial classes, as described by Magiorakos *et al* (69).

4.3 Phenotypic Characterization Methods

4.3.1 Screening Criteria for ESBL- and AmpC-producers

Any *E. coli* or *K. pneumoniae* isolate collected as part of the CANWARD study found to have a ceftriaxone and/or ceftazidime and/or aztreonam MIC of ≥1 µg/ml was classified as a putative ESBL-producer, and underwent phenotypic testing via the CLSI confirmatory disk test (68). An AmpC phenotype was defined in this study as any *E. coli* isolate demonstrating a ceftriaxone and/or ceftazidime and/or aztreonam MIC of ≥1 µg/ml as well as a cefoxitin MIC of ≥32 µg/ml that was negative for ESBL-production via the CLSI confirmatory disk test (29, 49, 70).

4.3.2 CLSI Confirmatory Disk Test

All putative ESBL-producing *E. coli* and *K. pneumoniae* were assessed by the CLSI confirmatory disk test to confirm the presence of an ESBL phenotype (68). For each isolate meeting the above-described criteria, a 0.5 McFarland standard (Remel, Lenexa Kansas) was prepared by the direct colony suspension method. A sterile cotton

swab was then placed into this adjusted suspension and the excess liquid was removed by wringing the swab against the side of the tube. This swab was then used to inoculate the surface of two MHA plates by streaking the swab over the entire surface in three different directions as well as the outer rim of the plate. Once dry, antimicrobial disks were applied to both plates (two disks per plate) ensuring adequate spacing between disks. On the first plate, disks containing 30 µg of cefotaxime, and 30/10 µg cefotaxime-clavulanic acid were applied, while the second plate received those containing 30 µg ceftazidime, and 30/10 µg ceftazidime-clavulanic acid. Plates were then incubated at 35±2^{o}C in ambient air for 16 to 18 hours and the zones of inhibition surrounding each respective disk were measured. A positive result is interpreted as a ≥5 mm increase in zone diameter for at least one of the two agents tested (cefotaxime and ceftazidime) in combination with clavulanic acid as compared to that agent alone. Quality control strains *E. coli* ATCC® 25922 (ESBL-negative) and *K. pneumoniae* ATCC® 700603 (ESBL-positive) were run in parallel with all tests. A valid result for *E. coli* ATCC® 25922 is defined as a ≤2mm increase in zone diameter for both cefotaxime-clavulanic acid and ceftazidime-clavulanic acid when compared to cefotaxime and ceftazidime, respectively. A valid test for *K. pneumoniae* ATCC® 700603 is defined as a ≥5 mm increase in zone diameter for ceftazidime-clavulanic acid when compared to ceftazidime alone, and a ≥3mm increase in zone diameter for cefotaxime-clavulanic acid when compared to cefotaxime alone.

4.3.3 Screening Criteria for Carbapenemase-production

Any *E. coli* or *K. pneumoniae* isolate collected as part of the CANWARD study found to have an ertapenem and/or meropenem MIC ≥0.5 µg/ml was further analyzed for

carbapenemase production by polymerase chain reaction (PCR) and sequencing as described below (49).

4.4 Genotypic Characterization Methods

4.4.1 Lysate Preparation

Bacterial DNA extraction for use in PCR-based methods was carried out by the boil preparation method. Bacterial isolates were subcultured from frozen stock on Trypticase soy agar plates with 5% sheep blood (5%-SBA), streaking for isolation. Once purity had been ensured, 1-3 isolated colonies were suspended with a sterile loop in 100 µl of sterile distilled H_2O (dH_2O) and vortexed. Samples were then boiled for 20 minutes at 90-100^oC to allow for cell lysis and DNA extraction. All lysates were stored at -20^oC.

4.4.2 PCR

PCR was used in this study for the detection of a variety of genes including those encoding resistance determinants, virulence factors, as well as other molecular markers used in the typing of isolates. In all cases, appropriate positive and negative controls were run for quality control purposes. Positive controls consisted of bacterial DNA containing the gene (or genes) of interest, while the negative control varied by reaction and was comprised of either bacterial DNA lacking the gene of interest or sterile dH_2O containing no DNA. Controls were run in parallel each time tests were conducted. For a given set of tests to be deemed viable, all control isolates were required to give the appropriate result.

4.4.3 Agarose Gel Electrophoresis

The results of PCR-based tests were determined by agarose gel electrophoresis using 1.5-2% gels. Gels were prepared by dissolving 1.5-2 g (1.5%: 1.5 g, 2%: 2 g) of

agarose (Invitrogen, Carlsbad, California) in 100 ml of 0.5X Tris-Borate-EDTA buffer (TBE; 45mM Tris-borate, 1mM EDTA [pH 8.3± 0.1]). This solution was brought to a boil and allowed to cool to ~50-55^{o}C with intermittent swirling. Once cool, 1 µl of ethidium bromide was added and the solution was poured into a casting tray with combs. The gel was then allowed to cool at room temperature for 30-45 minutes or at 4^{o}C for 15 minutes. Agarose gels were stored for a maximum of 24 hours at 4^{o}C prior to use.

Once prepared, gels were placed into an electrophoresis chamber containing 0.5X TBE buffer, and 10 µl of each PCR product was loaded into the wells. If Colorless GoTaq® Flexi Buffer (Invitrogen) was used, PCR products were mixed with 2 µl of Ficoll dye prior to loading, while in the case of Green GoTaq® Flexi Buffer (Invitrogen), no loading dye was required. A 123 bp molecular size standard (Invitrogen) was added to the first and last well of any row of wells, and gels were run at 100 V/cm for 35 to 60 minutes. All gels were visualized using the AlphaImager HP (Alpha Innotech, Santa Clara, California).

4.4.4 DNA Sequencing

4.4.4.1 Purification and Quantification of DNA Content for Sequencing

PCR products were purified using Amicon® Ultra Centrifugal Filter Units (Millipore Corporation, Etobicoke, Ontario). An Amicon® filter was first placed into a collection tube, followed by the addition of approximately 40 µl of PCR product to the filter. Next, the assembly was centrifuged at 11,500 revolutions per minute (RPM) (14,000 x g) for five minutes in an Eppendorf 5417C Microcentrifuge (Eppendorf, Hamburg, Germany), 500 µl of 1X Tris-EDTA (TE; 10mM Tris, 1mM EDTA [pH 8.0]) buffer was then added and the centrifugation step repeated (11,500 RPM (14,000 x g) for

five minutes). The filter was then removed from the collection tube, inverted into a new collection tube, and centrifuged at 3,100 RPM (1,000 x g) for two minutes to collect DNA. Purified DNA was stored at -20°C. The DNA content of each purified PCR product was determined using an Ultrospec 2100 Pro Spectrophotometer (Amersham Biosciences, Baie d'Urfe, Quebec) and UVette® cuvettes (Eppendorf) prior to sequencing. The concentration of DNA in each sample was determined according to the principle that 50 µg/ml of DNA has an optical density of 1 at 260 nm.

4.4.4.2 Sequencing Reaction

Sequencing reactions were performed using the ABI Prism® BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Purified DNA template was diluted to a concentration of ~50 ng/µl using sterile, nuclease free dH_2O. Each sequencing reaction was performed in a total volume of 10 µl and contained 2 µl of premix solution (Applied Biosystems), 2 µl of 5X sequencing buffer (Applied Biosystems), 1.6 µl of 0.1 µM primer, 1 µl of diluted template DNA, and 3.4 µl of dH_2O. Amplification was performed on a Perkin-Elmer 9700 Gene Amp® PCR system (Invitrogen) with the following cycling parameters: 25 cycles of 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), and 60°C for 4 minutes (extension).

4.4.4.3 Purification of Sequencing Products

Sequencing products were purified by ethanol/sodium acetate precipitation. All 10 µl of each sequencing reaction was added to 25 µl of 95% ethanol and 1 µl of 3M sodium acetate (pH 4.6) in a 0.6 ml tube and vortexed. Samples were then left in the dark at room temperature for at least 45 minutes and no more than 24 hours. Next, samples

were centrifuged for 20 minutes at 13,000 RPM (18,000 x g) to pellet DNA. The supernatant was then carefully aspirated, and the pellet was rinsed with 125 µl of 70% ethanol. Following a brief vortex, each sample was then centrifuged for five minutes at 13,000 RPM (18,000 x g). The supernatant was again removed carefully and samples were dried in a dry bath at 90^{o}C for one minute. Samples were then carefully inspected to ensure all traces of ethanol had been removed and the pellet was resuspended in 16 µl of Hi-Di™ Formamide (Applied Biosystems). Samples were then loaded directly onto 96-well sequencing plates for processing in the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Sequencing was run on the ABI PRISM® 3100 Genetic Analyzer according to the manufacturer's instructions.

4.4.4.4 Sequence Analysis

Sequence analysis was performed using the Lasergene Core Suite v8.1 (DNA Star, Madison, Wisconsin) software. Sequences were aligned and assembled using the SeqMan Pro (DNA Star) program, exported, and the EditSeq (DNA Star) program was then used to highlight the correct open reading frame (ORF). Reference sequences were selected from *www.lahey.org/studies* and all sequences of a given family were aligned with a reference in the MegAlign (DNA Star) program to ensure proper reading frame.

4.4.4.5 Sequence Identification

A blastx search was done on all ESBL, AmpC, and carbapenemase sequences using the Basic Local Alignment Search Tool (BLAST) (*blast.ncbi.nlm.nih.gov/Blast.cgi*). A positive identification was recorded only in cases of 100% amino acid identity. In cases where this was not obtained, the sequence of the new

variant was submitted to *www.lahey.org/studies* for cataloguing as well as to GenBank (*www.ncbi.nlm.nih.gov/genbank*).

4.4.5 Characterization of ESBL Genes

Primer sequences used for the PCR identification and sequencing of ESBL genes are listed in Table 8. All phenotypically confirmed ESBL-producing isolates were characterized by PCR and sequencing for the detection of genes encoding bla_{TEM} (71), bla_{SHV} (72), $bla_{CTX\text{-}M}$ (73), and bla_{OXA} (74). All isolates producing a positive result with primer set CTX-M-U1/CTX-M-U2 were further analyzed by PCR reactions specific for CTX-M genes of group 1 (CTX-M-1A/CTX-M-1B), group 2 (TOHO-1-1/TOHO-1-2), and group 9 (TOHO-2-1/TOHO-2-2) (73). Reactions were conducted in a total volume of 50 µl using 10 µl of 5X Colorless GoTaq® Flexi Buffer, 3 µl of 25mM $MgCl_2$, 1 µl 10mM dNTP mixture, and 0.5 µl GoTaq® Flexi DNA Polymerase (Promega, Madison, Wisconsin) as well as 0.5 µl of each primer (forward and reverse), and 10 µl of bacterial lysate. All products obtained were purified and sequenced as described in the previous sections. Positive control isolates were provided by the CANWARD and the Canadian Intensive Care Unit (CAN-ICU) surveillance studies. These included: *E. coli* 59096 ($bla_{CTX\text{-}M\text{-}15}$-positive: CTX-M-U1/CTX-M-U2, CTX-M-1A/CTX-M-1B), *E. coli* 62175 ($bla_{CTX\text{-}M\text{-}14}$-positive, $bla_{TEM\text{-}1}$-positive: TOHO-2-1/TOHO-2-2, TEM-1/TEM-2), *E. coli* 64539 ($bla_{CTX\text{-}M\text{-}2}$-positive: TOHO-1-1/TOHO-1-2), *E. coli* 77713 ($bla_{OXA\text{-}1}$-positive: OXA-1F/OXA-1R), and *E. coli* 80942 ($bla_{SHV\text{-}2a}$-positive: SHV-up/SHV-lo).

TABLE 8. Primers used for the identification and sequencing of ESBL genes in phenotypically confirmed isolates.

ESBL Gene	Primer Characteristics		
Primer	Primer Sequence	Size (bp)	Ref.
TEM			
TEM-1†	5′-ATAAAATTCTTGAAGAC-3′	1079	(71)
TEM-2†	5′-TTACCAATGCTTAATCA-3′		
TEM-B*	5′-AAAACTCTCAAGGATCTT-3′		
TEM-C*	5′-AAAGATGCTGAAGATCA-3′		
TEM-D*	5′-TTTGGTATGGCTTCATTC-3′		
TEM-F*	5′-TTTTTTGCACAACATGGG-3′		
SHV			
SHV-up†	5′-CGCCGGGTTATTCTTATTTGTCGC-3′	1016	(72)
SHV-lo†	5′-TCTTTCCGATGCCGCCGCCAGTCA-3′		
SHV-sooA*	5′-CTTTACTCGCCTTTATCG-3′		(75)
SHV-sooB*	5′-TCCCGCAGATAAATCACCA-3′		
CTX-M			
CTXMU1	5′-ATGTGCAGYACCAGTAARGTKATGGC- 3′	593	(73)
CTXMU2	5′-TGGGRRAARTARGTSACCAGAAYCAGCGG-3′		
CTX-M-1 Group			
CTXM1A†	5′-TGGTTAAAAAATCACTGCG-3′	876	(73)
CTXM1B†	5′-ATTACAAACCGTCGGTGAC-3′		
CTXM1C*	5′-ATAACGRGGCGATGAATAAG-3′		
CTXM1D*	5′-ATTCATCGCCACGTTATCG-3′		
CTX-M-2 Group			
TOHO-1-1	5′-ACTCAGAGCATTCGCCGCTCA-3′	879	(73)
TOHO-1-2	5′-TTATTGCATCAGAAACCGTG-3′		
CTX-M-9 Group			
TOHO-2-1†	5′-ATGGTGACAAAGAGAGTGCAACG-3′	837	(73)
TOHO-2-2†	5′-ACAGCCCTTCGGCGATGATTC-3′		
TOHO-2-3*	5′-CGATCGGCGATGAGACGTTT-3′		
TOHO-2-3*	5′-ACGTCTCATCGCCCGATCGC-3′		
OXA-1/4/10			
OXA-1F	5′-ACACAATACATATCAACTTCGC-3′	813	(74)
OXA-1R	5′-AGTGTGTTTAGAATGGTGATC-3′		

†: primer used for initial PCR screening; *: additional primer used for sequencing purposes.

4.4.6 Characterization of AmpC β-lactamase Genes

Primer sequences used for the PCR identification and sequencing of AmpC genes are listed in Table 9. Putative AmpC-producing *E. coli* were screened by PCR and sequencing for the presence of bla_{ENT}, bla_{DHA}, bla_{FOX}, and bla_{CIT} acquired AmpC β-lactamase enzymes as previously described (70). Those isolates producing a positive result with primers CIT-A/CIT-B were further amplified with primer set CMY-2-1/CMY-2-2 in order to identify bla_{CMY-2}. Sequencing reactions were carried out on bla_{CMY-2}-producing isolates using primers CMY-2-1, CMY-2-2, CMY-2-4, and CMY-2-5. Sequencing for the detection of promoter/attenuator mutations within the chromosomal *ampC* gene was carried out on any isolate that was PCR-negative for all acquired AmpC β-lactamases listed above, as previously described (76). The *E. coli* K-12 *ampC* promoter/attenuator sequence (accession number: U00096) was used for comparison of obtained sequences. Reactions were conducted in a total volume of 50 µl using 10 µl of 5X Colorless GoTaq® Flexi Buffer, 3 µl of 25mM $MgCl_2$, 1 µl 10mM dNTP mixture, and 0.5 µl GoTaq® Flexi DNA Polymerase (Promega, Madison, Wisconsin) as well as 0.5 µl of each primer (forward and reverse), and 10 µl of bacterial lysate. Positive controls were kindly provided by Dr. Michael Mulvey and the National Microbiology Laboratory. These included: *K. pneumoniae* N07-1535 (bla_{DHA}-positive: DHA-1/DHA-2), *K. pneumoniae* ESBL-199 (bla_{FOX}-positive: FOX-A/FOX-B), *E. coli* N02-080 (bla_{CMY-2}-positive: CIT-A/CIT-B, CMY-2-1/CMY-2-2), and *Enterobacter cloacae* N03-0087 (bla_{ENT}-positive: ENT-A/ENT-B).

TABLE 9. Primers used for the identification and sequencing of acquired and chromosomal AmpC β-lactamase genes in phenotypically confirmed isolates.

AmpC Gene	Primer Characteristics		
Primer	Primer Sequence	Size (bp)	Ref.
DHA, DHA-related genes			
DHA-1	5′-TTCTGCCGCTGATAATGTCGC-3′	1047	(70)
DHA-2	5′GGCTTTGACTCTTTCGGTATTC 3′		
ENT, ACT-1/MIR-1-related genes			
ENT-A	5′-AGTAAAACCTTCACCTTCACCG-3′	405	(70)
ENT-B	5′-ATGCGCCTCTTCCGCTTTC-3′		
FOX, FOX-related genes			
FOX-A	5′-TGTGGACGGCATTATCCAG-3′	877	(70)
FOX-B	5′-AAAGCGCGTAACCGGATTG-3′		
CIT, CMY-related genes			
CIT-A	5′-ATGCAGGAGCAGGCTATTC-3′	686	(70)
CIT-B	5′-TGGAGCGTTTTCTCCTGAAC-3′		
CMY-2			
CMY-2-1	5′-ACACTGATTGCGTCTGACG-3′		(N/A[a])
CMY-2-2	5′-AATATCCTGGGCCTCATCG-3′		
CMY-2-4	5′-TGCAACCATTAAAACTGGC-3′		
CMY-2-5	5′-TTGCTTTTAATTACGGAAC-3′		
ampC			
ampC1	5′-AATGGGTTTTCTACGGTCTG-3′	191	(76)
ampC2	5′-GGGCAGCAAATGTGGAGCAA-3′		

[a]N/A: not applicable (unpublished).

4.4.7 Characterization of Carbapenemase-producing Enterobacteriaceae

Primer sequences used for the PCR identification and sequencing of carbapenemase genes are listed in Table 10. All putative carbapenemase-producing *E. coli* and *K. pneumoniae* were screened for the presence of bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{GES}, and bla_{OXA-48} by multiplex PCR as described by Mataseje *et al.* (77, 78). Isolates found to be PCR-positive for bla_{KPC} were reamplified using the primers KPC-F and KPC-R to yield a 1000bp product, as described by Yigit *et al* (63). Sequencing was carried out using the primers KPC-F, KPC-R, KPC1, and KPC2. Multiplex PCR was carried out in a total volume of 25 µl and included 12.5 µl of Qiagen Multiplex PCR Kit (Qiagen Inc., Mississauga, Ontario), 0.5 µl of each IMP primer, 0.25 µl of each KPC, VIM, NDM, and GES primer, 0.125 µl of each OXA-48 primer, 6.75 µl of dH_2O, and 2.5 µl of bacterial lysate. Positive control isolates were kindly provided by Dr. Johann Pitout of the University of Calgary, and by International Health Management Associates Inc. (IHMA). These included: *K. pneumoniae* Kp1 (bla_{KPC}-positive: KPC1/KPC2), *P. aeruginosa* NTU-92/99 (bla_{IMP}-positive: IMP1/IMP2), *P. aeruginosa* PS-679/00 (bla_{VIM}-positive: VIM1/VIM2), *E. coli* MH01 (bla_{NDM}-positive: NDM-F/NDM-R), *K. pneumoniae Kp0R1* (bla_{GES}-positive: GES-2/GES-3), and *K. pneumoniae* Kp1514 (bla_{OXA-48}-positive: OXA-48.F/OXA-48.R).

TABLE 10. Primers used for the identification and sequencing of carbapenemase genes in putative carbapenemase-producing *E. coli* and *K. pneumoniae* isolates.

Gene	Primer Characteristics		
Primer	Primer Sequence	Size (bp)	Ref.
bla$_{KPC}$			
KPC1	5′-ATGTCACTGTATCGCCGTC-3′	863	(79)
KPC2	5′-AATCCCTCGAGCGCGAGT-3′		
KPC-F	5′-TGTCACTGTATCGCCGTC-3′	1000	(63)
KPC-R	5′-CTCAGTGCTCTACAGAAAACC-3′		
bla$_{IMP}$			
IMP1	5′-CCWGATTTAAAAATYGARAAGCTTG-3′	522	(79)
IMP2	5′-TGGCCAHGCTTCWAHATTTGCRTC-3′		
bla$_{VIM}$			
VIM1	5′-GTTTGGTCGCATATCGCAAC-3′	382	(79)
VIM2	5′-AATGCGCAGCACCAGGATAGAA-3′		
bla$_{NDM}$			
NDM-F	5′-GGTGCATGCCCGGTGAAATC-3′	660	(79)
NDM-R	5′-ATGCTGGCCTTGGGGAACG-3′		
bla$_{GES}$			
GES-2	5′-ATCAGCCACCTCTCAATGG-3′	302	(77)
GES-3	5′-TAGCATCGGGACACATGAC-3′		
bla$_{OXA-48}$			
OXA-48.F	5′-TTGGTGGCATCGATTATCGG-3′	744	(80)
OXA-48.R	5′-GAGCACTTCTTTTGTGATGGC-3′		

4.4.8 Co-resistance to Other Antimicrobial Classes: ESBL-producing *E. coli*

4.4.8.1 *aac(3')-IIa* and 16S rRNA Methylase Production

Any ESBL-producing *E. coli* isolate with a gentamicin MIC ≥8 μg/ml was assessed by PCR for the presence of the aminoglycoside modifying enzyme *aac(3')-IIa* using the primers *aac(3')IIa*.F (5′-GCCGACTGGCACTGTGATGGGATAC-3′) and *aac(3')IIa*.R (5′-TGCAATGCGGTAACGGAGTTTAGCG-3′), as described by Aggen *et al* (81). Any ESBL-producing *E. coli* isolate with a gentamicin MIC ≥16 μg/ml and an amikacin MIC ≥64 μg/ml was assessed by multiplex PCR for the presence of genes encoding the 16S rRNA methylases *armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD* as described by Berçot *et al* (82). Positive control isolates producing the above methylase variants were kindly provided by Dr. Ronald Jones and Dr. Mariana Castanheira of JMI Laboratories. These included: *E. cloacae* 243-50C (*armA*-positive, *rmtA*-positive), *E. coli* 10990-J (*rmtB*-positive), *E. cloacae* 246-5A (*rmtC*-positive), *E. coli* 10401-J (*rmtD*-positive), *E. coli* ATCC® 87415 (*aac(3')-IIa*-positive).

4.4.8.2 *aac(6')-Ib* Variants

All ESBL-producing *E. coli* isolates were analyzed by PCR for the presence of gene variants encoding *aac(6')-Ib* using the primers *aac(6')Ib*.F (5′-TTGCGATGCTCTATGAGTGGCTA-3′) and *aac(6')Ib*.R (5′-CTCGAATGCCTGGCGTGTTT-3′), as described by Park *et al* (83). Restriction digestion was used to distinguish between aminoglycoside acetylating variants and the *aac(6')-Ib-cr* variant, conferring reduced susceptibility to ciprofloxacin (83). Restriction digestion of all *aac(6')-Ib* positive PCR products was carried out by combining 2 μl of 10X NEBuffer 4 and 0.25 μl (5 units) *BtsCI* (20,000 U/ml) (New England Biolabs,

Pickering, Ontario), with 10 µl of PCR product and 7.75 µl dH_2O. This reaction mixture was incubated in a 37°C water bath for two hours, after which, results were determined by agarose gel electrophoresis in 2% gels (100 V/cm; 1 hour runtime). The presence of *aac(6')-Ib-cr* was confirmed in those isolates yielding a single band following restriction digestion with *BtsCI* (482 bp), while the presence of aminoglycoside acetylating variants *aac(6')-Ib/aacA4* was confirmed in those isolates yielding two bands following digestion. Control isolates were taken from the CAN-ICU and CANWARD studies. These included: *E. coli* 62188 (*aac-6'-Ib-cr*-positive), *E. coli* 72162 (*aac-6'-Ib-cr*-negative, *aacA4*-positive), and *E. coli* 88563 (*aac-6'-Ib/aacA4*-negative).

4.5 Phylogenetic Groups and Virulence: ESBL-producing *E. coli*

4.5.1 Virulence Factors and Extraintestinal Pathogenic *E. coli*

All ESBL-producing *E. coli* were screened by PCR for the presence of five key virulence factors associated with ExPEC. These included *papA/papC* (P fimbriae major structural subunit and assembly; analyzed collectively), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesions), *iutA* (aerobactin receptor), and *kpsMII* (group 2 capsule). Reactions were carried out as described by Pitout *et al*. and those isolates found to carry ≥2 of the above virulence factors were classified as ExPEC (84). Positive control isolates were provided by the CANWARD study. These included: *E. coli* 81987 (*papA/C*-positive), *E. coli* 84177 (*sfa*-positive), and *E. coli* 88242 (*afa*-, *iutA*-, and *kpsMII*-positive). In addition, *E. coli* 84693 (negative for all five virulence factors) from the CANWARD study was used as a negative control isolate.

4.5.2 Phylogenetic Grouping of *E. coli* Isolates

All ESBL-producing *E. coli* were classified by multiplex PCR into the one of the four (A, B1, B2, and D) main phylogenetic groups of *E. coli* (40). This multiplex reaction amplified 3 different DNA fragments: *(1) chuA*, a gene required for heme transport in enterohemorarhagic O157:H7 *E. coli*, *(2) yjaA*, a gene initially identified in the complete genome sequence of *E. coli* K-12, function of which is unknown, and *(3)* an anonymous DNA fragment designated TspE4.C2 (40). Results were interpreted as described by Clermont *et al*., based on specific combinations of the three DNA fragments amplified (40). *E. coli* 59096 (B2) from the CANWARD study was used as a positive control, as it produces a product for all three DNA fragments.

4.6 Genetic Relationships

4.6.1 Pulsed-field Gel Electrophoresis (PFGE)

PFGE was carried out in order to assess the genetic relatedness among the isolates included in this study. Following two subcultures from frozen stock on 5%-SBA, a single colony of each isolate was suspended in 3 ml of Brain-Heart Infusion (BHI) broth (Fischer Scientific, Ottawa, Ontario) and incubated at 35±2^{o}C in ambient air for 16-18 hours. Next, 200 µl of each broth culture was transferred to a labelled 1.5 ml tube and centrifuged for 1 minute at 14,000 RPM (20,800 x g). The supernatant was then carefully aspirated and the pellet of bacterial cells resuspended in 200 µl of cell suspension buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA [pH 8.0]), prior to the addition of 10 µl of Proteinase K (20 mg/ml) to each tube. In order to cast plugs, a 1% solution of SeaKem® Gold Agarose (Lonza, Basel, Switzerland) was either prepared by boiling 0.25 g in 25 ml of 1X TE (10mM Tris-HCl [pH 8.0] and 1mM EDTA [pH 9.0]) buffer, or by re-melting

previously made agarose. While in use, SeaKem® Gold Agarose was kept at 65-70°C to keep from solidifying. To make plugs, 200 µl of melted SeaKem® Gold Agarose was added to the previous cell suspension and mixed by slowly pipetting up and down to avoid the formation of bubbles. This mixture was then immediately dispensed (~100 µl per plug) into disposable plug molds (Bio-Rad Laboratories, Hercules, California) and allowed to solidify for 10-15 minutes at room temperature or for 5 minutes at 4°C.

Once solidified, plugs were added to 1 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0], 1% N-lauroylsarcosine) containing 5 µl of Proteinase K (20 mg/ml), and plugs were lysed for 2 hours at 55°C. Plugs were then washed a total of six times to remove any remaining cell lysis buffer: two times with 1 ml of dH_20 followed by four washes with 1 ml of 1X TE buffer. All wash steps included a 15-minute incubation period at 55°C, and following each wash the solution in each tube was aspirated, discarded, and replaced. Once all six washes were complete, plugs were stored in 1 ml of fresh 1X TE buffer and kept at 4°C for a maximum of 6 months.

In order to digest plugs, approximately one third of each plug was cut off lengthwise with a sterile scalpel and placed in a 0.6 ml tube. Next, 250 µl of 1X NEBuffer 4 (New England Biolabs) was prepared per plug, of which, 150 µl was added directly to each plug slice and left at room temperature for 10 minutes to allow plugs to equilibrate. This buffer was then aspirated and replaced with the remaining 100 µl of fresh 1X NEBuffer 4, 3 µl (60 units) of XbaI (20,000 U/ml) (New England Biolabs) was added, and plugs were incubated at in a 37°C water bath for four hours. Depending on the number of samples to be run, either a small (10 wells, 100 ml) or large (20 wells, 150 ml) 1% agarose gel was prepared using Seakem® Gold Agarose and 0.5X TBE. Gel

combs used for casting were kept 1-2mm from the bottom surface of the mold, and the gel was prepared by briefly boiling the agarose and TBE, allowing the solution to cool sufficiently, removing any solidified agarose from the top surface of the solution, and subsequently pouring the agarose into the casting mold. Gels were allowed to solidify for a minimum of 45-60 minutes before use. Once the gel had been made, 2000 ml of 0.5X TBE was prepared and 2 ml of 100mM thiourea was added. The buffer was then poured into the CHEF (Contour-clamped homogeneous electric field) DRIII electrophoresis chamber (Bio-Rad Laboratories).

Before processing plugs, the power supply, pump (set at ~80), and cooling module (14^{o}C) were turned on. Digested plugs were removed from the water bath and the restriction enzyme/buffer mixture was carefully aspirated. Plug slices were then inserted into the wells of the gel using a sterile spatula and scalpel, ensuring each slice was seated in the bottom of the well and that all bubbles had been removed. Again using a sterile spatula and scalpel, a 0.5-1mm slice of λ molecular size standard ladder (New England Biolabs) was added to the first and last well of each row of wells (small gel: 1 row/2 standards; large gel: 2 rows/4 standards). The gel was then placed into the CHEF and ran with the following parameters: initial switch time of 2.2 seconds, final switch time of 54.2 seconds, voltage of 6 V/cm (200 V), included angle of 120, runtime of 17 hours. The next day, the gel was stained in the dark for 60 minutes with SYBR® Green (Molecular Probes, Eugene, Oregon) in 1X TE buffer, after which it was destained as necessary using dH_2O. The gel was then visualized and photographed under UV light and an image was saved using the AlphaImager HP (Alpha Innotech)

All PFGE gels were photographed and digitized using the AlphaImager HP and analyzed using Bionumerics version 3.5 software (Applied Maths, Austin, Texas). The unweighted pair group method was used for cluster analysis and the dice coefficient for the calculation of percent similarity.

4.6.2 *pabB* Detection of the *E. coli* ST-131 Clone

Clermont *et al*. have developed an allele-specific PCR for the *pabB* gene of *E. coli*, able to distinguish ST-131 from all other STs (85). All *E. coli* isolates included in this study were characterized by this reaction, using the primers O25pabBspe.F (5′-TCCAGCAGGTGCTGGATCGT-3′) and O25pabBspe.R (5′-GCGAAATTTTTCGCCGTACTGT-3′).

4.7 Statistical Analysis

Statistical significance was calculated by the chi-squared test, binary logistic regression, or the Fisher exact test in the case of small sample sizes using the SPSS statistics (Version 20) program (IBM Corporation). Statistical significance in this study is defined as a *P*-value ≤0.05, any *P*-value >0.05 has been denoted NS (not statistically significant) throughout this thesis.

5. RESULTS

5.1 Prevalence of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* Isolated from Canadian Hospitals

The national and regional prevalence of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* isolated in Canadian hospitals from 2007 to 2012 is summarized in Table 11. Between 2007 and 2012, 29,931 bacterial isolates were collected as part of the ongoing CANWARD national surveillance study, including a total of 5,951 *E. coli* [2007: 1,560; 2008: 1,131; 2009: 1,097; 2010: 1,017; 2011: 646; 2012: 500] and 1,828 *K. pneumoniae* [2007: 455; 2008: 314; 2009: 356; 2010: 307; 2011: 227; 2012: 169], of which, 8.9% (531/5,951) of *E. coli* and 10.0% (183/1,828) of *K. pneumoniae* collected had a ceftriaxone and/or ceftazidime MIC of ≥1 μg/ml (*P*=NS). In total, 4.5% (269/5,951) and 3.0% (54/1,828) of *E. coli* and *K. pneumoniae*, respectively, were phenotypically confirmed as ESBL-producers by CLSI confirmatory disk test (*P*=0.003), while 2.5% (125/4,949) of *E. coli* demonstrated an AmpC phenotype (126/262).

The national prevalence of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* has been variable throughout the study period. Nationally, ESBL-producing *E. coli* demonstrated a statistically significant increase from 2007 to 2012 [2007: 3.4%; 2008: 4.9%; 2009: 4.3%; 2010: 2.9%; 2011: 7.1%; 2012: 7.6%] (*P*<0.001), as well as significant regional increases in Ontario (*P*=0.016) and Quebec (*P*=0.008). Non-significant increases were observed in the remaining regions (2007 compared to 2012) of British Columbia/Alberta, Saskatchewan/Manitoba, and the Maritimes.

Both AmpC-producing *E. coli* [2007: 0.7%; 2008: 3.1%; 2009: 2.7%; 2010: 2.7%; 2011: 2.9%; 2012: 2.2%] and ESBL-producing *K. pneumoniae* [2007: 1.5%; 2008: 3.2%; 2009: 3.4%; 2010: 3.3%; 2011: 4.0%; 2012: 3.6%] did not demonstrate an overall significant national increase in prevalence from 2007 to 2012. In the case of AmpC-producing *E. coli*, significance was obtained when comparison was limited to the study endpoints (2007 vs. 2012) ($P<0.001$) and it is notable that the prevalence of ESBL-producing *K. pneumoniae* reached peak incidence in 2011/2012. Regionally, the prevalence of AmpC-producing *E. coli* increased significantly in British Columbia/Alberta ($P=0.038$) and Quebec ($P=0.029$), while in the remaining regions of Saskatchewan/Manitoba, Ontario, and the Maritimes, significance was limited again to comparisons made at the study endpoints ($P=0.036$, $P=0.025$, $P=0.005$, respectively). No significant regional increases were observed for ESBL-producing *K. pneumoniae*; however, an increase in prevalence was observed for the British Columbia/Alberta region when comparing 2007 (1.3%) to 2012 (10.0%) ($P=0.019$).

TABLE 11. The national and regional prevalence of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae*: CANWARD 2007-2012.

Cohort (n)	CANWARD Study Year: % (no. in cohort/total no. of species collected)							
Region[a]	2007	2008	2009	2010	2011	2012	2007-2012	*P* value.[c]
ESBL-*E. coli* (269)								
National	3.4 (53/1560)	4.9 (55/1131)	4.3 (47/1097)	2.9 (30/1017)	7.1 (46/646)	7.6 (38/500)	4.5 (269/5951)	<0.001
BC/AB	4.4 (12/271)	7.6 (18/237)	9.4 (14/149)	1.9 (3/157)	7.1 (7/99)	6.6 (5/76)	6.0 (59/989)	NS
SK/MB	1.8 (5/285)	4.7 (11/235)	1.4 (2/143)	2.8 (4/141)	3.8 (3/79)	3.4 (3/87)	2.9 (28/970)	NS
ON	6.6 (29/442)	5.8 (17/292)	6.1 (20/328)	4.6 (12/259)	11.0 (23/210)	11.6 (20/172)	7.1 (121/1703)	0.016
QC	1.3 (6/456)	2.2 (6/270)	1.5 (5/335)	1.6 (5/320)	4.8 (8/167)	6.7 (5/75)	2.2 (35/1623)	0.008
MAR	0.9 (1/106)	3.1 (3/97)	4.2 (6/142)	4.3 (6/140)	5.5 (5/91)	5.6 (5/90)	3.9 (26/666)	NS
AmpC-*E. coli* (126)								
National	0.7 (4/558[b])	3.1 (35/1131)	2.7 (30/1097)	2.7 (27/1017)	2.9 (19/646)	2.2 (11/500)	2.5 (126/4949)	NS
BC/AB	0.8 (1/126)	3.8 (9/237)	5.4 (8/149)	7.6 (12/157)	1.0 (1/99)	3.9 (3/76)	4.0 (34/844)	0.038
SK/MB	1.3 (1/80)	2.6 (6/235)	1.4 (2/143)	2.8 (4/141)	5.1 (4/79)	2.3 (2/87)	2.5 (19/765)	NS
ON	0.0 (0/92)	3.8 (11/292)	1.8 (6/328)	1.9 (5/259)	2.4 (5/210)	1.7 (3/172)	2.2 (30/1353)	NS
QC	0.6 (1/154)	3.0 (8/270)	2.7 (9/335)	0.3 (1/320)	3.6 (6/167)	0.0 (0/75)	1.9 (25/1321)	0.029
MAR	0.9 (1/106)	1.0 (1/97)	3.5 (5/142)	3.6 (5/140)	3.3 (3/91)	3.3 (3/90)	2.7 (18/666)	NS
ESBL-*K. pneumoniae* (54)								
National	1.5 (7/455)	3.2 (10/314)	3.4 (12/356)	3.3 (10/307)	4.0 (9/227)	3.6 (6/169)	3.0 (54/1828)	NS
BC/AB	1.3 (1/76)	3.9 (3/77)	1.9 (1/54)	2.9 (1/34)	3.0 (1/33)	10.0 (3/30)	3.3 (10/304)	NS
SK/MB	0.0 (0/67)	0.0 (0/40)	0.0 (0/46)	4.4 (2/45)	0.0 (0/24)	0.0 (0/17)	0.8 (2/239)	NS
ON	1.4 (2/142)	5.2 (5/96)	7.9 (10/126)	6.3 (6/96)	6.8 (5/73)	3.3 (2/60)	5.1 (30/593)	NS
QC	3.2 (4/126)	2.7 (2/73)	0.0 (0/96)	1.2 (1/81)	4.4 (3/68)	3.1 (1/32)	2.3 (11/476)	NS
MAR	0.0 (0/44)	0.0 (0/28)	2.9 (1/34)	0.0 (0/51)	0.0 (0/29)	0.0 (0/30)	0.5 (1/216)	NS

[a]BC/AB: British Columbia/Alberta, SK/MB: Saskatchewan/Manitoba, ON: Ontario, QC: Quebec, MAR: Maritimes (New Brunswick/Nova Scotia); [b]Cefoxitin was tested against 558 of 1560 *E. coli* collected in 2007; [c]NS: not statistically significant ($P>0.05$).

5.2 Patient Demographics

Patient demographics associated with ESBL-producing *E. coli*, AmpC-producing *E. coli,* and ESBL-producing *K. pneumoniae* infections are summarized in Table 12, Table 13, and Table 14, respectively. Figure 7 summarizes the demographic factors associated with ESBL- and AmpC-producers as compared to non-ESBL- and non-ESBL/AmpC-producing isolates. Overall, the prevalence of ESBL-producing *E. coli* was greater among males as compared to females [5.5% (129/2,333) vs. 3.9% (140/3,618)]. Similarly, both AmpC-producing *E. coli* [2.7% (53/1,950) vs. 2.4% (73/2,999)] and ESBL-producing *K. pneumoniae* [3.3% (33/997) vs. 2.5% (21/831)] demonstrated a higher prevalence among males as well. The proportion of ESBL-producing *E. coli* isolates collected from males was found to be significantly greater when compared to non-ESBL-producing *E. coli* (Odds Ratio (OR): 1.45; 95% Confidence Interval (CI): 1.14-1.86) (P=0.003), while in the case of AmpC-producing *E. coli* and ESBL-producing *K. pneumoniae*, no such significant difference was observed.

Both ESBL- and AmpC-producing *E. coli* were isolated most frequently from the ≥65 year old age group [5.2% (150/2,888) and 2.6% (64/2,439), respectively], while ESBL-producing *K. pneumoniae* occurred most frequently in individuals 18-64 years of age [4.1% (30/740)]. When compared to non-ESBL-producing isolates, both ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae* demonstrated a significant difference in age distribution. In the case of ESBL-producing *E. coli*, this difference was observed at the extremes of age (≤17 years and ≥65 years age group), where a significantly larger proportion of ESBL-producing *E. coli* were isolated from individuals ≥65 years of age versus those ≤17 years of age (OR: 4.85; 95% CI: 2.26-10.41)

(P<0.001). Compared to non-ESBL-producing *K. pneumoniae*, patients 18-64 years of age demonstrated a significant association with ESBL-producing infections as compared to patients ≥65 years of age (OR: 2.11; 95% CI: 1.17-3.81) (P=0.040).

The prevalence of ESBL- and AmpC-producing *E. coli* was greatest in the ICU [7.4% (42/570) and 4.5% (22/484), respectively], while in both cases, the largest proportion of isolates were collected from general medical wards [39.0% (105/269) and 33.3% (42/126), respectively]. ESBL-producing *K. pneumoniae* demonstrated highest prevalence on general medical wards [4.2% (25/599)]. The proportion of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* infections was significantly greater among inpatients as compared to outpatients (P<0.001, P<0.001, P=0.007, respectively).

ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* were distributed across all specimen sources with the majority being isolated from blood [52.8% (142/269), 49.2% (62/126), 53.7% (29/54), respectively]. No significant demographic factors were observed in the case of AmpC-producing *E. coli* or ESBL-producing *K. pneumoniae* with regards to specimen source. Interestingly, from 2009 to 2012 as well as overall [7.6%, (33/435)], ESBL-producing *E. coli* demonstrated greatest prevalence among respiratory specimens. Furthermore, the proportion of ESBL-producing *E. coli* isolated from respiratory specimens differed significantly from non-ESBL-producing isolates [12.3% (33/269) vs. 7.1% (402/5,682)] (P=0.004), and the rate of ESBL-producing *E. coli* isolated from respiratory specimens was greater when compared to all other specimen sources (OR: 1.66; 95% CI: 1.12-2.45).

TABLE 12. Patient demographics associated with ESBL-producing *E. coli* isolated from Canadian hospitals.

Parameter	CANWARD Study Year: % (no. in cohort/total no. collected)						
Value	2007 (n=53)	2008 (n=55)	2009 (n=47)	2010 (n=30)	2011 (n=46)	2012 (n=38)	2007-2012 (n=269)
Gender							
Male	4.4 (28/636)	5.2 (22/420)	4.5 (18/396)	4.6 (18/392)	6.5 (18/276)	11.7 (25/213)	5.5 (129/2333)
Female	2.7 (25/924)	4.6 (33/711)	4.1 (29/701)	1.9 (12/625)	7.6 (28/370)	4.5 (13/287)	3.9 (140/3618)
Age (years)							
≤17	0.0 (0/171)	2.4 (3/124)	1.8 (2/111)	0.0 (0/107)	1.6 (1/63)	2.0 (1/51)	1.1 (7/627)
18-64	2.9 (19/651)	5.5 (26/476)	5.0 (23/460)	2.9 (12/407)	6.4 (16/250)	8.3 (16/192)	4.6 (112/2436)
≥65	4.6 (34/738)	4.9 (26/531)	4.2 (22/526)	3.6 (18/503)	8.7 (29/333)	8.2 (21/257)	5.2 (150/2888)
Hospital Location							
Clinic/Office	3.2 (8/247)	2.5 (4/159)	1.4 (3/221)	3.0 (6/203)	8.8 (10/113)	2.6 (2/77)	3.2 (33/1020)
Emergency Room	1.1 (7/620)	4.6 (21/453)	3.2 (12/375)	1.6 (6/383)	6.4 (16/250)	3.5 (7/198)	3.0 (69/2279)
Intensive Care Unit	2.5 (4/157)	8.0 (6/75)	11.4 (10/88)	6.8 (7/103)	4.8 (4/83)	17.2 (11/64)	7.4 (42/570)
Medical Ward	6.2 (26/421)	5.9 (22/374)	5.4 (18/335)	4.0 (10/250)	7.9 (13/164)	11.3 (16/141)	6.2 (105/1685)
Surgical Ward	7.0 (8/115)	2.9 (2/70)	5.1 (4/78)	1.3 (1/78)	8.3 (3/36)	10.0 (2/20)	5.0 (20/397)
Specimen Source							
Blood	4.3 (34/788)	5.4 (28/522)	4.1 (22/540)	2.3 (12/531)	5.7 (20/352)	9.5 (26/275)	4.7 (142/3008)
Urine	2.4 (15/634)	4.7 (24/509)	3.0 (13/437)	3.0 (11/371)	9.5 (18/190)	2.6 (4/154)	3.7 (85/2295)
Wound	2.6 (1/38)	2.5 (1/40)	7.1 (4/56)	2.5 (1/40)	4.2 (1/24)	6.7 (1/15)	4.2 (9/213)
Respiratory	3.0 (3/100)	3.3 (2/60)	12.5 (8/64)	8.0 (6/75)	8.8 (7/80)	12.5 (7/56)	7.6 (33/435)

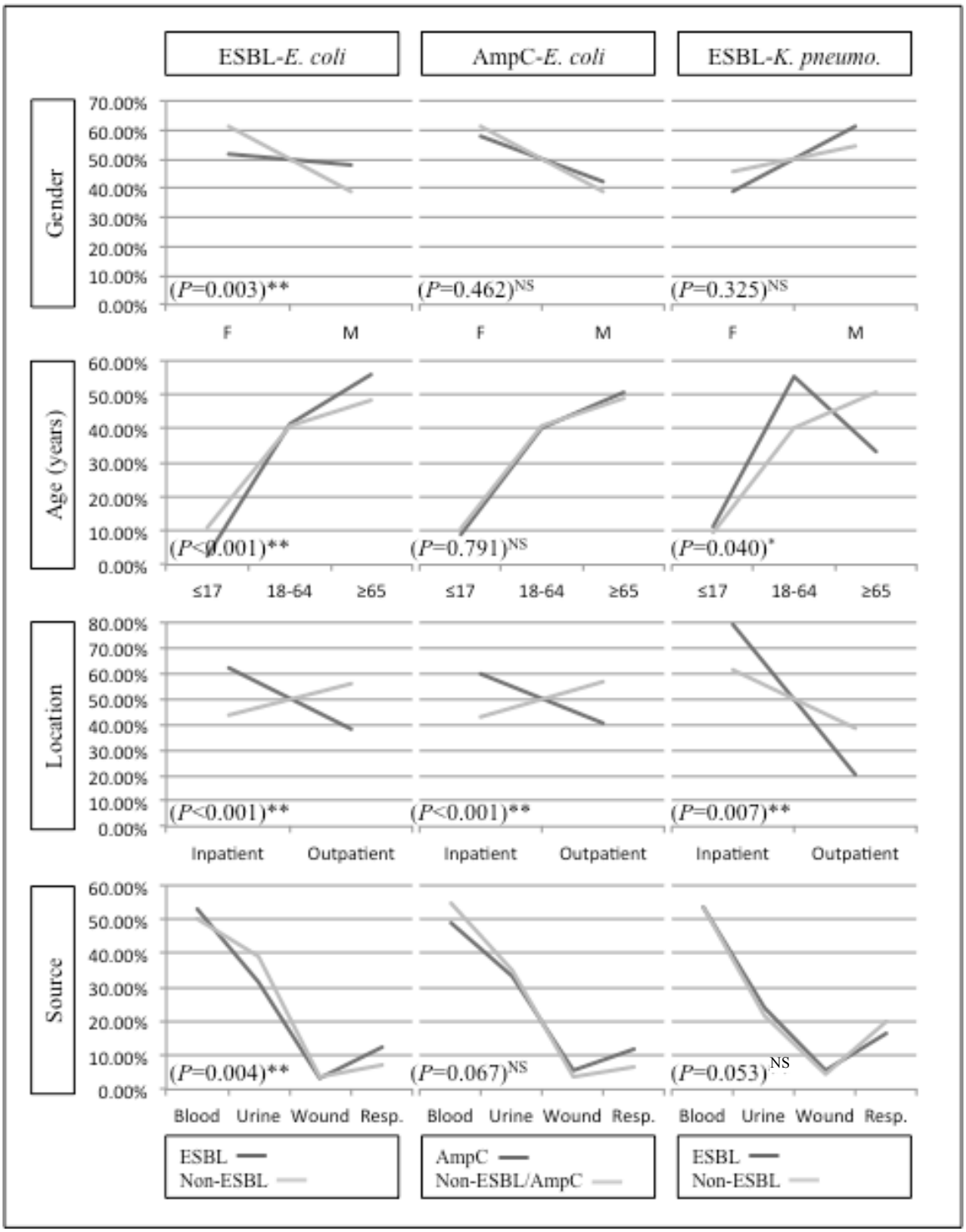
TABLE 13. Patient demographics associated with AmpC-producing *E. coli* isolated from Canadian hospitals.

Parameter	CANWARD Study Year: % (no. in cohort/total no. collected)						
Value	2007 (n=4)	2008 (n=35)	2009 (n=30)	2010 (n=27)	2011 (n=19)	2012 (n=11)	2007-2012 (n=126)
Gender							
Male	1.2 (3/253)	3.3 (14/420)	2.0 (8/396)	2.8 (11/392)	3.6 (10/276)	3.3 (7/213)	2.7 (53/1950)
Female	0.3 (1/305)	3.0 (21/711)	3.1 (22/701)	2.6 (16/625)	2.4 (9/370)	1.4 (4/287)	2.4 (73/2999)
Age (years)							
≤17	0.0 (0/41)	3.2 (4/124)	0.9 (1/111)	3.7 (4/107)	1.6 (1/63)	2.0 (1/51)	2.2 (11/497)
18-64	0.4 (1/228)	3.2 (15/476)	2.4 (11/460)	2.7 (11/407)	4.0 (10/250)	1.6 (3/192)	2.5 (51/2013)
≥65	1.0 (3/289)	3.0 (16/531)	3.4 (18/526)	2.4 (12/503)	2.4 (8/333)	2.7 (7/257)	2.6 (64/2439)
Hospital Location							
Clinic/Office	0.0 (0/59)	3.1 (5/159)	2.7 (6/221)	0.5 (1/203)	1.8 (2/113)	0.0 (0/77)	1.7 (14/832)
Emergency Room	0.8 (2/255)	2.6 (12/453)	1.3 (5/375)	2.3 (9/383)	2.8 (7/250)	1.0 (2/198)	1.9 (37/1914)
Intensive Care Unit	1.4 (1/71)	6.7 (5/75)	2.3 (2/88)	1.9 (2/103)	7.2 (6/83)	9.4 (6/64)	4.5 (22/484)
Medical Ward	0.7 (1/146)	3.2 (12/374)	3.9 (13/335)	4.4 (11/250)	1.8 (3/164)	1.4 (2/141)	3.0 (42/1410)
Surgical Ward	0.0 (0/27)	1.4 (1/70)	5.1 (4/78)	5.1 (4/78)	2.8 (1/36)	5.0 (1/20)	3.6 (11/309)
Specimen Source							
Blood	0.6 (3/468)	3.3 (17/522)	2.2 (12/540)	3.2 (17/531)	2.8 (10/352)	1.1 (3/275)	2.3 (62/2688)
Urine	0.0 (0/72)	2.8 (14/509)	3.0 (13/437)	2.7 (10/371)	1.6 (3/190)	1.3 (2/154)	2.4 (42/1733)
Wound	0.0 (0/3)	7.5 (3/40)	1.8 (1/56)	0.0 (0/40)	8.3 (2/24)	6.7 (1/15)	3.9 (7/178)
Respiratory	6.7 (1/15)	1.7 (1/60)	6.3 (4/64)	0.0 (0/75)	5.0 (4/80)	8.9 (5/56)	4.3 (15/350)

TABLE 14. Patient demographics associated with ESBL-producing *K. pneumoniae* isolated from Canadian hospitals.

Parameter	CANWARD Study Year: % (no. in cohort/total no. collected)						
Value	2007 (n=7)	2008 (n=10)	2009 (n=12)	2010 (n=10)	2011 (n=9)	2012 (n=6)	2007-2012 (n=54)
Gender							
Male	1.6 (4/248)	3.7 (6/161)	3.9 (8/203)	3.0 (5/164)	5.4 (7/130)	3.3 (3/91)	3.3 (33/997)
Female	1.4 (3/207)	2.6 (4/153)	2.6 (4/153)	3.5 (5/143)	2.1 (2/97)	3.8 (3/78)	2.5 (21/831)
Age (years)							
≤17	2.7 (1/37)	0.0 (0/42)	6.9 (2/29)	3.1 (1/32)	13.3 (2/15)	0.0 (0/17)	3.5 (6/172)
18-64	1.1 (2/186)	6.2 (7/113)	3.7 (6/162)	5.5 (7/127)	5.6 (5/89)	4.8 (3/63)	4.1 (30/740)
≥65	1.7 (4/232)	1.9 (3/159)	2.4 (4/165)	1.4 (2/148)	1.6 (2/123)	3.4 (3/89)	2.0 (18/916)
Hospital Location							
Clinic/Office	2.0 (1/49)	0.0 (0/28)	0.0 (0/58)	3.3 (1/30)	7.7 (2/26)	0.0 (0/19)	1.9 (4/210)
Emergency Room	0.0 (0/126)	1.3 (1/80)	1.3 (1/76)	0.0 (0/85)	4.5 (3/66)	3.8 (2/52)	1.4 (7/485)
Intensive Care Unit	1.3 (1/79)	1.6 (1/62)	5.6 (4/71)	10.9 (6/55)	2.0 (1/51)	2.8 (1/36)	4.0 (14/354)
Medical Ward	2.7 (4/148)	5.3 (6/114)	5.9 (7/119)	2.9 (3/104)	3.2 (2/62)	5.8 (3/52)	4.2 (25/599)
Surgical Ward	1.9 (1/53)	6.7 (2/30)	0.0 (0/32)	0.0 (0/33)	4.5 (1/22)	0.0 (0/10)	2.2 (4/180)
Specimen Source							
Blood	1.5 (4/264)	2.2 (3/138)	2.6 (5/195)	3.7 (6/164)	4.7 (6/129)	5.6 (5/90)	3.0 (29/980)
Urine	0.9 (1/112)	5.1 (5/98)	3.1 (2/64)	4.4 (3/68)	6.7 (2/30)	0.0 (0/28)	3.3 (13/400)
Wound	5.6 (1/18)	7.7 (1/13)	4.5 (1/22)	0.0 (0/17)	0.0 (0/10)	0.0 (0/3)	3.6 (3/83)
Respiratory	1.6 (1/61)	1.5 (1/65)	5.3 (4/75)	1.7 (1/58)	1.7 (1/58)	2.1 (1/48)	2.5 (9/365)

FIGURE 7. Demographic factors associated with ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* infections as compared to non-ESBL and non-ESBL/AmpC-producing strains.



NS: P>0.05; *: 0.25<*P*≤0.5; **: *P*≤0.025.

5.3 Antimicrobial Susceptibility

The activity of the antimicrobials tested against ESBL-producing *E. coli*, AmpC-producing *E. coli,* and ESBL-producing *K. pneumoniae* is summarized in Table 15. The antibiotics with the greatest activity against the isolates in this study were amikacin, meropenem, ertapenem, and colistin. Both ESBL- and AmpC-producing *E. coli* also demonstrated high susceptibility (>90%) to piperacillin/tazobactam. However, in the case of ESBL-producing *K. pneumoniae*, susceptibility decreased to 68.5% with a large number (16.7%) of isolates falling in the intermediate range. Similarly, while tigecycline demonstrated excellent activity against ESBL- and AmpC-producing *E. coli* (99.6% and 100% susceptibility, respectively), ESBL-producing *K. pneumoniae* were significantly less likely to be susceptible to this agent (85.2% susceptibility, $P<0.001$).

A MDR phenotype was observed in 78.8% (212/269) and 66.7% (36/54) of ESBL-producing *E. coli* and *K. pneumoniae*, respectively ($P=0.043$), while AmpC-producing *E. coli* were significantly less likely to be MDR when compared to ESBL-producing *E. coli* (34.9%, $P<0.001$). The frequency of multidrug resistance among ESBL-producing *E. coli* increased slightly during the study period from 77.4% in 2007 to 78.9% in 2012 (P=NS), with peak incidence occurring in 2010 (83.3%). Greater than 75% of MDR ESBL-producing *E. coli* demonstrated concomitant resistance to oxyimino-cephalosporins, ciprofloxacin, trimethoprim-sulfametoxazole, and/or gentamicin. Three percent (8/269) and 9.3% (5/54) of ESBL-producing *E. coli* and *K. pneumoniae*, respectively, were XDR ($P=0.048$), while no AmpC-producing *E. coli* demonstrated an XDR phenotype.

TABLE 15. Antimicrobial activity against ESBL-producing *E. coli*, AmpC-producing *E. coli,* and ESBL-producing *K. pneumoniae.*

Cohort (n)	MIC (μg/ml)				MIC Interpretation[a]		
Antibiotic	MIC_{50}	MIC_{90}	Min.	Max.	%S	%I	%R
ESBL-*E. coli* (269)							
AMC[b]	8	16	1	>32	57.2	35.7	7.1
Cefazolin	>128	>128	16	>128	0.0	0.0	100.0
Cefoxitin	8	16	0.5	>32	81.4	10.0	8.6
Ceftriaxone	>64	>64	≤0.25	>64	1.1	1.5	97.4
Ceftazidime	16	>32	≤0.5	>32	33.8	7.9	58.3
Cefepime	8	>32	≤1	>32	54.0	25.5	20.5
TZP[b]	4	16	≤1	>512	92.6	5.2	2.2
Ertapenem	≤0.06	0.25	≤0.06	4	97.8	1.1	1.1
Meropenem	≤0.12	≤0.12	≤0.12	1	100.0	0.0	0.0
Ciprofloxacin	>16	>16	≤0.06	>16	10.4	0.7	88.9
Amikacin	4	8	≤2	>64	95.9	3.7	0.4
Gentamicin	2	>32	≤0.5	>32	52.8	0.4	46.8
Tigecycline	0.5	1	0.12	4	99.6	0.4	0.0
SXT[b]	>8	>8	≤0.12	>8	31.6	0.0	68.4
Fosfomycin	2	4	≤1	>512	95.5	3.3	1.1
Nitrofurantoin	16	64	≤1	256	89.6	7.1	3.3
Colistin	0.5	1	≤0.06	>16	99.1	0.0	0.9
ESBL-*K. pneumoniae* (54)							
AMC[b]	8	32	2	>32	50.0	34.0	16.0
Cefazolin	>128	>128	8	>128	0.0	0.0	100.0
Cefoxitin	8	16	2	>32	80.0	10.0	10.0
Ceftriaxone	64	>64	≤0.25	>64	14.8	9.3	75.9
Ceftazidime	32	>32	1	>32	31.9	2.1	66.0
Cefepime	4	>32	≤1	>32	61.4	6.8	31.8
TZP[b]	8	256	2	>512	68.5	16.7	14.8
Ertapenem	0.06	0.5	≤0.06	1	98.0	2.0	0.0
Meropenem	≤0.12	≤0.12	≤0.12	0.12	100.0	0.0	0.0
Ciprofloxacin	4	>16	≤0.06	>16	33.3	9.3	57.4
Amikacin	≤2	16	≤2	>64	90.7	1.9	7.4
Gentamicin	1	>32	≤0.5	>32	53.7	0.0	46.3
Tigecycline	1	4	0.5	16	85.2	7.4	7.4
SXT[b]	>8	>8	≤0.12	>8	27.8	0.0	72.2
Colistin	0.5	1	0.25	>16	98.0	0.0	2.0
AmpC-*E. coli* (126)							
AMC[b]	32	>32	1	>32	26.2	21.4	52.4
Cefazolin	>128	>128	0.5	>128	0.8	3.2	96.0
Cefoxitin	>32	>32	32	>32	0.0	0.0	100.0
Ceftriaxone	8	32	≤0.25	>64	42.1	3.2	54.8
Ceftazidime	16	>32	1	>32	44.3	5.7	50.0
Cefepime	≤0.25	1	≤0.25	>32	97.0	1.0	2.0

Cohort (n)	MIC (μg/ml)				MIC Interpretation[a]		
Antibiotic	MIC_{50}	MIC_{90}	Min.	Max.	%S	%I	%R
AmpC-*E. coli* cont.							
TZP[b]	4	16	≤1	>512	90.5	6.3	3.2
Ertapenem	≤0.06	0.25	≤0.06	1	97.6	2.4	0.0
Meropenem	≤0.06	≤0.06	≤0.06	0.12	100.0	0.0	0.0
Ciprofloxacin	0.12	>16	≤0.06	>16	61.1	0.8	38.1
Amikacin	2	4	≤2	>64	98.4	0.0	1.6
Gentamicin	≤0.5	32	≤0.5	>32	83.3	0.0	16.7
Tigecycline	0.5	1	0.12	2	100.0	0.0	0.0
SXT[b]	0.25	>8	≤0.12	>8	65.1	0.0	34.9
Fosfomycin	2	8	≤1	>512	96.8	1.6	1.6
Nitrofurantoin	16	64	≤1	256	89.7	6.3	4.0
Colistin	0.25	0.5	0.12	1	100.0	0.0	0.0

[a]%S: % susceptible, %I: % intermediate, %R: % resistant; [b]AMC: amoxicillin/clavulanic acid, TZP: piperacillin/tazobactam, SXT: trimethoprim-sulfamethoxazole.

5.4. Phenotypic Characterization

In total, 8.9% (531/5,951) of the *E. coli* isolates collected as part of the CANWARD study had a ceftriaxone and/or ceftazidime MIC of ≥1 μg/ml, of which 50.7% (269/531) were phenotypically confirmed as ESBL-producers by the CLSI confirmatory disk test. Two hundred and thirteen (79.2%) ESBL-producing *E. coli* isolates were phenotypically identified as ESBL-producers (≥5 mm increase in zone diameter when combined with clavulanic acid) by both cefotaxime and ceftazidime when tested by the CLSI confirmatory disk test, while only 44 (16.4%) and 12 (4.5%) isolates were identified by cefotaxime and ceftazidime alone, respectively. Of those 44 isolates phenotypically identified by cefotaxime alone, 28 (63.6%) produced CTX-M-14, 10 (22.7%) produced CTX-M-15, three (6.8%) produced an unknown ESBL, one (2.3%) produced CTX-M-24, one (2.3%) produced CTX-M-27, and one (2.3%) produced SHV-2a. Of the remaining 12 isolates phenotypically identified by ceftazidime alone, six (50.0%) produced CTX-M-15, three (25.0%) produced an unknown ESBL, two (16.7%) produced CTX-M-14, and one (8.3%) produced TEM-12. In this study, cefotaxime alone was significantly more likely to give a positive result for ESBL-production as compared to ceftazidime alone (P=0.032). The characteristics of phenotypically confirmed ESBL-producing *E. coli* isolates with an unknown genotype are summarized in Table 16.

TABLE 16. Characteristics of phenotypically confirmed ESBL-producing *E. coli* isolates with unknown genotypes.

Isolate	Year	Genotype	Province[a]	Screening Criteria[b]		CLSI Confirmatory Disk Test[c]					
				CTR (µg/ml)	CAZ (µg/ml)	CTX	CTX/CLA	Diff.	CAZ	CAZ/CLA	Diff.
70106	2007	TEM-1	ON	>64	>32	8	29	21	16	21	5
80057	2008	Unknown	ON	>64	>32	12	12	0	6	12	6
80059	2008	TEM-1	ON	4	32	21	21	0	13	18	5
80835	2008	Unknown	AB	64	>32	12	30	18	7	11	4
87797	2009	TEM-1	ON	64	2	6	26	20	25	29	4
101610	2012	TEM-1	ON	64	> 32	16	16	0	12	18	6
101976	2012	TEM-1	SK	16	16	16	22	6	18	21	3
102174	2012	Unknown	NB	64	>32	6	11	5	6	14	8

[a]AB: Alberta, NB: New Brunswick, ON: Ontario, SK: Saskatchewan; [b]Minimum inhibitory concentration (MIC) of ceftriaxone (CTR) and ceftazidime (CAZ); [c]Zone of inhibition measured in mm surrounding 30 µg cefotaxime (CTX), 30/10 µg cefotaxime/clavulanic acid (CTX/CLA), 30 µg ceftazidime (CAZ), and 30/10 µg ceftazidime/clavulanic acid (CAZ) disks, Diff.: difference in mm between zone surrounding CTX and CAZ as compared to CTX/CLA and CAZ/CLA, respectively.

5.5 Genotypic Characterization

5.5.1 Genotypic Characterization of ESBL-producing *E. coli*

The genotypic characterization of ESBL-producing *E. coli* is summarized in Table 17. Among ESBL-producing *E. coli*, CTX-M [94.1% (253/269)] represented the dominant enzyme type identified with CTX-M-15 [66.5% (179/269)] being the dominant genotype. The identification of SHV- [2.6% (7/269)] and TEM-type [0.4% (1/269)] ESBLs was limited in this study. In total, six different CTX-M variants were identified in 253 isolates. These included, CTX-M-15 [70.8% (179/253)], CTX-M-14 [19.8% (50/253)], CTX-M-27 [7.5% (19/253)], CTX-M-24 [0.8% (2/253)], CTX-M-3 [0.8% (2/253)], and CTX-M-65 [0.4% (1/253)]. Those isolates producing SHV-type ESBLs were limited to either SHV-12 [57.1% (4/7)] or SHV-2a [42 .9% (3/7)], while the one TEM-producing isolate identified produced TEM-12. One hundred and ninety-six (72.9%) ESBL-producing *E. coli* isolates produced multiple β-lactamases, these included the narrow spectrum enzymes TEM-1 [30.6% (60/196)], OXA-1 [51.5% (101/196)], as well those isolates producing both TEM-1 and OXA-1 [17.9% (35/196)]. The proportion of isolates producing CTX-M-15 increased from 54.7% (29/53) in 2007 to 68.4% (26/38) in 2012 with a maximum incidence of 71.7% (33/46) in 2011 (*P*=NS).

TABLE 17. The genotypic characterization of ESBL-producing *E. coli* isolated from Canadian hospitals.

Family: % of total (n) Variant[a]: % of total (n)	% of ESBL-*E. coli* (n)
CTX-M: 94.1 (253)	
CTX-M-15: 66.5 (179)	
CTX-M-15	9.7 (26)
+ TEM-1	7.8 (21)
+ OXA-1	36.8 (99)
+ TEM-1, OXA-1	12.3 (33)
CTX-M-14: 18.6 (50)	
CTX-M-14	5.6 (15)
+ TEM-1	11.9 (32)
+ OXA-1	0.7 (2)
+ TEM-1, OXA-1	0.4 (1)
CTX-M-27: 7.1 (19)	
CTX-M-27	6.3 (17)
+ TEM-1	0.7 (2)
CTX-M-24: 0.7 (2)	
CTX-M-24	0.4 (1)
+ TEM-1	0.4 (1)
CTX-M-3: 0.7 (2)	
+ TEM-1	0.4 (1)
+ TEM-1, OXA-1	0.4 (1)
CTX-M-65: 0.4 (1)	
CTX-M-65	0.4 (1)
SHV: 2.6 (7)	
SHV-12: 1.5 (4)	
SHV-12	0.4 (1)
+ TEM-1	1.1 (3)
SHV-2a: 1.1 (3)	
SHV-2a	1.1 (3)
TEM: 0.4 (1)	
TEM-12: 0.4 (1)	
TEM-12	0.4 (1)
Unknown: 3.0 (8)	
Unknown	1.1 (3)
+ TEM-1	1.9 (5)

[a]TEM-1 is not an ESBL, however, it has been included due to frequent co-expression.

5.5.1.1 Molecular Basis of Co-resistance to Other Antimicrobial Classes

All ESBL-producing *E. coli* were screened for the presence *of aac(6')-Ib* variants, including *aac(6')-Ib-cr*. One hundred and eighty-one (67.3%) ESBL-producing *E. coli* isolates were positive for *aac(6')-Ib*. In total, 65.4% (176/269) were found to carry the *aac(6')-Ib-cr* variant, capable of conferring reduced susceptibility to ciprofloxacin. The remaining 1.9% (5/269) carried the *aac(6')-Ib/aac(A4)* variant, capable of N-acetylating aminoglycoside antibiotics such as gentamicin. The *aac(6')-Ib-cr* variant was found in 67.4% (161/239) of ciprofloxacin resistant ESBL-producing *E. coli* identified in this study.

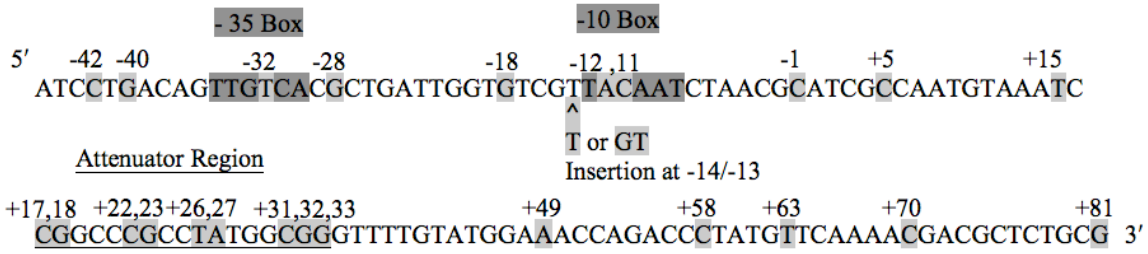
In total, 46.8% (126/269) and 0.4% (1/269) of ESBL-producing *E. coli* were found to be gentamicin and amikacin resistant, respectively. The aminoglycoside acetyltransferase *aac(3')-IIa* was identified in 84.9% (107/126) of gentamicin resistant ESBL-producing *E. coli*. As mentioned, *aac(A4)* was identified in five (4.0%) of 126 gentamicin resistant ESBL-producing *E. coli*, of which, four co-expressed *aac(3')-IIa*. The 16S rRNA methylase was not detected in the one isolate demonstrating high-level aminoglycoside resistance. The observed resistance pattern of this isolate is likely attributable to a combination of multiple aminoglycoside modifying enzymes.

5.5.2 Genotypic Characterization of AmpC-producing *E. coli*

Of 126 AmpC-producing *E. coli*, 68 (54.0%) were found to produce acquired AmpC β-lactamases which included CMY-2 [98.5% (67/68)] and FOX-5 [1.5% (1/68)]. The remaining 58 (46.0%) AmpC-producing isolates were screened for the presence of promoter/attenuator mutations within the chromosomal *ampC* gene, as compared to a 191 bp region of the *E. coli* K-12 promoter sequence. The number of mutations observed per

isolate ranged from a minimum of one to a maximum of seven. The *E. coli* K-12 promoter/attenuator sequence and the positions of observed mutations are outlined in Figure 8. In total, nine, nine, and eight different mutations were observed in the promoter, attenuator, and other regions, respectively (Table 18). The complete listing of mutations observed and the groupings of these mutations are organized by isolate number in Table 19.

FIGURE 8. Positions of mutations observed within the promoter/attenuator region of the chromosomal *ampC* gene of *E. coli.* Modified from (29).



Dark grey shading indicates the -35 and -10 box, light grey shading indicates the positions of the observed mutations.

TABLE 18. The genotypic characterization of AmpC-producing *E. coli* isolated from Canadian hospitals.

Region Position	Mutation	Count	% (Region)	Rank	% (Overall)
Promoter Region					
-42	C to T	7	11.7	4	12.1
-40	G to A	1	1.7	8	1.7
-32	T to A	10	16.7	3	17.2
-28	G to A	19	31.7	1	32.8
-18	G to T	11	18.3	2	19.0
-14/-13	Ins[a] GT	6	10.0	5	10.3
-13/-12	Ins[a] T	3	5.0	6	5.2
-12	A to T	1	1.7	8	1.7
-11	C to T	2	3.3	7	3.4
Attenuator Region					
+17	C to A	1	3.0	4	1.7
+18	G to A	1	3.0	4	1.7
+22	C to T	8	24.2	1	13.8
+23	C to A	1	3.0	4	1.7
+26	T to G	6	18.2	2	10.3
+27	A to T	6	18.2	2	10.3
+31	C to T	3	9.1	3	5.2
+32	G to A	6	18.2	2	10.3
+33	G to A	1	3.0	4	1.7
Other					
-1	C to T	7	11.1	4	12.1
+5	C to T	3	4.8	6	5.2
+15	C to T	1	1.6	7	1.7
+49	A to G	1	1.6	7	1.7
+58	C to T	14	22.2	2	24.1
+63	T to C	4	6.3	5	6.9
+70	C to T	9	14.3	3	15.5
+81	G to A	24	38.1	1	41.4

[a]Ins: insertion.

TABLE 19. Complete listing of promoter/attenuator mutations within the chromosomal *ampC* gene of AmpC-producing *E. coli*.

Isolate	Year	Province[a]	CTR[b] (μg/ml)	CAZ[b] (μg/ml)	FOX[b] (μg/ml)	Mutations within the chromosomal *ampC*
76325	2007	MB	16	N/A	>32	+58 C to T, +63 T to C, +81 G to A
78967	2008	SK	>64	>32	>32	+22 C to T, +26 T to G, +27 A to T, +32 G to A, +70 C to T
79692	2008	QC	≤0.25	1	32	-28 G to A, +81 G to A
80189	2008	QC	≤0.25	2	>32	+70 C to T
80208	2008	QC	≤0.25	1	>32	-28 G to A, +81 G to A
80240	2008	QC	≤0.25	1	32	-28 G to A, +81 G to A
80379	2008	BC	0.5	8	32	-42 C to T, -28 G to A, +81 G to A
80861	2008	AB	0.5	4	32	-13/-14 Ins[c] GT, +58 (C to T), +63 T to C
80952	2008	AB	≤0.25	2	>32	-32 T to A, -28 G to A, -18 G to T, +31 C to T, +81 G to A
81037	2008	SK	≤0.25	1	32	-32 T to A, +70 C to T
81063	2008	SK	1	2	>32	-32 T to A, -28 G to A, -11 C to T, +15 C to T, +22 C to A, +49 A to G, +81 G to A
81067	2008	SK	≤0.25	2	>32	-32 T to A, +18 G to A
81196	2008	ON	>64	>32	>32	-28 G to A, +17 C to T, +81 G to A
82294	2008	ON	≤0.25	1	32	-28 G to A, +81 G to A
82932	2008	BC	≤0.25	2	32	-12 A to T, +81 G to A
82940	2008	BC	>64	>32	>32	+70 C to T
82948	2008	BC	4	32	32	-32 T to A, -28 G to A, -18 G to T, +31 C to T, +81 G to A
82988	2008	BC	≤0.25	1	32	-28 G to A, +81 G to A
83018	2008	ON	≤0.25	2	32	-13/-14 Ins[c] GT, +70 C to T
83027	2008	ON	≤0.25	2	32	-13/-14 Ins[c] GT, +70 C to T
83030	2008	ON	≤0.25	2	32	-13/-14 Ins[c] GT, +70 C to T
83194	2008	SK	0.5	4	>32	-32 T to A, -28 G to A, +31 C to T, +81 G to A
84783	2009	AB	1	16	>32	-40 G to T, -18 G to A, -1 C to T, +58 C to T
85794	2009	SK	≤0.25	1	32	+22 C to T, +26 T to G, +27 A to T, +32 G to A
85863	2009	QC	1	2	>32	-32 T to A, -11 C to T, +58 C to T, +63 T to C
86471	2009	NB	≤0.25	1	32	-18 G to A, -1 C to T, +58 C to T
87422	2009	NS	≤0.25	1	32	+58 C to T, +63 T to C

Isolate	Year	Province[a]	CTR[b] (μg/ml)	CAZ[b] (μg/ml)	FOX[b] (μg/ml)	Mutations within the chromosomal *ampC*
87679	2009	ON	≤0.25	1	>32	+22 C to T, +26 T to G, +27 A to T, +32 G to A
88058	2009	ON	≤0.25	1	>32	-28 G to A
89128	2009	QC	1	2	32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
89168	2009	QC	≤0.25	2	>32	-32 T to A, +22 C to T, +26 T to G, +27 A to T, +32 G to A
90484	2010	ON	0.5	8	>32	-18 G to A, -1 C to A, +58 C to T
91137	2010	BC	1	8	32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
91150	2010	BC	≤0.25	1	32	+22 C to T, +26 T to G, + 27 A to T, +32 G to A
92394	2010	ON	≤0.25	4	>32	+22 C to T, +26 T to G, +27 A to T, +32 G to A
93999	2010	AB	≤0.25	1	32	+70 C to T
94561	2010	SK	≤0.25	1	32	-28 G to A
94787	2010	MB	≤0.25	1	32	+70 C to T
95595	2011	MB	2	8	>32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
95773	2011	MB	1	4	32	-12/-13 Ins[c] T, +5 C to T, +22 G to A, +81 G to A
95989	2011	QC	≤0.25	2	>32	-12/-13 Ins[c] T, +5 C to T, +81 G to A
96253	2011	NB	0.5	2	32	-32 T to A, + 81 G to A
96627	2011	BC	0.5	4	32	-12/-13 Ins[c] T, +5 C to T, +23 C to A, +81 G to A
96855	2011	QC	≤0.25	2	32	-28 G to A, +81 G to A
97405	2011	SK	≤0.25	1	32	-32 T to A, -28 G to A, +58 C to T, +81 G to A
98547	2011	ON	2	16	>32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
99773	2011	QC	≤0.25	1	>32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
100258	2012	NS	≤0.25	2	>32	+58 C to T, +63 T to C, +81 G to A
100532	2012	SK	≤0.25	1	32	-28 G to A, +81 G to A
100711	2012	BC	1	8	>32	-13/-14 Ins[c] GT, -28 G to A, +81 G to A
101134	2012	MB	≤0.25	1	>32	-28 G to A, +81 G to A
101563	2012	ON	≤0.25	1	32	-28 G ot A, +81 G to A
101587	2012	ON	2	32	32	-42 C to T, -18 G to A, -1 C to T, +58 C to T
103117	2012	NS	≤0.25	2	>32	-28 G to A, -13/-14 Ins GT, +33 G to A, +81 G ot A

[a]AB: Alberta, BC: British Columbia, MB: Manitoba, NB: New Brunswick, NS: Nova Scotia, ON: Ontario, QC: Quebec; [b]Minimum inhibitory concentration of ceftriaxone (CTR), ceftazidime (CAZ), and cefoxitin (FOX); [c]Ins: insertion.

5.5.3 Genotypic Characterization of ESBL-producing *K. pneumoniae*

The genotypic characterization of ESBL-producing *K. pneumoniae* is summarized in Table 20. Thirty-four (63.0%) ESBL-producing *K. pneumoniae* isolates produced CTX-M-type ESBLs, which included CTX-M-15 in 26 (76.5%) isolates, CTX-M-14 in five (14.7%) isolates, as well as CTX-M-2, CTX-M-3, and CTX-M-27 in one (2.9%) isolate each. SHV-type β-lactamases were produced by 94.4% (51/54) of ESBL-producing *K. pneumoniae*, of which 33.3% (17/51) produced a SHV variant with broad extended-spectrum activity (in accordance with *www.lahey.org/studies*). Those SHV variants with ESBL activity included SHV-12 [15.7% (8/51)], SHV-2a [11.8% (6/51)], SHV-2 [2.0% (1/51)], SHV-5 [2.0% (1/51)], and SHV-31 [2.0% (1/51)]. In total, 75.9% (41/54) of ESBL-producing *K. pneumoniae* produced multiple β-lactamases including the narrow spectrum β-lactamases SHV-1 in 29.6% (16/54) of isolates, TEM-1 in 42.6% (23/54) of isolates, and OXA-1 in 26.9% (16/54) of isolates.

TABLE 20. The genotypic characterization of ESBL-producing *K. pneumoniae* isolated from Canadian hospitals.

Genotype[a]	Count	%
CTX-M-15, SHV-11, TEM-1, OXA-1	5	9.3
CTX-M-15, SHV-1, OXA-1	4	7.4
SHV-12, TEM-1	4	7.4
SHV-2a	4	7.4
Unknown, SHV-1	4	7.4
CTX-M-14, SHV-11	3	5.6
CTX-M-15, SHV-1	3	5.6
SHV-12	3	5.6
CTX-M-15, OXA-1	2	3.7
CTX-M-15, SHV-1, TEM-1	2	3.7
CTX-M-15, SHV-1, TEM-1, OXA-1	2	3.7
CTX-M-15, SHV-11, TEM-1	2	3.7
CTX-M-14, SHV-1	1	1.9
CTX-M-14, SHV-11, TEM-1	1	1.9
CTX-M-15, SHV-11	1	1.9
CTX-M-15, SHV-12, TEM-1	1	1.9
CTX-M-15, SHV-168	1	1.9
CTX-M-15, SHV-28, OXA-1	1	1.9
CTX-M-15, SHV-28, TEM-1	1	1.9
CTX-M-15, SHV-5, TEM-1, OXA-1	1	1.9
CTX-M-2, SHV-11	1	1.9
CTX-M-27, SHV-11	1	1.9
CTX-M-3, SHV-108, TEM-1	1	1.9
SHV-2	1	1.9
SHV-2a, OXA-1	1	1.9
SHV-2a, TEM-1	1	1.9
SHV-31, TEM-1	1	1.9
Unknown, TEM-1	1	1.9

[a]TEM-1 and SHV-1 are not ESBLs, however, they have been included due to frequent co-expression.

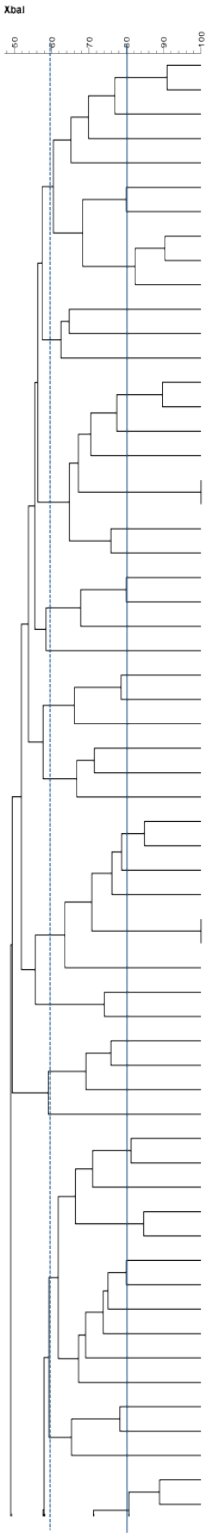
5.6 Genetic Relatedness of ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* in Canada

5.6.1 Genetic Relationships Among ESBL-producing *E. coli*

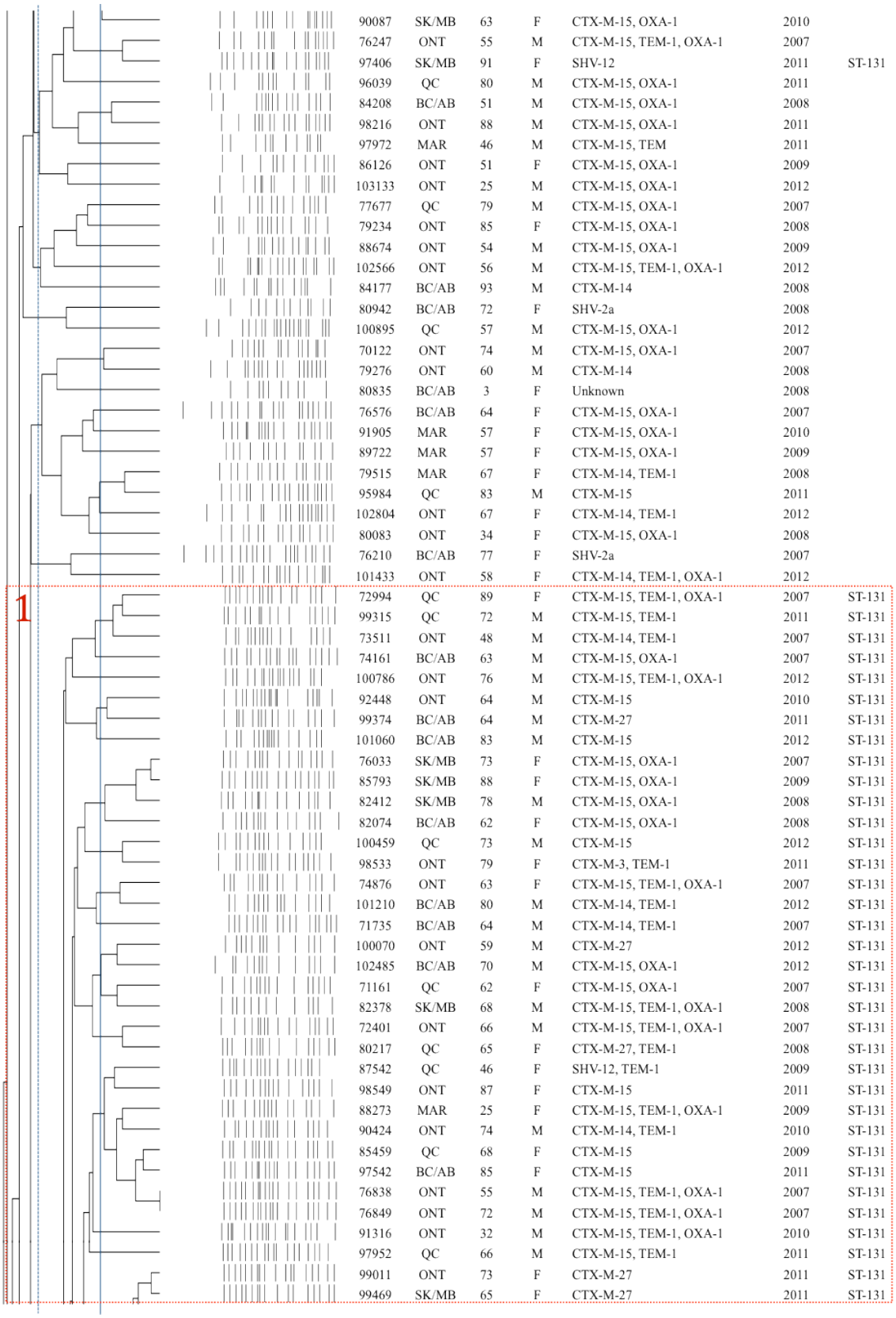
5.6.1.1 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was conducted in order to assess genetic relatedness among ESBL-producing *E. coli* isolated from Canadian hospitals (Figure 9). ESBL-producing *E. coli* were generally unrelated by PFGE (<80% similarity). However, a number of large clusters were identified and were comprised of ST-131 isolates (60% to >80% similarity).

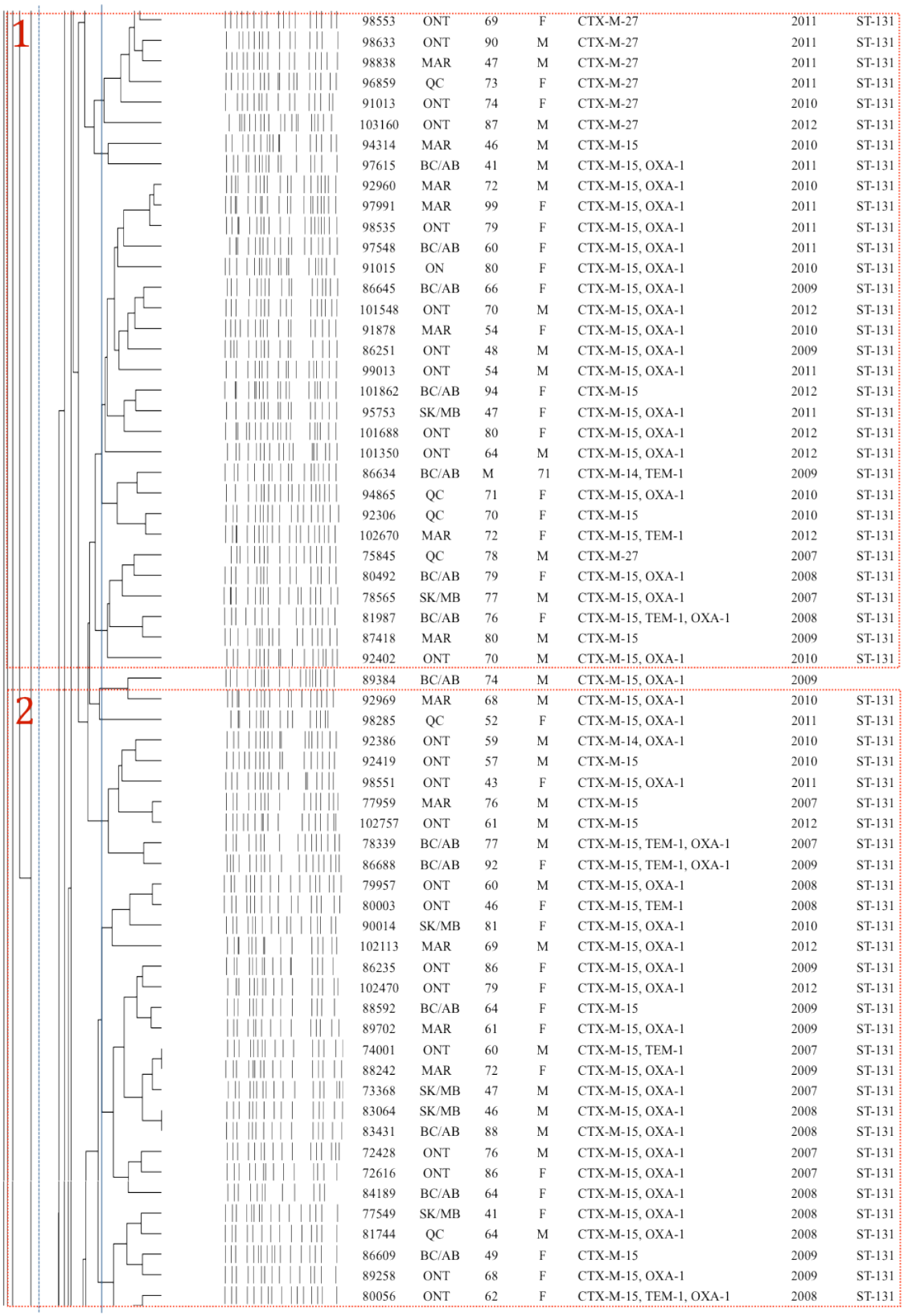
FIGURE 9. PFGE dendrogram of ESBL-producing *E. coli* isolated from Canadian hospitals: CANWARD 2007-2012[a,b].



STOCK	REGION	AGE	GENDER	GENOTYPE	YEAR	ST-131
91191	BC/AB	66	M	CTX-M-14, TEM-1	2010	
92756	BC/AB	66	M	CTX-M-14, TEM-1	2010	
101610	ONT	52	M	Unkown, TEM-1	2012	
72495	ONT	68	M	CTX-M-14, TEM-1	2007	
88563	BC/AB	72	M	CTX-M-27, TEM-1	2009	
72612	ONT	23	M	CTX-M-14, TEM-1	2007	
74764	SK/MB	81	M	CTX-M-14	2007	
87797	ONT	62	F	Unknown, TEM-1	2009	
98552	ONT	90	F	SHV-12, TEM-1	2011	
102772	ONT	43	M	CTX-M-14, TEM-1	2012	
101093	SK/MB	56	M	CTX-M-15, TEM-1, OXA-1	2012	
98543	ONT	83	M	CTX-M-14, OXA-1	2011	
91012	ONT	90	F	CTX-M-14, TEM-1	2010	
73548	SK/MB	88	F	CTX-M-15, OXA-1	2007	
99599	MAR	80	F	CTX-M-15, TEM-1	2011	
82929	BC/AB	77	M	CTX-M-15, TEM-1, OXA-1	2008	
87673	ONT	69	F	CTX-M-14	2009	
76789	ONT	61	F	CTX-M-15, OXA-1	2007	
76791	ONT	59	M	CTX-M-15, OXA-1	2007	
94929	BC/AB	72	M	CTX-15, OXA-1	2010	
98356	ONT	56	F	CTX-M-14	2011	
72511	ONT	82	F	CTX-M-15, TEM-1	2007	
89439	ONT	80	F	CTX-M-15, OXA-1	2009	
76819	ONT	80	F	CTX-M-15, OXA-1	2007	
86655	BC/AB	84	M	CTX-M-14, OXA-1	2009	
70094	ONT	74	F	CTX-M-14, TEM-1	2007	
81100	SK/MB	78	M	CTX-M-14, TEM-1	2008	
74855	ONT	85	F	CTX-M-15, OXA-1	2007	
79170	QC	54	F	CTX-M-15, OXA-1	2008	
80057	ONT	81	F	Unknown	2008	
81270	ONT	81	F	CTX-M-15, TEM-1	2008	
71546	ONT	33	F	CTX-M-27	2007	
75417	QC	82	M	CTX-M-14	2007	
85332	ONT	45	F	CTX-M-14, TEM-1	2009	
82395	SK/MB	54	M	CTX-M-14, TEM-1	2008	
84664	BC/AB	36	M	CTX-M-15, OXA-1	2009	
84693	BC/AB	36	M	CTX-M-15, OXA-1	2009	
81758	QC	55	M	CTX-M-15, TEM-1, OXA-1	2008	
79443	MAR	66	M	CTX-M-15, OXA-1	2008	
85143	ONT	80	F	CTX-M-14, TEM-1	2008	
87344	ONT	73	M	CTX-M-27	2009	
88040	ONT	34	F	CTX-M-14	2009	
87191	ONT	1	F	CTX-M-14, TEM-1	2009	
80960	BC/AB	76	M	CTX-M-15, TEM-1	2008	
100019	ONT	46	F	CTX-M-15, OXA-1	2012	
98151	QC	67	M	CTX-M-15, OXA-1	2011	
89386	BC/AB	62	M	CTX-M-15, OXA-1	2009	ST-131
89306	ONT	54	F	CTX-M-15, TEM-1	2009	
89946	SK/MB	62	F	CTX-M-15, OXA-1	2009	
88753	QC	61	M	CTX-M-15, TEM-1, OXA-1	2009	
102112	MAR	69	M	CTX-M-15, TEM-1	2012	
90900	QC	54	F	CTX-M-24	2010	
98557	ONT	70	F	CTX-M-15, OXA-1	2011	
98537	ONT	80	M	CTX-M-15	2011	
70589	BC/AB	49	F	CTX-M-15	2007	
81300	ONT	34	M	CTX-M-15, OXA-1	2008	
81687	MAR	78	M	CTX-M-15, OXA-1	2008	
80059	ONT	81	F	Unknown, TEM-1	2008	
80939	BC/AB	22	M	CTX-M-15	2008	
100270	MAR	40	M	CTX-M-15, OXA-1	2012	



90087	SK/MB	63	F	CTX-M-15, OXA-1	2010		
76247	ONT	55	M	CTX-M-15, TEM-1, OXA-1	2007		
97406	SK/MB	91	F	SHV-12	2011	ST-131	
96039	QC	80	M	CTX-M-15, OXA-1	2011		
84208	BC/AB	51	M	CTX-M-15, OXA-1	2008		
98216	ONT	88	M	CTX-M-15, OXA-1	2011		
97972	MAR	46	M	CTX-M-15, TEM	2011		
86126	ONT	51	F	CTX-M-15, OXA-1	2009		
103133	ONT	25	M	CTX-M-15, OXA-1	2012		
77677	QC	79	M	CTX-M-15, OXA-1	2007		
79234	ONT	85	F	CTX-M-15, OXA-1	2008		
88674	ONT	54	M	CTX-M-15, OXA-1	2009		
102566	ONT	56	M	CTX-M-15, TEM-1, OXA-1	2012		
84177	BC/AB	93	M	CTX-M-14	2008		
80942	BC/AB	72	F	SHV-2a	2008		
100895	QC	57	M	CTX-M-15, OXA-1	2012		
70122	ONT	74	M	CTX-M-15, OXA-1	2007		
79276	ONT	60	M	CTX-M-14	2008		
80835	BC/AB	3	F	Unknown	2008		
76576	BC/AB	64	F	CTX-M-15, OXA-1	2007		
91905	MAR	57	F	CTX-M-15, OXA-1	2010		
89722	MAR	57	F	CTX-M-15, OXA-1	2009		
79515	MAR	67	F	CTX-M-14, TEM-1	2008		
95984	QC	83	M	CTX-M-15	2011		
102804	ONT	67	F	CTX-M-14, TEM-1	2012		
80083	ONT	34	F	CTX-M-15, OXA-1	2008		
76210	BC/AB	77	F	SHV-2a	2007		
101433	ONT	58	F	CTX-M-14, TEM-1, OXA-1	2012		
72994	QC	89	F	CTX-M-15, TEM-1, OXA-1	2007	ST-131	
99315	QC	72	M	CTX-M-15, TEM-1	2011	ST-131	
73511	ONT	48	M	CTX-M-14, TEM-1	2007	ST-131	
74161	BC/AB	63	M	CTX-M-15, OXA-1	2007	ST-131	
100786	ONT	76	M	CTX-M-15, TEM-1, OXA-1	2012	ST-131	
92448	ONT	64	M	CTX-M-15	2010	ST-131	
99374	BC/AB	64	M	CTX-M-27	2011	ST-131	
101060	BC/AB	83	M	CTX-M-15	2012	ST-131	
76033	SK/MB	73	F	CTX-M-15, OXA-1	2007	ST-131	
85793	SK/MB	88	F	CTX-M-15, OXA-1	2009	ST-131	
82412	SK/MB	78	M	CTX-M-15, OXA-1	2008	ST-131	
82074	BC/AB	62	F	CTX-M-15, OXA-1	2008	ST-131	
100459	QC	73	M	CTX-M-15	2012	ST-131	
98533	ONT	79	F	CTX-M-3, TEM-1	2011	ST-131	
74876	ONT	63	F	CTX-M-15, TEM-1, OXA-1	2007	ST-131	
101210	BC/AB	80	M	CTX-M-14, TEM-1	2012	ST-131	
71735	BC/AB	64	M	CTX-M-14, TEM-1	2007	ST-131	
100070	ONT	59	M	CTX-M-27	2012	ST-131	
102485	BC/AB	70	M	CTX-M-15, OXA-1	2012	ST-131	
71161	QC	62	F	CTX-M-15, OXA-1	2007	ST-131	
82378	SK/MB	68	M	CTX-M-15, TEM-1, OXA-1	2008	ST-131	
72401	ONT	66	M	CTX-M-15, TEM-1, OXA-1	2007	ST-131	
80217	QC	65	F	CTX-M-27, TEM-1	2008	ST-131	
87542	QC	46	F	SHV-12, TEM-1	2009	ST-131	
98549	ONT	87	F	CTX-M-15	2011	ST-131	
88273	MAR	25	F	CTX-M-15, TEM-1, OXA-1	2009	ST-131	
90424	ONT	74	M	CTX-M-14, TEM-1	2010	ST-131	
85459	QC	68	F	CTX-M-15	2009	ST-131	
97542	BC/AB	85	F	CTX-M-15	2011	ST-131	
76838	ONT	55	M	CTX-M-15, TEM-1, OXA-1	2007	ST-131	
76849	ONT	72	M	CTX-M-15, TEM-1, OXA-1	2007	ST-131	
91316	ONT	32	M	CTX-M-15, TEM-1, OXA-1	2010	ST-131	
97952	QC	66	M	CTX-M-15, TEM-1	2011	ST-131	
99011	ONT	73	F	CTX-M-27	2011	ST-131	
99469	SK/MB	65	F	CTX-M-27	2011	ST-131	



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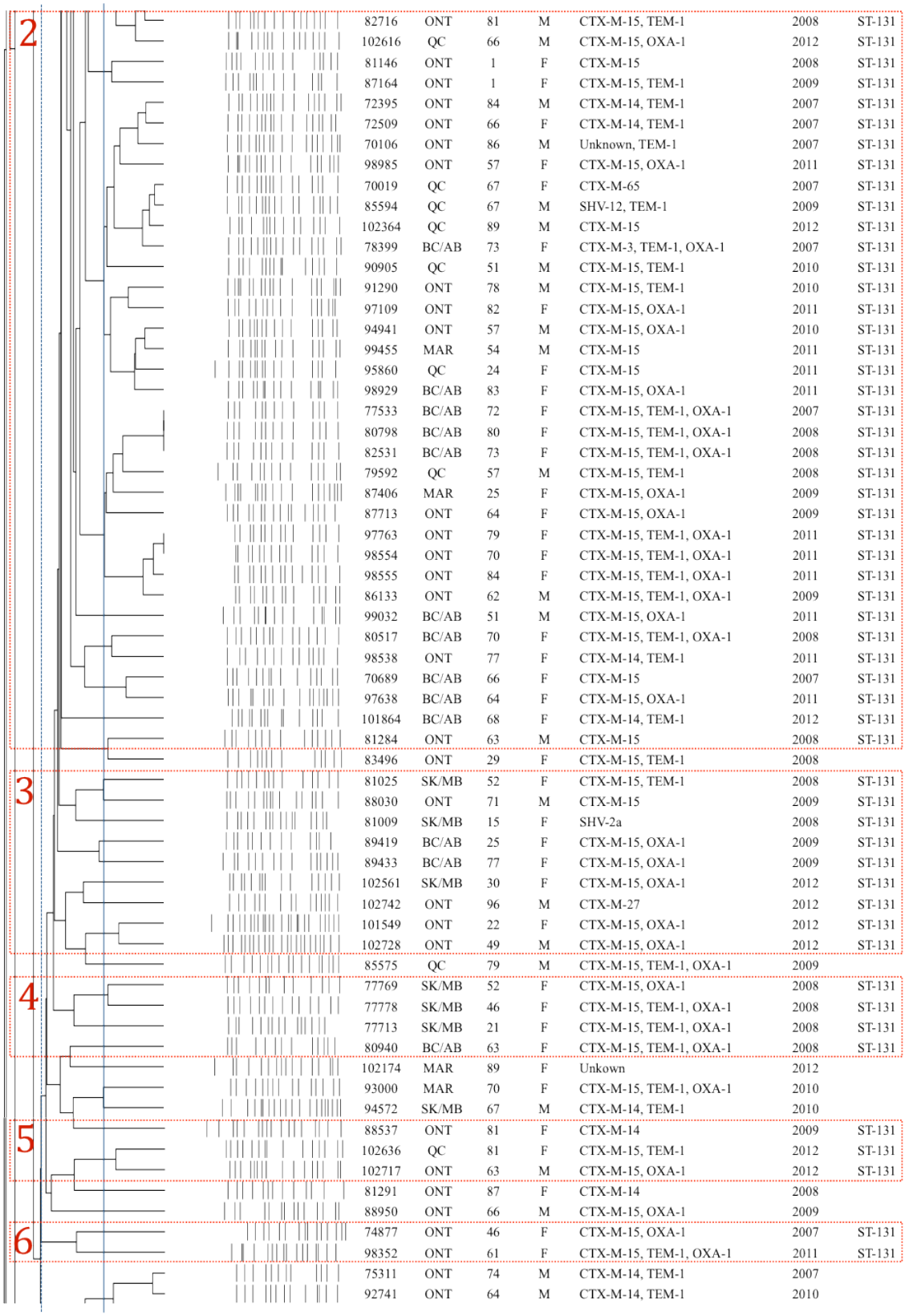
98838	MAR	47	M	CTX-M-27	2011	ST-131
96859	QC	73	F	CTX-M-27	2011	ST-131
91013	ONT	74	F	CTX-M-27	2010	ST-131
103160	ONT	87	M	CTX-M-27	2012	ST-131
94314	MAR	46	M	CTX-M-15	2010	ST-131
97615	BC/AB	41	M	CTX-M-15, OXA-1	2011	ST-131
92960	MAR	72	M	CTX-M-15, OXA-1	2010	ST-131
97991	MAR	99	F	CTX-M-15, OXA-1	2011	ST-131
98535	ONT	79	F	CTX-M-15, OXA-1	2011	ST-131
97548	BC/AB	60	F	CTX-M-15, OXA-1	2011	ST-131
91015	ON	80	F	CTX-M-15, OXA-1	2010	ST-131
86645	BC/AB	66	F	CTX-M-15, OXA-1	2009	ST-131
101548	ONT	70	M	CTX-M-15, OXA-1	2012	ST-131
91878	MAR	54	F	CTX-M-15, OXA-1	2010	ST-131
86251	ONT	48	M	CTX-M-15, OXA-1	2009	ST-131
99013	ONT	54	M	CTX-M-15, OXA-1	2011	ST-131
101862	BC/AB	94	F	CTX-M-15	2012	ST-131
95753	SK/MB	47	F	CTX-M-15, OXA-1	2011	ST-131
101688	ONT	80	F	CTX-M-15, OXA-1	2012	ST-131
101350	ONT	64	M	CTX-M-15, OXA-1	2012	ST-131
86634	BC/AB	M	71	CTX-M-14, TEM-1	2009	ST-131
94865	QC	71	F	CTX-M-15, OXA-1	2010	ST-131
92306	QC	70	F	CTX-M-15	2010	ST-131
102670	MAR	72	F	CTX-M-15, TEM-1	2012	ST-131
75845	QC	78	M	CTX-M-27	2007	ST-131
80492	BC/AB	79	F	CTX-M-15, OXA-1	2008	ST-131
78565	SK/MB	77	M	CTX-M-15, OXA-1	2007	ST-131
81987	BC/AB	76	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
87418	MAR	80	M	CTX-M-15	2009	ST-131
92402	ONT	70	M	CTX-M-15, OXA-1	2010	ST-131
89384	BC/AB	74	M	CTX-M-15, OXA-1	2009	
92969	MAR	68	M	CTX-M-15, OXA-1	2010	ST-131
98285	QC	52	F	CTX-M-15, OXA-1	2011	ST-131
92386	ONT	59	M	CTX-M-14, OXA-1	2010	ST-131
92419	ONT	57	M	CTX-M-15	2010	ST-131
98551	ONT	43	F	CTX-M-15, OXA-1	2011	ST-131
77959	MAR	76	M	CTX-M-15	2007	ST-131
102757	ONT	61	M	CTX-M-15	2012	ST-131
78339	BC/AB	77	M	CTX-M-15, TEM-1, OXA-1	2007	ST-131
86688	BC/AB	92	F	CTX-M-15, TEM-1, OXA-1	2009	ST-131
79957	ONT	60	M	CTX-M-15, OXA-1	2008	ST-131
80003	ONT	46	F	CTX-M-15, TEM-1	2008	ST-131
90014	SK/MB	81	F	CTX-M-15, OXA-1	2010	ST-131
102113	MAR	69	M	CTX-M-15, OXA-1	2012	ST-131
86235	ONT	86	F	CTX-M-15, OXA-1	2009	ST-131
102470	ONT	79	F	CTX-M-15, OXA-1	2012	ST-131
88592	BC/AB	64	F	CTX-M-15	2009	ST-131
89702	MAR	61	F	CTX-M-15, OXA-1	2009	ST-131
74001	ONT	60	M	CTX-M-15, TEM-1	2007	ST-131
88242	MAR	72	F	CTX-M-15, OXA-1	2009	ST-131
73368	SK/MB	47	M	CTX-M-15, OXA-1	2007	ST-131
83064	SK/MB	46	M	CTX-M-15, OXA-1	2008	ST-131
83431	BC/AB	88	M	CTX-M-15, OXA-1	2008	ST-131
72428	ONT	76	M	CTX-M-15, OXA-1	2007	ST-131
72616	ONT	86	F	CTX-M-15, OXA-1	2007	ST-131
84189	BC/AB	64	F	CTX-M-15, OXA-1	2008	ST-131
77549	SK/MB	41	F	CTX-M-15, OXA-1	2008	ST-131
81744	QC	64	M	CTX-M-15, OXA-1	2008	ST-131
86609	BC/AB	49	F	CTX-M-15	2009	ST-131
89258	ONT	68	F	CTX-M-15, OXA-1	2009	ST-131
80056	ONT	62	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131



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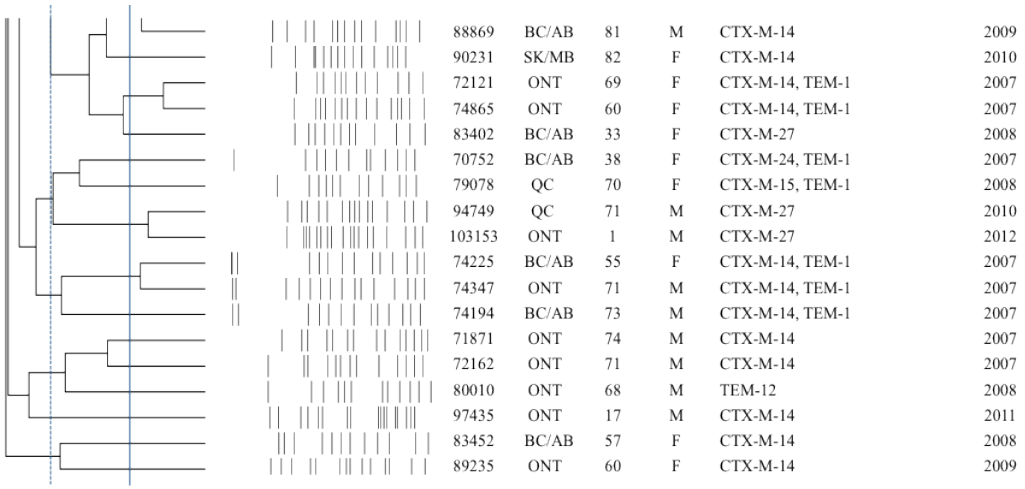
102616	QC	66	M	CTX-M-15, OXA-1	2012	ST-131
81146	ONT	1	F	CTX-M-15	2008	ST-131
87164	ONT	1	F	CTX-M-15, TEM-1	2009	ST-131
72395	ONT	84	M	CTX-M-14, TEM-1	2007	ST-131
72509	ONT	66	F	CTX-M-14, TEM-1	2007	ST-131
70106	ONT	86	M	Unknown, TEM-1	2007	ST-131
98985	ONT	57	F	CTX-M-15, OXA-1	2011	ST-131
70019	QC	67	F	CTX-M-65	2007	ST-131
85594	QC	67	M	SHV-12, TEM-1	2009	ST-131
102364	QC	89	M	CTX-M-15	2012	ST-131
78399	BC/AB	73	F	CTX-M-3, TEM-1, OXA-1	2007	ST-131
90905	QC	51	M	CTX-M-15, TEM-1	2010	ST-131
91290	ONT	78	M	CTX-M-15, TEM-1	2010	ST-131
97109	ONT	82	F	CTX-M-15, OXA-1	2011	ST-131
94941	ONT	57	M	CTX-M-15, OXA-1	2010	ST-131
99455	MAR	54	M	CTX-M-15	2011	ST-131
95860	QC	24	F	CTX-M-15	2011	ST-131
98929	BC/AB	83	F	CTX-M-15, OXA-1	2011	ST-131
77533	BC/AB	72	F	CTX-M-15, TEM-1, OXA-1	2007	ST-131
80798	BC/AB	80	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
82531	BC/AB	73	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
79592	QC	57	M	CTX-M-15, TEM-1	2008	ST-131
87406	MAR	25	F	CTX-M-15, OXA-1	2009	ST-131
87713	ONT	64	F	CTX-M-15, OXA-1	2009	ST-131
97763	ONT	79	F	CTX-M-15, TEM-1, OXA-1	2011	ST-131
98554	ONT	70	F	CTX-M-15, TEM-1, OXA-1	2011	ST-131
98555	ONT	84	F	CTX-M-15, TEM-1, OXA-1	2011	ST-131
86133	ONT	62	M	CTX-M-15, TEM-1, OXA-1	2009	ST-131
99032	BC/AB	51	M	CTX-M-15, OXA-1	2011	ST-131
80517	BC/AB	70	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
98538	ONT	77	F	CTX-M-14, TEM-1	2011	ST-131
70689	BC/AB	66	F	CTX-M-15	2007	ST-131
97638	BC/AB	64	F	CTX-M-15, OXA-1	2011	ST-131
101864	BC/AB	68	F	CTX-M-14, TEM-1	2012	ST-131
81284	ONT	63	M	CTX-M-15	2008	ST-131
83496	ONT	29	F	CTX-M-15, TEM-1	2008	
81025	SK/MB	52	F	CTX-M-15, TEM-1	2008	ST-131
88030	ONT	71	M	CTX-M-15	2009	ST-131
81009	SK/MB	15	F	SHV-2a	2008	ST-131
89419	BC/AB	25	F	CTX-M-15, OXA-1	2009	ST-131
89433	BC/AB	77	F	CTX-M-15, OXA-1	2009	ST-131
102561	SK/MB	30	F	CTX-M-15, OXA-1	2012	ST-131
102742	ONT	96	M	CTX-M-27	2012	ST-131
101549	ONT	22	F	CTX-M-15, OXA-1	2012	ST-131
102728	ONT	49	M	CTX-M-15, OXA-1	2012	ST-131
85575	QC	79	M	CTX-M-15, TEM-1, OXA-1	2009	
77769	SK/MB	52	F	CTX-M-15, OXA-1	2008	ST-131
77778	SK/MB	46	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
77713	SK/MB	21	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
80940	BC/AB	63	F	CTX-M-15, TEM-1, OXA-1	2008	ST-131
102174	MAR	89	F	Unkown	2012	
93000	MAR	70	F	CTX-M-15, TEM-1, OXA-1	2010	
94572	SK/MB	67	M	CTX-M-14, TEM-1	2010	
88537	ONT	81	F	CTX-M-14	2009	ST-131
102636	QC	81	F	CTX-M-15, TEM-1	2012	ST-131
102717	ONT	63	M	CTX-M-15, OXA-1	2012	ST-131
81291	ONT	87	F	CTX-M-14	2008	
88950	ONT	66	M	CTX-M-15, OXA-1	2009	
74877	ONT	46	F	CTX-M-15, OXA-1	2007	ST-131
98352	ONT	61	F	CTX-M-15, TEM-1, OXA-1	2011	ST-131
75311	ONT	74	M	CTX-M-14, TEM-1	2007	
92741	ONT	64	M	CTX-M-14, TEM-1	2010	



88869	BC/AB	81	M	CTX-M-14	2009
90231	SK/MB	82	F	CTX-M-14	2010
72121	ONT	69	F	CTX-M-14, TEM-1	2007
74865	ONT	60	F	CTX-M-14, TEM-1	2007
83402	BC/AB	33	F	CTX-M-27	2008
70752	BC/AB	38	F	CTX-M-24, TEM-1	2007
79078	QC	70	F	CTX-M-15, TEM-1	2008
94749	QC	71	M	CTX-M-27	2010
103153	ONT	1	M	CTX-M-27	2012
74225	BC/AB	55	F	CTX-M-14, TEM-1	2007
74347	ONT	71	M	CTX-M-14, TEM-1	2007
74194	BC/AB	73	M	CTX-M-14, TEM-1	2007
71871	ONT	74	M	CTX-M-14	2007
72162	ONT	71	M	CTX-M-14	2007
80010	ONT	68	M	TEM-12	2008
97435	ONT	17	M	CTX-M-14	2011
83452	BC/AB	57	F	CTX-M-14	2008
89235	ONT	60	F	CTX-M-14	2009

[a]The dashed and solid blue lines indicate 60% and 80% similarity, respectively; [b]red dashed boxes have been used to highlight related clusters of ST-131 isolates with each cluster being assigned a number of 1 through 6.

5.6.1.2 Detection of the O25b:H4 ST-131 Clone Among ESBL-producing *E. coli*

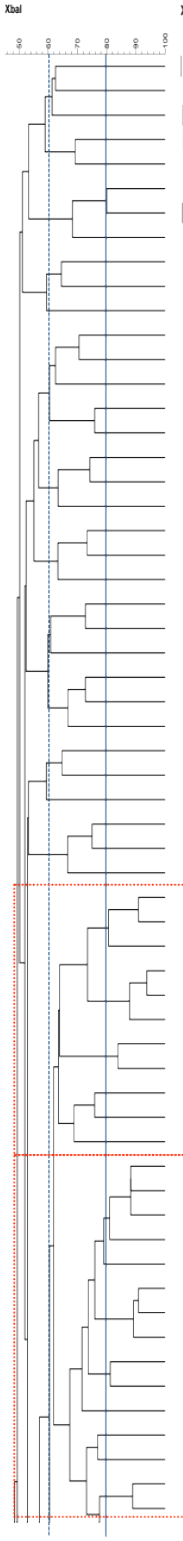
The ST-131 clone was detected among 56.9% (153/269) of ESBL-producing *E. coli*. The proportion of ST-131 isolates increased from 49.1% (26/53) in 2007 to 63.2% (24/38) in 2012, reaching peak incidence in 2011 at 71.7% (33/46, *P*=0.018). Of the 153 ST-131 ESBL-producing *E. coli* isolates, 121 (79.1%) produced CTX-M-15, 13 (8.5%) produced CTX-M-27, 11 (7.2%) produced CTX-M-14, three (2.0%) produced SHV-12, two (1.3%) produced CTX-M-3, one (0.7%) produced CTX-M-65, one (0.7%) produced SHV-2a, and one (0.7%) isolate had an unknown genotype. One hundred and fifty-one (98.7%) of 153 ST-131 isolates clustered together with at least one other ST-131 isolate by PFGE. In total, six clusters (numbered 1-6) were identified containing 67 (43.8%), 66 (43.1%), nine (5.9%), four (2.6%), three (2.0%), and two (1.3%) isolates, respectively.

5.6.2 Genetic Relationship Among AmpC-producing *E. coli*

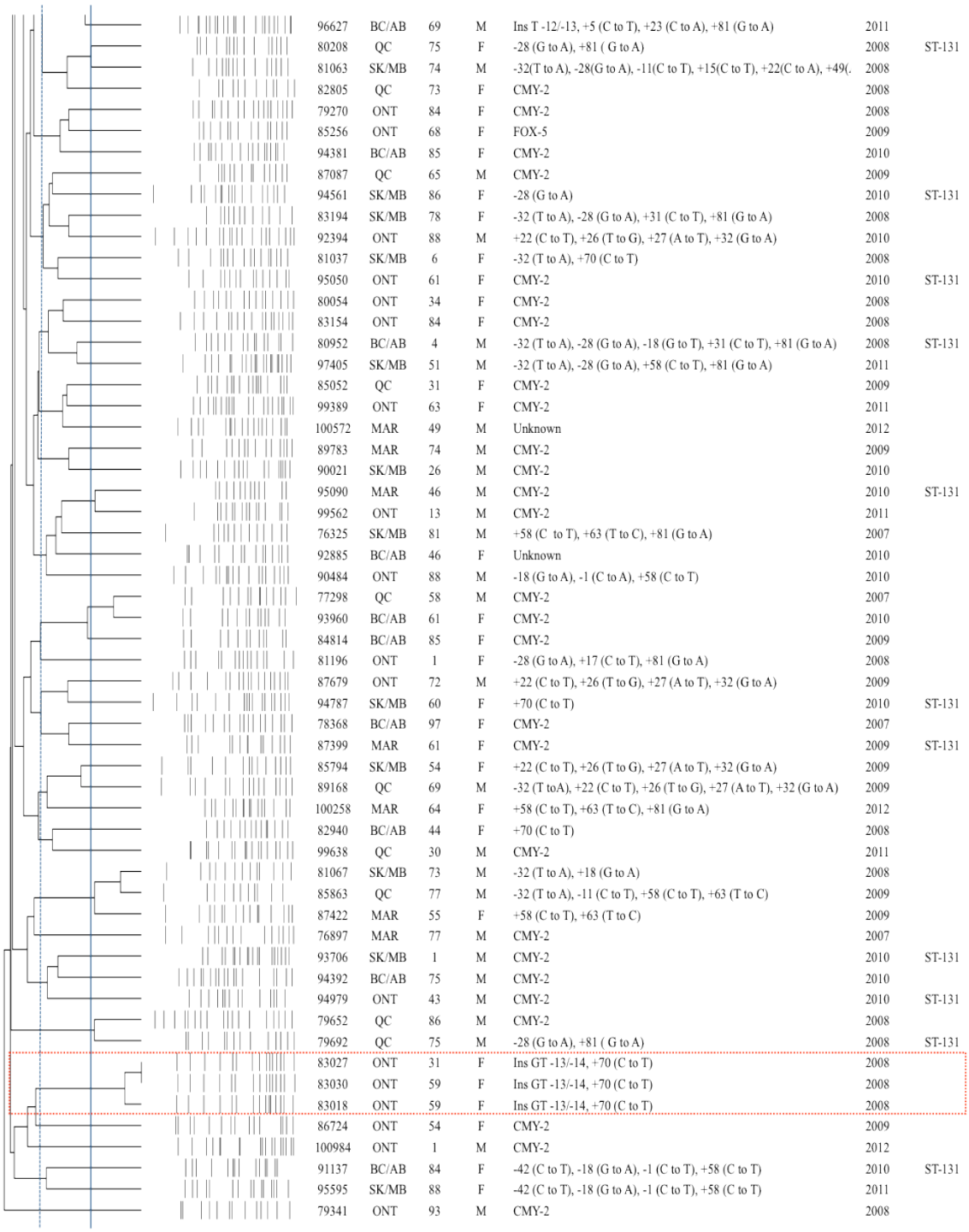
5.6.2.1 Pulsed-Field Gel Electrophoresis

PFGE was conducted in order to assess genetic relatedness among AmpC-producing *E. coli* isolated from Canadian hospitals (Figure 10). AmpC-producing *E. coli* were generally unrelated by PFGE (<80% similarity). Two clusters of ST-131 AmpC-producing *E. coli* were identified demonstrating 60% to >80% similarity. One possible outbreak was identified from an Ontario hospital in 2008 and contained three isolates all demonstrating the same mutations within the chromosomal *ampC* gene.

FIGURE 10. PFGE dendrogram of AmpC-producing *E. coli* isolated from Canadian hospitals: CANWARD 2007-2012[a,b].



STOCK	REGION	AGE	GENDER	GENOTYPE	YEAR	ST-131
80851	BC/AB	22	F	CMY-2	2008	
82713	ONT	59	M	CMY-2	2008	
86995	BC/AB	62	F	CMY-2	2009	
78967	SK/MB	60	F	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	2008	
84816	BC/AB	81	F	CMY-2	2009	
86864	QC	78	F	CMY-2	2009	
89128	QC	74	F	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2009	
83204	SK/MB	67	M	CMY-2	2008	
84783	BC/AB	73	F	-40 (G to T), -18 (G to A), -1 (C to T), +58 (C to T)	2009	ST-131
92722	ONT	2	M	CMY-2	2010	
80861	BC/AB	63	M	Ins GT -13/14, +58 (C to T), +63 (T to C)	2008	
89414	BC/AB	53	M	CMY-2	2009	
94008	BC/AB	10	M	CMY-2	2010	ST-131
92966	MAR	89	F	CMY-2	2010	
84081	MAR	32	M	CMY-2	2008	
89403	BC/AB	67	F	CMY-2	2009	
85067	QC	74	F	CMY-2	2009	
91151	BC/AB	33	F	CMY-2	2010	ST-131
101587	ONT	65	F	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2012	
98642	ONT	40	F	CMY-2	2011	
95447	QC	85	F	CMY-2	2011	ST-131
88937	ONT	2	F	CMY-2	2009	
96253	MAR	62	M	-32 (T to A), +81 (G to A)	2011	
98002	MAR	90	F	CMY-2	2011	
87509	QC	47	F	CMY-2	2009	
97136	QC	65	F	CMY-2	2011	
98547	ONT	71	M	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2011	
92936	BC/AB	28	F	CMY-2	2010	
91150	BC/AB	88	F	+22 (C to T), + 26 (T to G), +27 (A to T), +32 (G to A)	2010	ST-131
98855	MAR	83	M	CMY-2	2011	
93999	BC/AB	67	M	+70 (C to T)	2010	
94301	MAR	63	F	CMY-2	2010	
95586	SK/MB	56	F	CMY-2	2011	
83341	QC	47	M	CMY-2	2008	
101192	BC/AB	53	M	CMY-2	2012	ST-131
103117	MAR	87	F	Ins GT -13/-14, -28 (G to A), +33 (G to A), +81 (G to A)	2012	ST-131
99773	QC	55	F	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2011	ST-131
92995	MAR	47	F	CMY-2	2010	ST-131
93983	BC/AB	1	F	CMY-2	2010	ST-131
96969	ONT	55	M	CMY-2	2011	ST-131
80379	BC/AB	34	F	-42 (C to T), -28 (G to A), +81 (G to A)	2008	ST-131
82294	ONT	14	M	-28 (G to A), +81 (G to A)	2008	ST-131
101854	BC/AB	79	M	CMY-2	2012	ST-131
83724	SK/MB	58	F	CMY-2	2009	ST-131
80189	QC	53	F	+70 (C to T)	2008	
101134	SK/MB	65	M	-28 (G to A), +81 (G to A)	2012	ST-131
96855	QC	84	F	-28 (G to A), +81 (G to A)	2011	ST-131
88058	ONT	79	F	-28 (G to A)	2009	ST-131
100532	SK/MB	70	F	-28 (G to A), +81 (G to A)	2012	ST-131
91874	MAR	79	M	Unknown	2010	
80240	QC	82	F	-28 (G to A), +81 (G to A)	2008	ST-131
82988	BC/AB	78	M	-28 (G to A), +81 (G to A)	2008	ST-131
82948	BC/AB	88	F	-32 (T to A), -28 (G to A), -18 (G to T), +31 (C to T), +81 (G to A)	2008	ST-131
100711	BC/AB	85	M	Ins GT -13/-14, -28 (G to A), +81 (G to A)	2012	ST-131
86625	BC/AB	65	M	CMY-2	2009	ST-131
84251	QC	80	F	CMY-2	2008	ST-131
101563	ONT	82	M	-28 (G to A), +81 (G to A)	2012	ST-131
95773	SK/MB	48	M	Ins T -12/-13, +5 (C to T), +22 (G to A), +81 (G to A)	2011	
82932	BC/AB	51	F	-12 (A to T), +81 (G to A)	2008	
95989	QC	60	F	Ins T -12/-13, +5 (C to T), +81 (G to A)	2010	



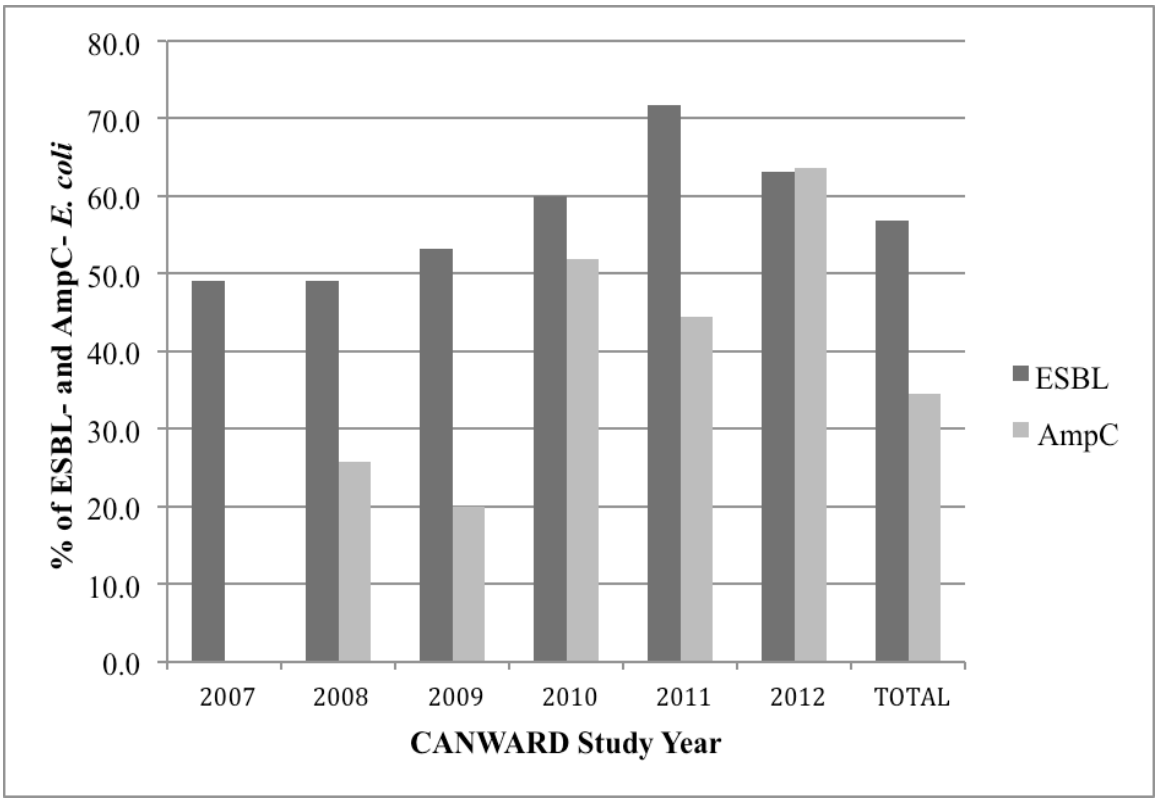
96627	BC/AB	69	M	Ins T -12/-13, +5 (C to T), +23 (C to A), +81 (G to A)	2011		
80208	QC	75	F	-28 (G to A), +81 (G to A)	2008	ST-131	
81063	SK/MB	74	M	-32(T to A), -28(G to A), -11(C to T), +15(C to T), +22(C to A), +49(.	2008		
82805	QC	73	F	CMY-2	2008		
79270	ONT	84	F	CMY-2	2008		
85256	ONT	68	F	FOX-5	2009		
94381	BC/AB	85	F	CMY-2	2010		
87087	QC	65	M	CMY-2	2009		
94561	SK/MB	86	F	-28 (G to A)	2010	ST-131	
83194	SK/MB	78	F	-32 (T to A), -28 (G to A), +31 (C to T), +81 (G to A)	2008		
92394	ONT	88	M	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A)	2010		
81037	SK/MB	6	F	-32 (T to A), +70 (C to T)	2008		
95050	ONT	61	F	CMY-2	2010	ST-131	
80054	ONT	34	F	CMY-2	2008		
83154	ONT	84	F	CMY-2	2008		
80952	BC/AB	4	M	-32 (T to A), -28 (G to A), -18 (G to T), +31 (C to T), +81 (G to A)	2008	ST-131	
97405	SK/MB	51	M	-32 (T to A), -28 (G to A), +58 (C to T), +81 (G to A)	2011		
85052	QC	31	F	CMY-2	2009		
99389	ONT	63	F	CMY-2	2011		
100572	MAR	49	M	Unknown	2012		
89783	MAR	74	M	CMY-2	2009		
90021	SK/MB	26	M	CMY-2	2010		
95090	MAR	46	M	CMY-2	2010	ST-131	
99562	ONT	13	M	CMY-2	2011		
76325	SK/MB	81	M	+58 (C to T), +63 (T to C), +81 (G to A)	2007		
92885	BC/AB	46	F	Unknown	2010		
90484	ONT	88	M	-18 (G to A), -1 (C to A), +58 (C to T)	2010		
77298	QC	58	M	CMY-2	2007		
93960	BC/AB	61	F	CMY-2	2010		
84814	BC/AB	85	F	CMY-2	2009		
81196	ONT	1	F	-28 (G to A), +17 (C to T), +81 (G to A)	2008		
87679	ONT	72	M	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A)	2009		
94787	SK/MB	60	F	+70 (C to T)	2010	ST-131	
78368	BC/AB	97	F	CMY-2	2007		
87399	MAR	61	F	CMY-2	2009	ST-131	
85794	SK/MB	54	F	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A)	2009		
89168	QC	69	M	-32 (T toA), +22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A)	2009		
100258	MAR	64	F	+58 (C to T), +63 (T to C), +81 (G to A)	2012		
82940	BC/AB	44	F	+70 (C to T)	2008		
99638	QC	30	M	CMY-2	2011		
81067	SK/MB	73	M	-32 (T to A), +18 (G to A)	2008		
85863	QC	77	M	-32 (T to A), -11 (C to T), +58 (C to T), +63 (T to C)	2009		
87422	MAR	55	F	+58 (C to T), +63 (T to C)	2009		
76897	MAR	77	M	CMY-2	2007		
93706	SK/MB	1	M	CMY-2	2010	ST-131	
94392	BC/AB	75	M	CMY-2	2010		
94979	ONT	43	M	CMY-2	2010	ST-131	
79652	QC	86	M	CMY-2	2008		
79692	QC	75	M	-28 (G to A), +81 (G to A)	2008	ST-131	
83027	ONT	31	F	Ins GT -13/-14, +70 (C to T)	2008		
83030	ONT	59	F	Ins GT -13/-14, +70 (C to T)	2008		
83018	ONT	59	F	Ins GT -13/-14, +70 (C to T)	2008		
86724	ONT	54	F	CMY-2	2009		
100984	ONT	1	M	CMY-2	2012		
91137	BC/AB	84	F	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2010	ST-131	
95595	SK/MB	88	F	-42 (C to T), -18 (G to A), -1 (C to T), +58 (C to T)	2011		
79341	ONT	93	M	CMY-2	2008		

[a]The dashed and solid blue lines indicate 60% and 80% similarity, respectively; [b]red dashed boxes have been used to highlight related clusters of ST-131 isolates.

5.6.2.2 Detection of the O25b:H4 ST131 Clone Among AmpC-producing *E. coli*

The rate of ST-131 among ESBL- and AmpC-producing *E. coli* isolated between 2007 and 2012 is summarized in Figure 11. In total, 40 (31.7%) of 126 AmpC-producing *E. coli* belonged to the ST-131 clone. AmpC-producing *E. coli* were significantly less likely to belong to the ST-131 clone as compared to ESBL-producing *E. coli* (56.8%, $P<0.001$). The majority [52.5% (21/40)] of ST-131 AmpC-producing *E. coli* belonged to one of two PFGE clusters demonstrating >60% similarity. The rate of ST-131 among AmpC-producing *E. coli* increased across the study period from 0.0% (0/4) in 2007 to 63.6% (7/11) in 2012 ($P=0.014$). Twenty-three (57.5%) of 40 ST-131 AmpC-producing *E. coli* contained promoter/attenuator mutations within the chromosomal *ampC*, while the remaining 42.5% (17/40) produced CMY-2.

FIGURE 11. The proportion of ST-131 ESBL-producing *E. coli* and AmpC-producing *E. coli* isolated from Canadian hospitals by study year.

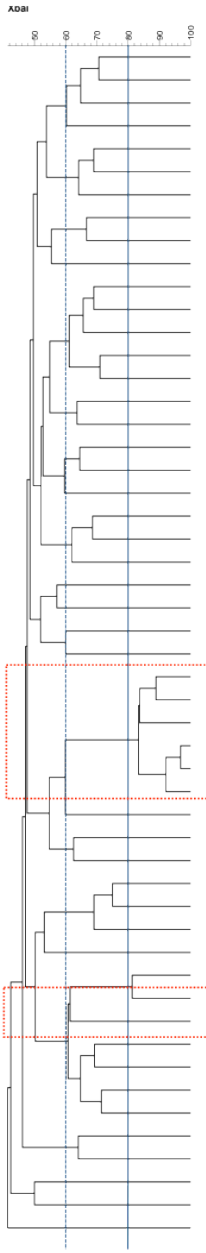


5.6.3 Genetic Relationship Among ESBL-producing *K. pneumoniae*

5.6.3.1 Pulsed-Field Gel Electrophoresis

PFGE was conducted in order to assess genetic relatedness among ESBL-producing *K. pneumoniae* isolated from Canadian hospitals (Figure 12). ESBL-producing *K. pneumoniae* were generally unrelated by PFGE, with 85.2% (46/54) of isolates demonstrating less than 80% similarity. However, two clusters of related isolates (>80% similarity) were identified at two different submitting centres in Ontario. The first cluster contained two isolates that were collected in 2008 and 2009, respectively, both found to produce SHV-12. The second cluster was somewhat larger, containing six isolates all of which produced CTX-M-15 and SHV-1, collected between February of 2009 and February of 2010.

FIGURE 12. PFGE dendrogram of ESBL-producing *K. pneumoniae* isolated from Canadian hospitals: CANWARD 2007-2012[a,b].



STOCK	REGION	AGE	GENDER	GENOTYPE	YEAR
102665	QC	32	F	CTX-M-15, SHV-28, TEM-1	2012
93739	QC	42	M	Unknown, TEM-1	2010
87728	ONT	50	F	SHV-12	2009
79105	QC	44	M	Unknown, SHV-1	2008
93881	ONT	56	M	CTX-M-14, SHV-1	2010
98662	ONT	36	M	CTX-M-15, SHV-1, TEM-1, OXA-1	2011
79260	ONT	33	M	SHV-2a	2008
71631	QC	76	M	CTX-M-15, SHV-1	2007
73960	ONT	30	F	CTX-M-15, SHV-5, TEM -1, OXA-1	2007
87195	ONT	1	F	CTX-M-14, SHV-11	2009
71301	QC	5	F	Unknown, SHV-1	2007
100691	BC/AB	79	M	SHV-2a	2012
95864	QC	42	F	CTX-M-15, SHV-1	2011
102484	BC/AB	35	F	SHV-12	2012
88846	BC/AB	83	F	CTX-M-15, OXA-1	2009
98556	ONT	76	M	CTX-M-15, SHV-1, TEM-1	2011
99014	ONT	60	M	SHV-12	2011
102729	ONT	61	M	Unknown, SHV-1	2012
91509	BC/AB	1	F	SHV-12, TEM-1	2010
99613	QC	1	M	CTX-M-15, SHV-1, TEM-1	2011
101870	BC/AB	67	F	SHV-2a, TEM-1	2012
76790	ONT	50	M	CTX-M-15, SHV-28, OXA-1	2007
82077	BC/AB	37	M	SHV2a, OXA-1	2008
77808	QC	80	M	Unknown, SHV-1	2007
91246	ONT	73	M	CTX-M-15, OXA-1	2010
77575	QC	66	M	CTX-M-2, SHV-11	2007
83054	ONT	52	F	SHV-12, TEM-1	2008
86717	ONT	41	M	CTX-M-15, SHV-1, OXA-1	2009
88648	ONT	41	M	CTX-M-15, SHV-1, OXA-1	2009
86781	ONT	41	M	CTX-M-15, SHV-1	2009
90500	ONT	42	F	CTX-M-15, SHV-1, OXA-1	2010
92409	ONT	42	M	CTX-M-15, SHV-1, OXA-1	2010
88117	ONT	89	M	CTX-M-15, SHV-1, TEM-1, OXA-1	2009
99282	QC	57	M	CTX-M-15, SHV-1, TEM0-1	2011
94296	ONT	55	F	CTX-M-15, SHV-11, TEM-1, OXA-1	2010
96687	BC/AB	26	M	CTX-M-27, SHV-11	2011
80817	BC/AB	62	F	SHV2a	2008
81261	ONT	26	M	CTX-M-15, SHV-11, TEM-1, OXA-1	2008
88378	ONT	2	F	CTX-M-15, SHV-11	2009
99230	ONT	2	F	SHV-2	2011
80026	ONT	76	F	CTX-M-15, SHV-12, TEM-1	2008
86231	ONT	75	M	SHV-12, TEM-1	2009
80008	ONT	81	M	CTX-M-15, SHV-11, TEM-1, OXA-1	2008
88624	ONT	64	M	CTX-M-14, SHV-11	2009
90476	ONT	74	F	CTX-M-14, SHV-11	2010
103159	ONT	68	M	CTX-M-15, SHV-11, TEM-1	2012
89263	ONT	67	M	SHV-31, TEM-1	2009
80103	QC	41	F	CTX-M-14, SHV-11, TEM-1	2008
90088	SK/MB	63	F	CTX-M-15, SHV-168	2010
89787	MAR	35	M	SHV-2a	2009
93690	SK/MB	40	M	CTX-M-15, SV-11, TEM-1, OXA-1	2010
80566	BC/AB	68	M	CTX-M-15, SHV-11, TEM-1	2008

[a]The dashed and solid blue lines indicate 60% and 80% similarity, respectively; [b]red dashed boxes have been used to highlight closely related clusters of isolates.

5.7 Phylogenetic Grouping and Virulence: ESBL-producing *E. coli*

Of the ESBL-producing *E. coli* collected in this study, 14.9% (40/269) belonged to the commensal phylogenetic group A, while 62.8% (169/269) and 22.3% (60/269) belonged to phylogenetic group B2 and D, respectively, associated with virulent extra-intestinal strains. No ESBL-producing *E. coli* were found to belong to phylogenetic group B1. The proportion of ESBL-producing *E. coli* belonging to phylogenetic groups A, B2, and D is summarized by study year in Figure 13. No statistically significant change in the proportion of isolates belonging to any one specific phylogenetic group was observed from 2007 to 2012. However, it is notable that the proportion of ESBL-producing *E. coli* belonging to group B2 increased from 2007 (54.7%) to 2012 (65.8%), while the proportion of isolates belong to group D decreased (2007: 34.0%; 2012: 18.4%). In total, 99.3% (152/213) of ST-131 isolates belonged to phylogenetic group B2, comprising 89.9% (152/169) of all group B2 isolates.

The five key virulence factors associated with ExPEC were detected among ESBL-producing *E. coli* in the following proportions: 83.3% (224/269) *iutA*, 62.8% (169/269) *kpsMII*, 26.4% (71/269) *papA/C*, 21.2% (57/269) *afa*, 0.7% (2/269) *sfa*. In total, 68.4% (184/269) of ESBL-producing *E. coli* were classified as ExPEC (≥2 of the above virulence factors). Within each group, 12.5% (5/40), 41.7% (25/60), and 91.1% (154/169) of those isolates belonging to phylogenetic groups A, D, and B2 were classified as ExPEC ($P<0.001$). ExPEC was identified among 94.8% (145/153) of ST-131 ESBL-producing *E. coli* isolates, while only 50.6% of non-ST-131 isolates were ExPEC ($P<0.001$). The virulence genotypes of ESBL-producing *E. coli* are further summarized in Table 21 and have been organized according to sequence type.

FIGURE 13. The proportion of ESBL-producing *E. coli* isolated from Canadian hospitals that belong to phylogenetic groups A, B2, and D.

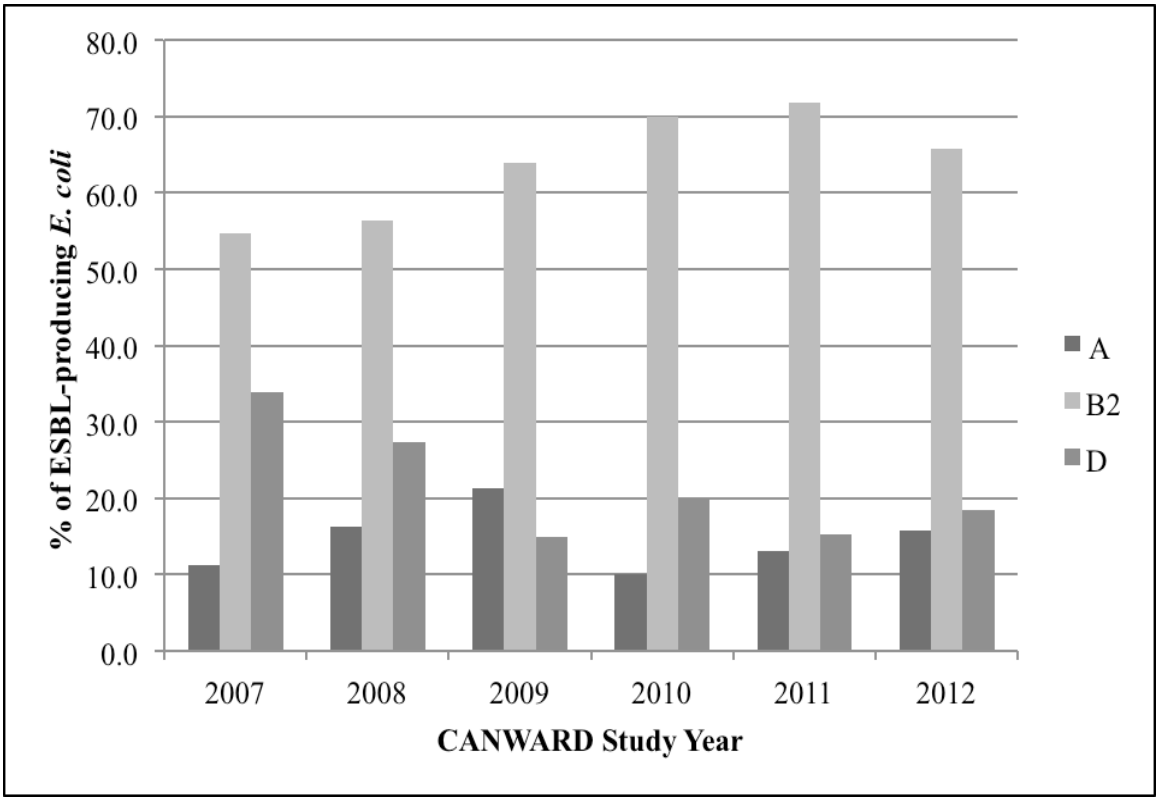


TABLE 21. Virulence genotype of ST-131 and non-ST-131 ESBL-producing *E. coli* isolated from Canadian Hospitals.

ST (% overall, n) ExPEC? (% cohort, n)	Genotype	Count	% cohort
ST-131 (56.9, 153)			
ExPEC (94.8, 145)	*iutA, kpsII*	53	36.6
	papA/C, iutA, kpsII	39	26.9
	afa, iutA, kpsII	28	19.3
	afa, iutA	10	6.9
	papA/C, iutA	6	4.1
	papA/C, afa, iutA	3	2.1
	papA/C, afa, iutA, kpsII	3	2.1
	papA/C, afa, kpsII	1	0.7
	papA/C, kpsII	1	0.7
	papAC, iutA, kpsII	1	0.7
Non-ExPEC (5.2, 8)	Non-ExPEC	8	100.0
Non-ST-131 (43.1, 116)			
ExPEC (33.6, 39)	*iutA, kpsII*	11	28.2
	papA/C, iutA	8	20.5
	afa, iutA, kpsII	7	17.9
	papA/C, iutA, kpsII	4	10.3
	afa, iutA	3	7.7
	papA/C, kpsII	3	7.7
	papA/C, afa, kpsII	1	2.6
	papA/C, sfa, iutA	1	2.6
	sfa, kpsII	1	2.6
Non-ExPEC (66.4, 77)	Non-ExPEC	77	100.0

5.8 Carbapenem Resistant Enterobacteriaceae: *E. coli* and *K. pneumoniae*

Nationally, 0.03% (2/5,951) of *E. coli* and 0.05% (1/1,828) of *K. pneumoniae* were found to produce a carbapenemase (all harboring bla_{KPC}). Both KPC-producing *E. coli* isolates were collected from Quebec in temporally independent cases (2010 and 2011, respectively). The one KPC-producing *K. pneumoniae* identified was isolated in 2009 from a patient in Ontario.

Both KPC-producing *E. coli* were isolated from respiratory specimens collected from male patients aged 77 and 74, respectively, located in the intensive care unit. One KPC-producing *K. pneumoniae* isolate was obtained from the blood culture of a 67 year-old female patient located on a medical ward.

Detailed antimicrobial susceptibility testing information for KPC-producing isolates is summarized in Table 22. All KPC-producing isolates demonstrated *in vitro* resistance to amoxicillin/clavulanate, cefazolin, ceftriaxone, ceftazidime, piperacillin/tazobactam, ertapenem, ciprofloxacin, and trimethoprim-sulfamethoxazole, while all remained susceptible to colistin and tigecycline. Only the one KPC-producing *K. pneumoniae* isolate demonstrated *in vitro* resistance to meropenem according to current CLSI breakpoints (MIC: 4 μg/ml), with both *E. coli* isolates having meropenem MICs of 1 μg/ml.

All KPC-producing *E. coli* (n=2) and *K. pneumoniae* (n=1) produced KPC-3. The two KPC-producing *E. coli* isolates co-expressed TEM-1 and were found to be genetically-related (80% similarity) by PFGE.

TABLE 22. Antimicrobial activity against KPC-producing *E. coli* and KPC-producing *K. pneumoniae.*

Antibiotic	MIC (µg/ml)		MIC Interpretation[b]		
	E. coli (n=2)[a]	*K. pneumo.* (n=1)	*E. coli* 1	*E. coli* 2	*K. pneumo.*
AMC[c]	>32, >32	>32	R	R	R
Cefazolin	>128, >128	>128	R	R	R
Cefoxitin	16, 16	>32	I	I	R
Ceftriaxone	32, 64	>64	R	R	R
Ceftazidime	>32, >32	>32	R	R	R
Cefepime	4, 16	16	S	I	I
TZP[c]	128, 256	512	R	R	R
Ertapenem	2, 8	16	R	R	R
Meropenem	1, 1	4	S	S	R
Ciprofloxacin	>16, >16	>16	R	R	R
Amikacin	32, 8	32	I	S	I
Gentamicin	>32, 2	8	R	S	I
Tigecycline	0.5, 0.25	2	S	S	S
SXT[c]	>8, >8	>8	R	R	R
Fosfomycin	2, 2	N/A[d]	S	S	N/A[d]
Nitrofurantoin	8, 16	N/A[d]	S	S	N/A[d]
Colistin	0.5, 0.25	0.25	S	S	S

[a]MIC *E. coli* isolate 1, MIC *E. coli* isolate 2; [b]S: susceptible (green), I: intermediate (grey), R: resistant (red); [c]AMC: amoxicillin/clavulanic acid, TZP: piperacillin/tazobactam, SXT: trimethoprim-sulfamethoxazole; [d]Fosfomycin and nitrofurantoin were not tested against KPC-producing *K. pneumoniae* and are therefore not applicable (N/A).

6. DISCUSSION

The central hypothesis of this thesis was that the prevalence of oxyimino-cephalosporin resistance would increase among *E. coli* and *K. pneumoniae* isolates causing infectious diseases in Canadian hospitals. We speculated that oxyimino-cephalosporin resistance in these species is primarily driven by ESBL-production and that any increase in resistance to these agents would be largely attributable to an increase in the prevalence of ESBL-producing isolates. We believed that ESBL-producing *E. coli* and *K. pneumoniae* would mainly produce CTX-M-type ESBLs, frequently display an MDR phenotype, and ST-131 would continue to drive increases in ESBL-production among *E. coli* isolates.

6.1 Cephalosporin Resistance Among *E. coli* and *K. pneumoniae* isolated from Canadian Hospitals

In the time since their release, oxyimino-cephalosporins have become workhorse antibiotics in hospitals all across the world for a variety of clinical presentations (20). Resistance to these agents has been monitored closely in the last 10 years by the Study for Monitoring Antimicrobial Resistance Trends (SMART), where in 2003, worldwide susceptibility rates to ceftriaxone/ceftazidime were estimated at 90.2%/89.7% and 84.3%/84.3% in *E. coli* and *K. pneumoniae*, respectively (86). More recently, SMART has reported statistically significant decreases in ceftriaxone and ceftazidime susceptibility from 2005 to 2010 among North American *E. coli* and *K. pneumoniae* isolates (87). In this study, ceftriaxone/ceftazidime susceptibility decreased from 96.1%/96.1% and 96.8%/96.8% in 2005 to 89.5%/91.1% and 85.5%/86.2% in 2010 among *E. coli* and *K. pneumoniae*, respectively (87).

In Canada, resistance to these agents has been slower to develop, where for example, it was estimated that >99% of *E. coli* and >98.5% of *K. pneumoniae* isolated from Canadian hospitals between 1997 and 1998 were susceptible to ceftriaxone and ceftazidime as reported by the SENTRY Antimicrobial Surveillance Program (88). It is notable that susceptibility rates to oxyimino-cephalosporins from the United States were slightly lower in this study as compared to the Canadian data (88). The cause of this difference is likely multifactorial and attributable to differences in population, antimicrobial usage, and infection control practices. Results from the CAN-ICU Study reported that by 2005-2006, ceftriaxone resistance rates had reached 3.7% and 0.4% in *E. coli* and *K. pneumoniae* isolated from Canadian ICUs, respectively (89). The results of this thesis indicate that these rates continue to climb, with overall resistance to ceftriaxone increasing in *E. coli* from 5.1% in 2007 to 8.4% in 2012. Perhaps even more astounding, ceftriaxone resistance among ICU *E. coli* and *K. pneumoniae* isolates increased approximately five- (18.8% vs. 3.7%) and seven-fold (2.8% vs. 0.4%), respectively, when 2012 CANWARD data is compared to that of CAN-ICU (2005-2006). It is notable that temporal comparisons of antimicrobial susceptibly are limited by variations in the breakpoints used. This limitation is specifically relevant to the lowering of the breakpoints for ceftriaxone and ceftazidime by CLSI in 2010.

6.2 The Prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in Canadian Hospitals

E. coli was the top ranked organism collected during the CANWARD study, comprising 20.1% of all isolates, while *K. pneumoniae* ranked fifth overall and third among Gram-negative organisms, comprising 6.1% of all isolates collected (3). The

national rates of ESBL-producing *E. coli* and *K. pneumoniae* from 2007 to 2012 were found to be 4.5% (Minimum: 2.9%; Maximum: 7.6%) and 3.0% (Minimum: 1.5%; Maximum: 4.0%), respectively. These rates remain lower that those reported by the SMART (2009-2010) study, where 8.5% and 8.8% of North American *E. coli* and *K. pneumoniae* isolates were found to produce an ESBL, respectively (90). With respect to Canadian data, the rate of ESBL-producing *E. coli* is comparable to that published by Peirano *et al.* in a study of *E. coli* blood culture isolates from the Calgary Health Region collected between 2000 and 2010 (39). In a similar study by the same group, the rate of ESBL-producing *K. pneumoniae* was found to be much lower [0.6% (89/15371)] than what is reported here (91). Nationally, the proportion of *E. coli* and *K. pneumoniae* isolates collected that produced an ESBL increased significantly during the study period, and in the case of *E. coli*, all regions demonstrated an increase in prevalence with the increases in Ontario and Quebec attaining statistically significance. Though the proportion of ESBL-producing *K. pneumoniae* demonstrated variability, at least in part due to the decreased overall number of *K. pneumoniae* collected in comparison to *E. coli*, the prevalence of these organisms also appears to be trending up in Canadian hospitals.

6.3 Molecular Epidemiology of ESBL-producing *E. coli* and *K. pneumoniae*

We speculated that the observed increase in ESBL-producing *E. coli* was largely driven by the continued success of the O25b:H4 ST-131 clone, reflected in the growing proportion of ST-131 isolates among our cohort. Similarly, Peirano *et al.* reported a clear association between ST-131 and a significant increase in the rate of ESBL-producing *E. coli* bloodstream infections since 2007 in the Calgary health region (39). In addition, this clone represents the major factor influencing the spread of CTX-M-15 in Canadian

hospitals, with 121 of 179 (68%) CTX-M-15-producers belonging to ST-131. These data are consistent with reports from the USA by Johnson *et al.* where ST-131 comprised 56% of CTX-M-15-producing isolates (92).

Factors driving the observed increase in ESBL-producing *K. pneumoniae* were less clear due in part to genotypic diversity and a lack of ST data. A recent report from Canada does however indicate the absence of any one dominant ST promoting the spread of ESBL-producing *K. pneumoniae*, where the major sequence types ST-17, ST-20, ST-573, and ST-575 comprised only 32% of isolates (91). While a large number of ESBL-producing *K. pneumoniae* (48%) were found to produce CTX-M-15, it is difficult to justify that any one successful plasmid or mobile element is currently influencing spread, as 13 different genotypes were identified among 26 CTX-M-15-producing isolates. Though indicative of a largely polyclonal cohort, this does not rule out the possibility that one or more dominant STs are responsible for the observed trends. For example, in a study by Ko *et al*., 70% of ESBL-producing *K. pneumoniae* from Korea belonged to ST-11, while multiple genotypes were identified within this ST indicating several acquisition events by this clone (93). In order to further delineate the molecular basis underlying the spread of ESBL-producing *K. pneumoniae* in Canadian hospitals, additional investigations are required.

6.4 Lowered CLSI Cephalosporin Breakpoints and the Clinical Detection of ESBL-producing Isolates

Recently, there has been considerable debate regarding whether it is necessary to utilize ESBL detection methods in the case of isolates with elevated MICs to extended-spectrum cephalosporins and the clinical relevance of such testing (94). We feel that it is

important to highlight that 1.1%, 33.8%, and 54.0% of ESBL-producing *E. coli* and 14.8%, 31.9%, and 61.4% of ESBL-producing *K. pneumoniae* in this study demonstrated *in vitro* susceptibility to ceftriaxone, ceftazidime, and cefepime, respectively, based on current cephalosporin breakpoints. These data are relevant to the recent CLSI and EUCAST recommendations that lowered cephalosporin breakpoints are sufficient for the detection of resistance genes and clinical decision making with regards to antibiotic selection (68, 95). Though the combination of MIC-based screening criteria and phenotypic methods for the detection of ESBL production does not represent a perfect system, the use of such tests in a clinical setting would provide further clarity when administering antimicrobial therapy and is important to the implementation of proper infection control. In addition, the recent push towards rapid diagnostics has brought a number of alternative detection methods such as real-time PCR, microarray technology, and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), which may be better suited for ESBL and β-lactamase detection within the clinical microbiology laboratory. It should be noted however that widespread implementation of some of these "newer" methods is still a few years away.

6.5 Prevalence and Molecular Epidemiology of AmpC-producing *E. coli* in Canadian Hospitals

The prevalence of AmpC-producing *E. coli* increased nationally between 2007 and 2012 including regional increases in British Columbia/Alberta and Quebec. With an overall rate of 2.5% among all *E. coli* collected, AmpC-producing isolates remain a relevant cause of antimicrobial resistant infections in Canadian hospitals and require continued surveillance. The molecular basis of AmpC-mediated resistance in this study

resulted from approximately equal proportions of isolates harboring mutations within the chromosomal *ampC* gene and those producing plasmid-mediated CMY-2, consistent with previous reports (29, 47, 70).

Studies investigating AmpC-producing *E. coli* in Canadian hospitals are limited. Initial work was conducted over a nine-month period in 1998/1999 where Forward *et al*. carried out an investigation of AmpC-producing *E. coli* at the nine-south central Ontario medical centres of the Toronto Invasive Bacterial Disease Network (96). In this study, a total of 30 AmpC-producing strains were identified, of which 22 contained chromosomal *ampC* promoter mutations, 26 contained mutations within the attenuator region, and only two isolates contained no mutations in either region (96). It is important to note however, that this study was limited by an absence of data regarding acquired AmpC β-lactamase genes. Shortly afterwards, Mulvey *et al.* carried out the first national investigation on AmpC-producing *E. coli*, where 29,323 *E. coli* strains were screened at 12 participating medical centres between 1 October 1999 and 30 September 2000 (97). In total, 182 unique strains were identified from a total of 232 putative AmpC-producing *E. coli*, of which 166 were found to contain at least one mutation within the chromosomal *ampC* gene, while only 25 strains were found to produce an acquired AmpC β-lactamase, all of which produced CMY-2 (97). More recently, Pitout *et al*. reported on the prevalence and molecular characteristics of AmpC-producing *E. coli* isolated from the Calgary health region from January 2000 to December 2003, and again from April 2006 to March 2007 (98, 99). From 2000 to 2003, the incidence of AmpC-producing *E. coli* increased each year from 0.1% in 2000 to 1.3% in 2003, and CMY-type acquired AmpC β-lactamases were identified in 34% of isolates (98). Promoter/attenuator mutations were not sought in

this study and only a random sample of the CMY-producing *E. coli* underwent further genotypic testing, all of which were found to produce CMY-2 (98). Subsequent study indicated the prevalence of AmpC-producing *E. coli* has not continued to climb in the Calgary health region, as a rate of 1.2% was reported for 2006/2007, however more recent study is required (99).

A subset of isolates from the Mulvey *et al*. study were further analyzed by Tracz *et al*. in order to investigate the role promoter/attenuator mutations play on the development of *ampC* overexpression and cefoxitin resistance (100). Here it was determined that mutations within the -35 box, the alternate -35 box (position -42), and those insertion mutations idealizing the spacer region between the -10 and -35 boxes were most important for *ampC* hyperproduction, generating eight- to 46-fold increases in expression (100). In relation to the data from this thesis, 40.7% of isolates contained at least one of these "key" mutations, with 18.5% of isolates containing a -32 mutation reported to be the greatest contributor of increased *ampC* promoter strength. Interestingly, it was noted that mutations within the -10 box and the attenuator had little impact on gene expression unless combined with -35 box or spacer mutations and that in these strains, overexpression was usually attributable to the insertion element IS10 and the strong pOUT promoter (100).

The observed increase in the prevalence of AmpC-producers among *E. coli* isolates collected in this study is likely multifactorial, resulting from clonal spread of virulent strains and the dissemination of conjugative plasmids as previously described by Baudry *et al.*, as well as increased selection pressure for chromosomal *ampC* hyperproducers (47). While the emergence and continued proliferation of ST-131 is

undoubtedly a key factor driving increased prevalence among ESBL-producing isolates, the data with regards to AmpC-producing isolates is less clear. Though there is some data from this thesis that would suggest the proportion of ST-131 AmpC-producing *E. coli* has increased in recent years, it is difficult to draw valid conclusions due to the relatively small number of AmpC-producing isolates collected, temporal intra-study variations in isolate collection, and a lack of MLST data. Furthermore, ST- analysis of AmpC-producing *E. coli* is lacking and recent data for comparison is extremely limited. It is notable that in one recent study from 2012, eight different STs were identified among 12 CMY-2-producing *E. coli* isolates, suggesting no major clone is influencing spread (101). The applicability of this data is limited as the isolates were collected from medical centres in France, Israel, Spain, and Italy, and are potentially of little relevance to Canada. Additionally, the bacterial isolates were cultured from rectal swabs and are not necessarily indicative of those isolates causing clinical disease in humans.

6.6 Carbapenemases and the Shifting Paradigm: Seeing the World Beyond Your Front Door

The carbapenems have long been considered agents of last resort for the treatment of MDR Enterobacteriaceae (50, 102). In the last decade, however, carbapenem resistance has grown among Enterobacteriaceae and is largely attributable to the production of carbapenemase enzymes. Such organisms are typically resistant to multiple antimicrobials and in some cases, no suitable treatment options remain (26, 65). Globally, the epidemiology and prevalence of carbapenemase-producing Enterobacteriaceae is highly varied and while such organisms have been slow to emerge within Canadian hospitals, they have become well established in a number of other

countries. As ESBL-producing organisms reach significant rates in Canada, reports from other parts of the world indicate a more serious problem is on the horizon.

6.6.1 KPC-producing Enterobacteriaceae

K. pneumoniae isolates of ST-258 and other members of the Enterobacteriaceae producing KPC-type carbapenemases are prevalent in the United States and many European countries, are highly resistant, spread easily form patient-to-patient, and are a significant cause of morbidity and mortality (51). The ability of such organisms to emerge and spread rapidly is well evidenced by reports from Israel. At one medical centre in Tel Aviv, KPC-producing *K. pneumoniae* isolates were isolated from a total of six patients in 2004 and 2005 combined (103). This was followed by a dramatic increase in 2006, where KPC-producing *K. pneumoniae* were isolated from 45 different patients at the same medical centre and at other hospitals throughout the country leading to a nationwide outbreak with considerable mortality (103-105). In addition, Europe does not represent the closest source for the "importation" of KPC-producing organisms into Canada, as the United States represents a major source of such organisms (51). Within the United States, the northeastern region represents a considerable reservoir of KPC-producing Enterobacteriaceae, where for example, it was estimated that 38% of *K. pneumoniae* isolated from New York hospitals in 2009 possessed the KPC enzyme (106).

6.6.2 NDM-producing Enterobacteriaceae

Though the NDM-1 carbapenemase was first described as recently as 2008, pathogens harboring this enzyme are now arguably the most significant threat in terms of antibiotic resistance worldwide (64). The Indian subcontinent (India, Pakistan, Bangladesh) represents the largest source of NDM-producing Enterobacteriaceae, where

Perry *et al*. have gone so far as to estimate that over 100 million of the citizens of India contain NDM-1-producing gut flora (107). In addition, extensive environmental contamination with NDM-1-producing organisms has been demonstrated in India by Kumarasamy *et al.* and is largely attributable to an inadequate infrastructure and unsanitary conditions (26). Considering the extensive population, foreign travel, and the popularity of the Indian Subcontinent for 'medical tourism' purposes, it is no surprise that NDM -1 has spread so rapidly to Europe, the United States, and Canada (51, 79).

In the case of KPC-, NDM-, and other carbapenemase-producing organisms, it is likely that with time, such organisms will become highly prevalent in Canadian hospitals. Forebodingly, cases of NDM-1-producing infection have already been reported in Canada with no obvious connections to foreign travel (108). As is often the case, by the time a problem presents itself it is often too late. It is for this reason that we must be mindful of the epidemiology of antibiotic resistant pathogens in other countries as to ensure the proper action and precautions are taken. While no one solution exists, infection control and the proper isolation of infected patients is central to minimizing the impact of these organisms in our country. Additionally, it is essential that new antimicrobials be investigated, developed, and brought to market, as the sustainability of our current armamentarium is in question.

6.7 Carbapenemase-producing Enterobacteriaceae in Canadian Hospitals

The identification of carbapenemase-producing isolates was minimal in this study and is limited by the low overall prevalence of such organisms in Canadian hospitals combined with the overall number of isolates collected. The first carbapenemase-producer in our cohort was received in January of 2009, shortly after KPC-producing

Enterobacteriaceae first appeared in Canadian hospitals (109). Globally, the prevalence of carbapenemase-producing *E. coli* and *K. pneumoniae* is largely dependent on region. From 2007 to 2009, the rate of carbapenemase-producing *E. coli* and *K. pneumoniae* was estimated to range between 1.8% and 2.4% based on USA, European, and Latin American data as reported by the SENTRY study (110). The national prevalence of carbapenemase resistance among clinical Enterobacteriaceae isolates in Canada is estimated by the Canadian Nosocomial Infection Surveillance Program (CNISP) to be significantly lower at 0.1% (59/52,078) with only 10 isolates confirmed to produce a carbapenemase enzyme (77). If we consider the years 2009 to 2012 of this study, corresponding with the emergence of carbapenemase-producers in Canadian hospitals, the CNISP data are in agreement with findings reported here [0.07% (3/4319)]. The detection of $bla_{\text{KPC-3}}$ in all carbapenemase-producers in this study is consistent with reports from Mataseje *et al.* where 7 of 10 carbapenemase-producing Enterobacteriaceae isolated from Canadian hospitals produced this variant (77).

Though the overall prevalence of carbapenemase-producing Enterobacteriaceae is low in Canada, some interesting case reports have appeared. In one such report, an outbreak of five NDM-1-producing *K. pneumoniae* occurred at a tertiary care medical centre in Brampton, Ontario, from October 2011 to November 2011 (108). Here, no source patient with travel to an endemic area was discovered. In addition, four of the five isolates demonstrated *in vitro* susceptibility to tigecycline only while the other isolate was susceptible to tigecycline and colistin (108). Other reports have also described the appearance of OXA-48/OXA-181 in Canadian hospitals (78, 111). Isolates harboring

these enzymes represent a threat to patient outcome as well as infection control as treatment options are limited and laboratory detection is difficult (78).

6.8 Study Limitations

Inherently, surveillance-based research has a number of limitations. Specific to the CANWARD study, such limitations included intrastudy variability in collection criteria and turnover in the participating medical centres. In the CANWARD study there were nine medical centres that participated in all study years from 2007 to 2012. As described in Table 11, statistically significant increases in prevalence were observed in three different regions for ESBL-producing *E. coli* (National, Ontario, and Quebec) and in two different regions for AmpC-producing *E. coli* (British Columbia/Alberta, Quebec). When the analysis of this data is restricted to only those centres present in all study years, significant increases are maintained in all cases except for AmpC-producing *E. coli* isolated from Quebec. This suggests variations in the participating medical centres did not drastically alter the trends observed in this study, though the effects appear to be more influential in the case of AmpC-producing *E. coli* where the overall cohort is smaller. It is notable that the overall number of *E. coli* and *K. pneumoniae* isolates collected decreased in the latter years of this study, therefore reducing statistical power. This trend was due to decreased isolate collection and is likely more influential in the case of *K. pneumoniae*, as a relatively small number of isolates were collected in 2011 and 2012 specifically. Completion of MLST on ESBL-producing *K. pneumoniae* and non-ST-131 *E. coli* isolates would provide further insight into the epidemiology of ESBL- and AmpC-producing organisms in Canada; however, such testing is time-consuming and expensive, as mentioned previously. The CANWARD study also received little demographical

information as part of the data collection. Accordingly, epidemiological assessments were limited to the observation of clusters; true outbreaks could not be determined. Previous hospital stays, antimicrobial usage and patient outcomes could not be assessed as part of this study. Lastly, as the prevalence of carbapenemase-producing Enterobacteriaceae is low in Canada, it is likely that sample size was a limiting factor in the accurate study of these organisms. Other investigations that are more specifically designed to study carbapenemase-producers are therefore required, such as the CNISP study described previously.

7. CONCLUSIONS

This thesis presents data on the prevalence of ESBL-producing *E. coli*, AmpC-producing *E. coli*, ESBL-producing *K. pneumoniae*, and carbapenemase-producing *E. coli* and *K. pneumoniae* isolated from Canadian hospitals from 2007 to 2012. Infections caused by ESBL-producing isolates increased dramatically during this study and such isolates now represent a major cause of antimicrobial resistant infections in Canada. From 2007 to 2012, the prevalence of ESBL-producing *E. coli* increased from 3.4% to 7.6%, while the prevalence of ESBL-producing *K. pneumoniae* increased from 1.5% to 3.6%. Though the incidence of AmpC-producing infections was slightly lower, the prevalence of these organisms also increased from 0.7% in 2007 to 2.2% in 2012.

Infections caused by ESBL-producing *E. coli*, AmpC-producing *E. coli*, and ESBL-producing *K. pneumoniae* were demographically diverse. ESBL- and AmpC-producing *E. coli* infections occurred most frequently in females, while ESBL-producing *K. pneumoniae* were isolated more frequently from males. A significant proportion of ESBL- and AmpC-producing infections occurred in patients ≥65 years of age. ESBL- and AmpC-producing infections were distributed across all specimen sources and all hospital locations, though they were more commonly isolated from blood and urine specimens in an inpatient setting.

Treatment options for ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae* were limited for the isolates in this study with 78.8% and 66.7%, respectively, demonstrating a MDR phenotype. AmpC-producing *E. coli* were generally found to be less resistant to antimicrobials, with 34.9% demonstrating a MDR phenotype.

Antimicrobials with the greatest activity against the isolates tested in this study include amikacin, ertapenem, meropenem, colistin, and tigecycline.

In this study, CTX-M-15 was identified as the major ESBL enzyme circulating within Canadian hospitals, though it is notable that ESBL-producing *K. pneumoniae* were more genotypically diverse as compared to ESBL-producing *E. coli*. The ST-131 clone is an important driving force behind the expansion of ESBL-producing *E. coli* within Canadian hospitals, specifically those producing CTX-M-15. While the proportion of ST-131 ESBL-producing *E. coli* increased along with the prevalence of these organisms, this clone does not appear to be as important in the case of AmpC-producing *E. coli*. CMY-2 has consistently remained the dominant acquired AmpC β-lactamase in Canada, both prior to and throughout this study.

Currently, the prevalence of carbapenemase-producing *E. coli* and *K. pneumoniae* is low in Canada, with 0.06% and 0.09% of *E. coli* and *K. pneumoniae*, respectively, found to produce a carbapenemase enzyme from 2009 to 2012. Though these organisms first appeared in Canada as recently as 2009, we believe that through increased selection pressure due to the rising incidence of ESBL-producing isolates and the influence of foreign travel, it is highly likely that the rate of carbapenemase-producers in Canadian hospitals will continue to increase.

8. FUTURE STUDIES

This thesis provides a thorough report on the prevalence and molecular epidemiology of ESBL-producing *E. coli*, AmpC-producing *E. coli*, ESBL-producing *K. pneumoniae*, and carbapenemase-producing *E. coli* and *K. pneumoniae* isolated from Canadian hospitals during a six year time period. Future studies are required in order to address additional questions raised by this research. Such studies should be conducted as follows:

(A) Continued surveillance of ESBL- and AmpC-producing isolates

This study has shown the prevalence and molecular epidemiology of ESBL- and AmpC-producing *E. coli* and *K. pneumoniae* to be dynamic within Canadian hospitals as well as nationally varied. It is important to continue to monitor the emergence of these organisms within Canada in order to understand how the trends identified by this thesis continue in the future. Such trends include the continued proliferation of ST-131 and CTX-M-15 among ESBL-producing *E. coli*, as well as the potential emergence of other genes such as CTX-M-27. Continued independent research of ESBL-producing organisms is also important within the context of the recent CLSI recommendation that lowered cephalosporin breakpoints are sufficient for the detection of resistance determinants. As this recommendation is adopted within clinical microbiology laboratories, it is likely that ESBL-producing organisms will be under reported at those centres, causing a potential lapse in infection control.

(B) Continued surveillance of carbapenem resistant Enterobacteriaceae

Carbapenemase-mediated resistance is an emerging issue within Canadian hospitals with serious implications for patient outcome. It is therefore essential that these organisms be

monitored closely over time to better understand their distribution and prevalence in Canada. A complete understanding of these organisms is also important to the clinical microbiology laboratory in order to ensure that the lowered CLSI carbapenem breakpoints are effective in identifying putative carbapenemase-producers and predicting treatment success *in vivo*.

(C) Sequence typing of ESBL- and carbapenemase-producing isolates

The implementation of MLST on non-ST-131 *E. coli* isolates and all ESBL-producing *K. pneumoniae* would yield a fuller understanding of the organisms circulating within Canadian hospitals. In the case of ESBL-producing *K. pneumoniae*, sequence typing data is lacking within the literature and would provide valuable insight into their epidemiology. Similarly, as a much smaller proportion of AmpC-producing *E. coli* were found to be ST-131 as compared to ESBL-producing *E. coli*, sequence typing would again yield valuable insight.

(D) Sequencing of ESBL-producing plasmids

This thesis identified a large number of ST-131 CTX-M-15-producing *E. coli* isolates that are difficult to differentiate based on the various methods used. Through advances in next generation sequencing it is now possible to sequence and annotate large DNA molecules. The study of ESBL-producing plasmids would provide considerable information on the genetic relatedness and spread of these organisms in Canada, specifically at the nucleotide level.

(E) *In vitro* testing of investigational antimicrobials

The isolates in this study represent a diverse cohort of genotypically characterized clinical strains demonstrating both MDR and XDR phenotypes. As resistance continues

to accumulate among Gram-negative organisms, specifically within the Enterobacteriaceae, our current armamentarium has become compromised. Though the development of new antimicrobials has slowed, multiple new agents are currently being developed specifically targeting those organisms described here. This cohort provides a useful tool in the evaluation of new investigational antimicrobials in order to better assess which candidates demonstrate adequate *in vitro* activity and warrant further investment.

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