Characterization of *Escherichia coli* and *Klebsiella pneumoniae* with Resistance or Reduced Susceptibility to Carbapenems isolated from Canadian Hospitals from 2007-2010

By

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Abstract

Escherichia coli and *Klebsiella pneumoniae* are among the most prevalent organisms causing infections in Canada. Their frequent association with multi-drug resistance (MDR-defined as concomitant resistance to ≥ 3 different antimicrobial classes) has challenged traditional treatment options which include the use of β-lactam antibiotics. Although, still rare, carbapenem resistance in Enterobacteriaceae (defined as meropenem MIC ≥ 4ug/ml) has emerged in Canada. Among *E. coli* and *K. pneumoniae*, the most common carbapenem-resistant mechanism is by *Klebsiella pneumoniae* carbapenemase (KPC) production, although resistance is not limited to this mechanism solely. Other methods of resistance include extended-spectrum β-lactamase (ESBL) and/or AmpC production coupled with outer membrane porin (OMP) alterations. This thesis focuses on *E. coli* and *K. pneumoniae* that demonstrate elevated minimum inhibitory concentrations (MIC's) to ertapenem (≥0.12 μg/ml). Although these are not "carbapenem-resistant" strains they are not wild-type carbapenem susceptible strains and are designated as "carbapenem reduced susceptible-CRS" *E. coli* and *K. pneumoniae* throughout this thesis.

In this study, all bacterial isolates were obtained from the Canadian Ward Surveillance Study (CANWARD). All isolates first underwent *in vitro* susceptibility testing to determine prevalence and antimicrobial resistance patterns. Any *E. coli* or *K. pneumoniae* isolate with an ertapenem MIC of ≥0.12 μg/ml were selected for further analysis. Isolates with an MIC of 0.12 and 0.25 μg/ml were termed CRS; those ≥0.5 μg/ml were termed CIR (carbapenem intermediate/resistant). The prevalence was found to be relatively stable over the years although there was an increase in prevalence among the *K. pneumoniae* isolates; 1.1% to 1.3% to 2.5% to 2.6% in 2007, 2008, 2009, and 2010, respectively.

To understand the molecular epidemiology of these isolates, genotypic characterization was conducted on ESBL, AmpC, carbapenemase genes, as well as the major nonspecific porins. The highest proportion of isolates were found to produce CTX-M-15 β-lactamase. Only 1 KPC-producing *E. coli* and 1 KPC-producing *K. pneumoniae* was found. Porin alteration was found to be a factor leading to carbapenem reduced susceptibility among isolates, especially among *K. pneumoniae* when combined with CTX-M β-lactamase production. In Canada, porin alteration combined with ESBL and/or AmpC β-lactamase production has been shown to be the major mechanism of carbapenem resistance which is consistent with the above findings.

To determine the genetic relatedness of CRS/CIR *E. coli* and *K. pneumoniae*, pulsed-field gel electrophoresis was carried out. The spread of these organisms was mainly due to polyclonal spread rather than one specific clone. This may explain why the prevalence in Canada has remained low.

Carbapenem resistance in Canada is an emerging issue to which attention must be given as very few treatment options remain once carbapenem resistance develops. This shows the need for ongoing surveillance and an understanding of the molecular mechanisms leading to carbapenem resistance. Research in this area may help contribute to infection control as well as to help guide therapy and help in the area of new drug development.

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EC	*Escherichia coli*
KP	*Klebsiella pneumoniae*
ETEC	Enterotoxigenic EC
EPEC	Enteropathogenic EC
EHEC/STEC	Enterohemorrhagic EC/Shiga toxin producing EC
EAEC	Enteroaggregative EC
EIEC	Enteroinvasive EC
DAEC	Diffure Adhearing EC
NAG	*N*-acetyl Glucosamine
NAM	*N*-acetly Muramic acid
PBP	Penicillin Binding Protein
ESBL	Extended-Spectrum β-lactamase
AmpC	AmpC β-lactamase
MDR	Multidrug Resistant
CARB	Carbenicillin-hydrolyzing β-lactamase
KPC	*Klebsiella pneumoniae* Carbapenemase
CRS	Carbapenem Reduced Susceptible
CIR	Carbapenem Intermediate/Resistant
OMP	Outer Membrane Protein
MBL	Metallo-β-Lactamase
ATCC	American Type Culture Collection
CLSI	Clinical Laboratory Standards Institute
BLAST	Basic Local Alignment Tool
CANWARD	Canadian Ward Surveillance Study
MIC	Minimum Inhibitory Concentration
MHT	Modified Hodge Test
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
MLST	Multilocus Sequence Typing
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
bp	Base Pair
kb	KiloBases
V/cm	Volts/Centimeter
rpm	Revolutions/Minute
xg	Specific Gravity
mm	Millimeter
L	Liter
ml	Milliliter
µl	Microliter
M	Molar
mM	Millimolar
ng/ml	Nanogram/ml
EDTA	Ethylene Diamine Tetra Acetic Acid
TE	Tris-HCl EDTA

TBE	Tris-Borate EDTA
TSA-5%SB	Trypticase Soy Agar with 5% Sheeps Blood
APS	Ammonium Persulfate
TEMED	Tetramethylethylenediamine
P/A	Promoter and/or Attenuator Mutations
UTI	Urinary Tract Infection
ER	Emergency Room
CLA	Clavulanic acid
TZP	Piperacillin-Tazobactam
AMC	Amoxicillin-Clavulanic acid
SXT	Trimethoprim-Sulfamethoxazole
ETP	Ertapenem
MEM	Meropenem
DOR	Doripenem
CRO	Ceftriaxone
CAZ	Ceftazidime
FEP	Cefepime
FOX	Cefoxitin
KIVNGO	KPC-2, IMP-4, VIM-2, NDM-1, GES-5, OXA-48

1. Introduction

1.1. Enterobacteriaceae

Enterobacteriaceae fall within the phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales (30). They make up a large family of Gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacteria, most being motile by means of peritrichous flagella (30, 33). This family includes many clinically significant genera and species including *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Salmonella* spp., *Serratia* spp., and *Shigella* spp. Members of this family can cause a wide variety of infections in both the community and the hospital setting, affecting normal hosts and those with other underlying illnesses. Enterobacteriaceae may account for 80% of clinically significant Gram-negative bacilli and 50% of clinically significant bacteria recovered in the diagnostic microbiology laboratories (33). In Canada, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *P. mirabilis*, and *S. marcescens* are amongst the top 20 organisms causing infections (16). These include urinary tract infections (UTIs), bacteremia, pneumonia, meningitis and gastrointestinal infections (30, 33). They account for more than 70% of UTIs, up to 50% of blood infections and a high percentage of intestinal infections. They may also be isolated from numerous other sites including the peritoneal cavity, respiratory tract, cerebrospinal fluid, synovial fluid, and abscesses. The term “enterics” comes from the fact that the natural habitat of many of the organisms in this family is the lower gastrointestinal tract.

1.2. *Escherichia coli* and *Klebsiella pneumoniae*

1.2.1. Characteristics of the organisms

1.2.1.1. *Escherichia coli*

Escherichia coli is the type species of the genus *Escherichia* which is the type genus of the family Enterobacteriaceae (30). A few of the main distinguishing features of *E. coli* is that most strains are motile and most can ferment lactose plus other sugars and produce indole from tryptophan. *E. coli* can grow on mostly any type of media but can be differentiated from other organisms by its characteristic appearance on blood and MacConkey agar (33). On both media, *E. coli* appear as smooth colonies ranging from 2-3 mm in diameter. Some strains are haemolytic, defined as the breakdown of red blood cells, which can be visualized on blood agar as clearing around the colonies. On MacConkey agar, colonies are red as a result of neutral red dye which stains organisms producing lactose and usually are surrounded by precipitated bile. Optimal conditions for growth on media is at 37°C for 16-18 hours although there are few strains which grow much slower (25, 33).

1.2.1.2. *Klebsiella pneumoniae*

Klebsiella pneumoniae is the species of the genus *Klebsiella* which is the type genus of the family Enterobacteriaceae. Most *K. pneumoniae* strains, like *E .coli*, have the ability to ferment lactose (30). However, one major distinguishing factor setting it apart from *E. coli* is that all *K. pneumoniae* strains are non-motile and do not produce indole. Like *E. coli, K. pneumoniae* can grow on most types of media and can be differentiated from other organisms by its characteristic appearance on blood and MacConkey agar (30, 33). On both types of media, colonies are mucoid and 3-4 mm in diameter. Colonies on MacConkey agar are pink in appearance. Growth conditions are again optimal at 37°C for

16-18 hours and like *E. coli,* a few strains may be slow growing and require additional incubation (25, 33).

1.2.2. Pathogenesis

While most strains of *E. coli* and *K. pneumoniae* make up part of the normal intestinal flora, some strains can cause disease in both healthy individuals as well as individuals with underlying disease. In terms of diarrheagenic *E. coli,* at least five pathotypes exist (Table 1) (30, 66, 95). The virulence traits are distinct for each pathotype of *E. coli* and contribute to the pathogenic nature of the organism (95). These include adherence factors (for attachment to intestinal wall), toxins (interrupt normal intestinal cell secretion and absorption), and cytotoxins (damage intestinal cells). In *K. pneumoniae,* five major virulence factors contribute to pathogenesis (95). These include capsular serotype, hypermucoviscosity phenotype, lipopolysaccharide, siderophores, and pili. Table 2 summarizes these virulence factors and their major functions.

TABLE 1: *E. coli* (EC) pathotypes, epidemiology, clinical features, and pathogenesis. Modified from (30, 95)**.**

Pathotype	Epidemiology	Clinical Features	Pathogenesis
ETEC, enterotoxigenic EC	-contaminated water and food -major cause of childhood diarrhea (developing countries) -leading cause of travellers diarrhea	-acute watery diarrhea (occasionally severe)	-large number of fimbrial adhesions -heat-stable and heat-labile enterotoxins
EPEC, enteropathogenic EC	-person-to-person transmission -leading cause of infantile diarrhea (developing countries)	-severe acute diarrhea -vomiting -may be persistent	-localized adherence (bundle-forming pilus) -attaching and effacing (intimin-Tir)
EHEC/STEC, enterohemorrhagic EC/Shiga toxin-producing EC	-food, water, and person-to-person transmission -major cause of bloody diarrhea (developed countries)	-watery and bloody diarrhea -haemolytic uremic syndrome	-shiga toxins -attaching and effacing (intimin-Tir-mediated)
EAEC, enteroaggregative EC	-unknown mode of transmission -cause of chronic diarrhea (developing countries) -emerging cause of travelers diarrhea	-mucoid diarrhea -often persistent	-aggregative adherence (several fimbriae) -pet and other toxins
EIEC, enteroinvasive EC	-contaminated food -cause of outbreaks (developed countries)	-watery diarrhea -dysentery	-cellular invasion -intracellular motility -cell-to-cell spread
DAEC, diffuse adhering EC	-unknown mode of transmission -cause of diarrhea in older children (developing countries)	-poorly described	-unknown

TABLE 2: Major virulence factors contributing to the pathogenicity of *K. pneumoniae.* Modified from (1, 95)**.**

Virulence factor	Main components	Major functions(s)
Capsule (77 K serotypes)	-K1 or K2 capsular polysaccharide	-antiphagocytosis
Hypermucoviscosity phenotype	-extracapsular polysaccharide	-serum resistance
Lipopolysaccharide (O-antigen 9 serotypes: O1-O5, O7, O8, O12, O2ac)	-lipopolysaccharide -O side chain	-endotoxin -serum resistance
Siderophores	-enterobactin -aerobactin -aerobactin receptor -kfu iron uptake system	-enhanced growth
Pili	-adhesion -type 1 pili -type 3 pili	-attachment -mannose-sensitive -mannose-resistant

1.2.3. Infections and Treatment

Enterobacteriaceae are important causes of UTIs, bloodstream infections, hospital- and health-care-associated pneumonias, and various intra-abdominal infections and may be sporadic or occur in outbreaks (30). *E. coli* is the most common cause of UTIs and a leading cause of neonatal bacteremia, sepsis, and meningitis. It is also the cause of a wide variety of extraintestinal infections including nosocomial pneumonia, cholecystitis and cholangitis, peritonitis, cellulitis, osteomyelitis, and infectious arthritis (30, 33, 66, 74). *K. pneumoniae* is also considered a primary pathogen causing UTIs and bacteremia, coming in second to *E. coli.* Other common infections include pneumonia, wound infections, infections of intravascular and other invasive devices, biliary tract infections, peritonitis, and meningitis (30). Although these are all extraintestinal infections, *E. coli* and *K. pneumoniae* are best known for their ability to cause gastrointestinal infections. Both organisms are part of the normal intestinal flora and can cause gastrointestinal infections in both healthy and immune suppressed individuals. Each type of *E. coli* diarrhea is associated with a different pathotype (Table 1) and can be described by no less than six different mechanisms (30, 95). Treatment for gastroenteritis is supportive and usually only requires rehydration with dietary management without the use of an antimicrobial agent (95). However, for the extraintestinal infections listed above, empiric therapy is required. Antimicrobials used to treat infections caused by *E. coli* and *K. pneumoniae* include β-lactams, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole. Resistant organisms may need susceptibility testing done in order to determine appropriate treatment and management for patients.

1.3. β-lactam Antibiotics

β-lactam antibiotics date back to the 1940s and 50s when only two β-lactam agents were known; penicillin G and penicillin V. Penicillin G was the first β-lactam antibiotic introduced into clinical practice. It wasn't until the 1960s that semisynthetic penicillin was developed followed by semisynthetic cephalosporins and other β-lactam antibiotics (87). β-lactam antibiotics now include penicillins, cephalosporins, carbapenems, and monobactam (Figure 1) (5). These are bactericidal agents which inhibit cell wall synthesis. To understand how they work, a good understanding of bacterial cell wall synthesis is a requisite (Figure 2) (5, 107). The bacterial cell wall is a complex structure formed by cross-links of peptidoglycan, maintaining cell shape and structure. In Gram-negatives, the peptidoglycan layer is within the periplasmic space in which the glycan component of a polymer of *N*-acetyl-glucosamine (NAG) and *N*-acetyl-muramic acid (NAM) are cross-linked via the peptide chain. Penicillin-binding proteins (PBPs) are the transpeptidases that catalyze this reaction. The PBP serine hydroxyl group will first attack the carbonyl group of D-ala on one stem peptide causing the release of the other D-ala. This acyl-enzyme complex is then attacked by a primary amine of another stem peptide to form the crosslink (Figure 2a). As shown in Figure 2, β-lactam antibiotics (Figure 2c, penicillin backbone; Figure 2d, cephalosporin backbone) mimic the D-ala-D-ala component of the cell wall (Figure 2b). The PBP serine mistakenly attacks the carbonyl group of the β-lactam ring (characteristic to all β-lactam antibiotics) creating a covalent acyl-enzyme complex. This complex hydrolyzes very slowly therefore the PBPs remain bound and cell wall synthesis is disrupted.

FIGURE 1: Chemical structures of β-lactams (1-4), site of action of β-lactamases (5), and chemical structures of β-lactamase inhibitors (6-8) (5)**.**

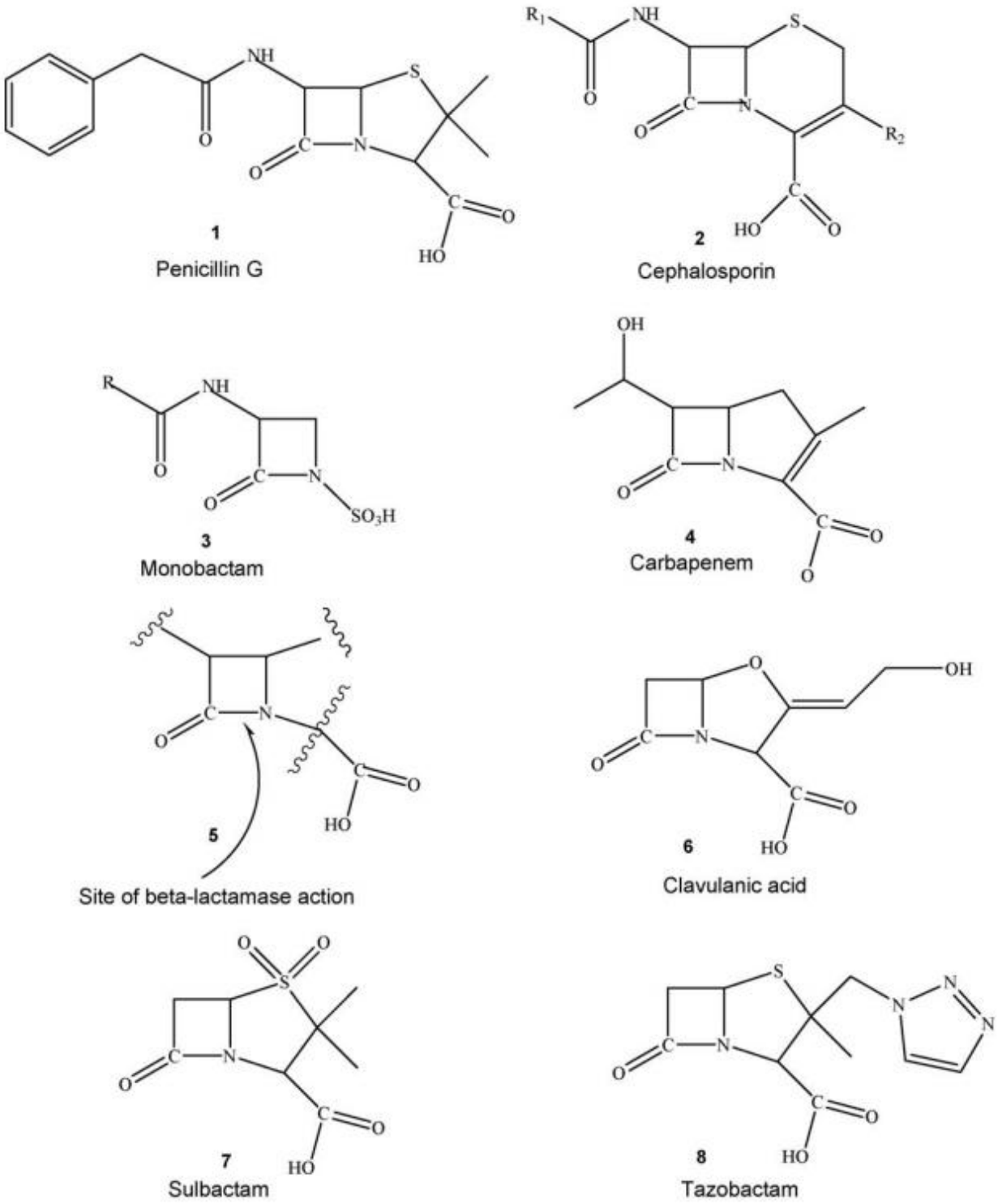
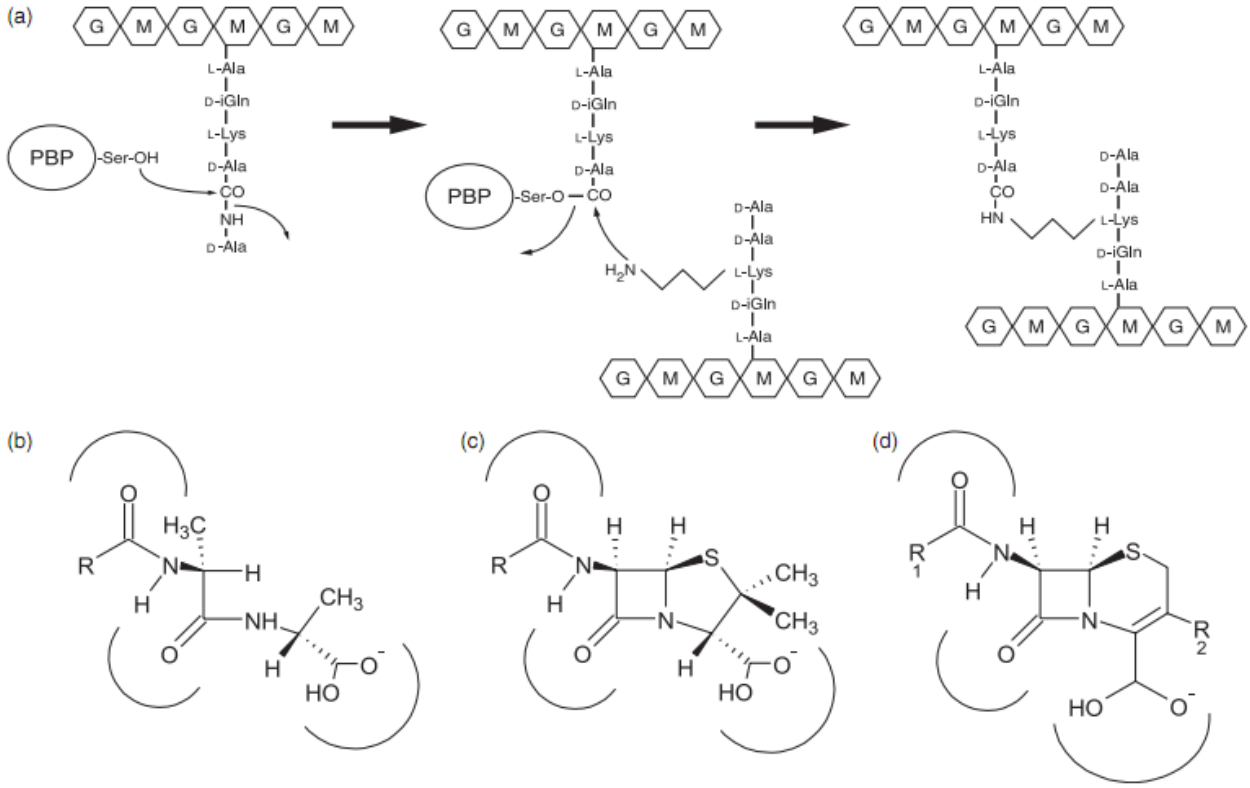


FIGURE 2: Peptidoglycan synthesis (a), N-Acyl-D-Ala peptide (b), Penicillin backbone (c), and Cephalosporin backbone (d) (107).



In terms of global usage, β-lactams account for greater than 60% of all antimicrobial consumption (52, 55). They are the preferred agents because of their efficacy and safety profile and because their activity can be extended or restored by chemical manipulation. No other classes of antibiotics have such chemical malleability and versatility (14, 55, 92). However, heavy usage of these agents has selected strongly for resistance.

1.3.1. Carbapenems

Carbapenems are a class of β-lactam antibiotics with an exceptionally broad spectrum of activity (110). They are stable to most β-lactamases including extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases. Like all β-lactams, carbapenems exhibit bactericidal activity by binding to PBPs. Imipenem binds preferentially to PBP2, followed by PBP1a and 1b. Meropenem and ertapenem bind most strongly to PBP2, followed by PBP3 then PBP1a and 1b. Doripenem has been reported to have strong affinity for species-specific PBP targets; PBP2 in *E. coli*. Resistance to the carbapenems among Gram-negatives can arise through hyperproduction of class C β-lactamases or ESBLs and porin loss, augmented drug efflux, alterations in PBPs, and carbapenemase production (serine carbapenemase or metallo-β-lactamase) (72, 110). This is of particular interest since carbapenems are considered the last-line therapy for severe infections.

1.4. Antibiotic Resistance and Resistance to β-lactam Antibiotics

Ten years ago, antibiotic resistance was a major concern in Gram-positive bacteria, particularly methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp (45). However, in recent times, there has been a major shift and clinical microbiologists increasingly agree that MDR Gram-negative bacteria pose the greatest risk

to public health. Not only is the increase in resistance faster in Gram-negative bacteria, but also there are fewer new and developmental antimicrobials active against Gram-negative bacteria.

Antibiotic resistance has been known ever since the discovery of penicillin (13). Since then, numerous classes of antimicrobials have been successfully developed and used in the treatment of infectious diseases. These include drugs that exploit cell wall synthesis, protein synthesis, DNA synthesis, cell membrane, and folate synthesis (37). However, the development and marketing approval of new agents has not kept up with the growing worldwide problem of antibiotic resistance and so infectious diseases remains one of the leading causes of death worldwide (95, 103). The importance of antimicrobial stewardship needs to be stressed as antibiotic misuse and overuse has driven the evolution of antibiotic resistance leading to increased morbidity and mortality, increased healthcare costs, and longer hospitalization (92).

In terms of antibiotic resistance to β-lactam agents, there are four major ways in which it can develop in Gram-negatives (5, 87). First is by the production of β-lactamases which have the ability to hydrolyze the β-lactam ring and render the antibiotic inactive. Second is through the alteration of the target site or PBP so the antibiotic cannot bind. Third is through outer membrane porin protein alteration through decreased or a complete absence of expression which prevents the antibiotic from getting into the cell. And fourth is through the production of efflux pumps which pump the antibiotic out of the cell as soon as it enters. One or a combination of these mechanisms can lead to the development of drug resistance with β-lactamase production and porin alteration being the most common.

1.5. β-lactamases

β-lactamase mediated hydrolysis of β-lactam agents is the most common mechanism of resistance for this class of antimicrobials in clinically important Gram-negative bacteria (15, 51). To date, there are over 900 unique protein sequences for β-lactamases (15, 48). These enzymes function by first forming a noncovalent complex with the antibiotic. The β-lactam ring is then attacked by the free hydroxyl group on the side chain of a serine residue at the active site of the enzyme to yield a covalent acyl ester. Hydrolysis of the ester bond releases the active enzyme and the hydrolyzed, inactive drug. This mechanism is followed by β-lactamases of molecular class A, C, and D. Class B enzymes are metalloenzymes and utilize an active site zinc ion to attack the β-lactam ring (15, 51). The β-lactamase classification scheme used above is called the Ambler classification scheme which categorizes enzymes based on amino acid sequence similarity (classes A through D). This along with the Bush-Jacoby classification scheme, which groups them based on substrate and inhibitor profiles (groups 1-4), are the two most widely used classification systems (Table 3) (5, 15, 51). For simplicity, the Ambler classification will be used for this thesis.

TABLE 3: β-lactamase classification schemes. Modified from (2, 15, 72)**.**

Molecular classification (ambler)	Function group(s) (Bush-Jacoby 2009)	Common Name	β-lactam resistance	Representative examples
A	2a	Staphylococcal penicillinase	Penicillins	PC1
A	2b	Penicillinase	Penicillins, first-generation cephalosporins (narrow-spectrum)	SHV-1, TEM-1, TEM-2
A	2be	ESBL[a]	Penicillins, cephalosporins, aztreonam	SHV-2, TEM-3, TEM-26, CTX-M
A	2ber	ESBL	Penicillins, cephalosporins, aztreonam, not inhibited by β-lactamase inhibitors	TEM-50
A	2br	Penicillinase	Penicillins, not inhibited by β-lactamase inhibitors	SHV-10, TEM-30
A	2c	CARB[b]	Carbenicillins	CARB
A	2ce	CARB	Cefepime, carbenicillins	RTG-4, CARB-1 (PSE-4)
A	2e	Cephalosporinases	Penicillins, cephalosporins	CepA
A	2f	Carbapenemase	Penicillins, cephalosporins, aztreonam, β-lactam/β-lactamase inhibitor combinations, carbapenems, variable inhibition by β-lactamase inhibitors	NMC-A, IMI-1, SME-1, SFC, BIC-1, GES-2, KPC
B	3 (3a, 3b, 3c)	Metallo-β-lactamases	All β-lactams except aztreonam, not inhibited by β-lactamase inhibitors	IMP-1, VIM-1, SPM-1, GIM-1, SIM-1, DIM-1, AIM-1, KHM-1, NDM-1
C	1, 1c	Cephalosporinase	Penicillins, cephalosporins, not inhibited by β-lactamase inhibitors	AmpC, CMY-2, ACT-1, DHA-1, ACC-1, FOX-1
D	2d, 2de	Oxacillinase	Penicillins, variable inhibition by β-lactamase inhibitors	OXA-1, OXA-10, OXA-15
D	2df	Carbapenemase	Penicillins, cephalosporins, aztreonam, β-lactam/β-lactamase inhibitor, variable	OXA-23, OXA-24/40, OXA-48, OXA-51/66/69,

			inhibition by β-lactamase inhibitors combinations, carbapenems	OXA-58, OXA-143

[a]ESBL, extended-spectrum β-lactamase.
[b]CARB, carbenicillin-hydrolyzing β-lactamase.

1.5.1. Extended-Spectrum β-lactamases

The introduction of third-generation cephalosporins into clinical practice in the early 1980s was a considered a major breakthrough in the fight against β-lactamase-mediated resistance to antibiotics (75). These expanded-spectrum agents were developed to battle the increased prevalence of β-lactamases in certain organisms, for example, ampicillin hydrolyzing TEM-1 and SHV-1 β-lactamases in *E. coli* and *K. pneumoniae*. Soon after, in 1983, the first report of a plasmid-encoded β-lactamase capable of hydrolyzing extended-spectrum cephalosporins was published. This β-lactamase showed a single nucleotide mutation in the gene encoding SHV-1 and not long after other β-lactamases with the same spectrum of activity started to appear, related to TEM-1. Hence these new β-lactamases were termed extended-spectrum β-lactamases (ESBLs) (5, 9, 75).

By definition, ESBLs are enzymes capable of conferring resistance to the penicillins, first-, second-, and third-generation cephalosporin, and aztreonam. Cephamycins (cefoxitin and cefotetan) and carbapenems still remain active as well as commonly used inhibitors like clavulanic acid, sulbactam, and tazobactam (53, 75). With the exception of OXA-type enzymes, which are class D enzymes, the ESBLs are of molecular class A and can be divided into three groups: TEM, SHV, and CTX-M types. Most are located on plasmids which allow for efficient and rapid dissemination. This is a major concern as most plasmids also confer resistance to other classes of drugs including aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole allowing organisms to present a multidrug resistant phenotype (MDR-defined as concomitant resistance to ≥3 different antimicrobial classes).

In the 1990s, TEM and SHV type ESBLs were the most predominant. However, since 2000, CTX-M enzymes have emerged worldwide and are now the most predominant type of ESBLs found in not only the nosocomial setting but in the community setting as well (7, 17). A combination of multiple factors has contributed to the rapid dissemination of the CTX-M enzymes such as co-selection due to MDR phenotypes, virulence factors, mobile genetic elements, highly mobile CTX-M-bearing plasmids and clonal spread. In particular, CTX-M-15 is the most wide spread and is thought to be the result of the spread of a highly successful clone, *E. coli* O25:H4-ST131 (19). *E. coli* and *K. pneumoniae* are the most common organisms producing ESBL enzymes but they have also been found in many other members part of the Enterobacteriaceae family as well as other families (80).

1.5.2. AmpC β-lactamases

The first bacterial enzyme reported to hydrolyze penicillin was the AmpC β-lactamase of *E. coli,* although it was not given this name in the 1940s (40). AmpC enzymes are grouped into Ambler class C. Organisms expressing AmpCs confer high level resistance to many β-lactam agents including the third-generation cephalosporins and cephamycins (cefoxitin and cefotetan). Fourth-generation cephalosporins (cefepime) and carbapenems still have good activity against these enzymes. However, unlike ESBLs, AmpC β-lactamases are not inhibited by the commonly used inhibitors such as clavulanic acid, sulbactam, and tazobactam but may be inhibited by cloxacillin or boronic acid (78, 99). AmpC enzymes can either be chromosomal or plasmid encoded.

In *E. coli,* the *ampC* gene is poorly expressed (42). The resistant phenotype is the result of constitutive overexpression of the AmpC β-lactamase. This can happen in two ways. One is through the deregulation of the *ampC* chromosomal gene by mutations in the

promoter and/or attenuator region of the gene (AmpC hyperproducers) and the other is through the acquisition of a plasmid-mediated *ampC* gene which is also what we see in *K. pneumoniae* (6, 62, 77, 81, 88). Many Enterobacteriaceae have inducible AmpC β-lactamases meaning they are induced in the presence of β-lactams (40). *E. coli* has a chromosomal non-inducible *ampC* gene with a weak promoter and transcriptional attenuator.

1.5.3. Treatment for ESBL and AmpC Producing Organisms

Given the ability of ESBL- and AmpC-producing organisms to hydrolyze many β-lactam antibiotics, it is not surprising that treatment options are very limited (40, 75). Furthermore, plasmids encoding ESBL and/or AmpC genes also carry genes encoding resistance to other agents like aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. One viable option for treating patients with an organism producing a single ESBL is a β-lactam/β-lactamase inhibitor combination although many organisms now produce multiple ESBLs and/or AmpCs therefore effectiveness may be reduced. The best treatment option seems to the use of carbapenems (imipenem, meropenem, doripenem, and ertapenem), confirmed not only by *in vitro* susceptibility but also by the increasingly extensive clinical experience (75). More recently, with the emergence of carbapenem resistance through acquired carbapenem hydrolyzing enzymes and/or production of other plasmid-mediated β-lactamases coupled with porin loss, treatment with a carbapenem may no longer be an option making choices for therapy extremely limited (59).

2. Carbapenemases

The growing increase in the rates of antibiotic resistance worldwide is a major cause for concern in both nonfermenting bacilli and isolates of the Enterobacteriaceae family (106). β-lactams have been the main choice of treatment for severe infections with carbapenems being the most active. Carbapenems have been the main choice especially for infections caused by ESBL- and AmpC-producing Enterobacteriaceae, particularly *E. coli* and *K. pneumoniae*. Carbapenem resistance can arise through productions of chromosomally encoded, molecular class A, carbapenemases like SME, NMC, and IMI as well as plasmid-encoded carbapenemases of class A (KPC and GES) and class B metallo-β-lactamases (active site divalent zinc cation-VIM, IMP, and NDM) (31, 63). However, resistance can also arise in organisms already producing ESBLs and/or AmpC β-lactamases coupled with alteration or complete loss of major outer membrane porins as has been reported in *E. coli* and *K. pneumoniae* (32, 41).

Carbapenemases are considered the most versatile family of β-lactamases with a very broad spectrum of activity (82, 85). Many of the carbapenemases are able to hydrolyze the full spectrum of β-lactam antibiotics which include the penicillins, cephalosporins, carbapenems, and monobactams and can also display inhibitor resistance to commercially available β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam.

Until the early 1990s, all carbapenemases were described as chromosomally encoded enzymes which were species-specific (85). This problem of clonal spread has now changed into a problem of global interspecies spread with the emergence of plasmid borne carbapenemases including class B IMP-1 in *Pseudomonas aeruginosa*, class D OXA-23 in

Acinetobacter baumannii, and the class C KPC-1 in *K. pneumoniae*. Increased attention has been given to organisms producing carbapenemases, not only for their clinical significance but also in the area of structure/function relationships, possibly in the hopes to design new agents that are active against these enzymes and/or to enhance the longevity of clinically available carbapenems (97).

2.1. Molecular Class A Carbapenemases

2.1.1. Chromosomally Encoded: SME, NMC, and IMI

The chromosomal carbapenemases occur in *Serratia marcescens* and *Enterobacter* spp. (5). These enzymes are inducible by imipenem and cefoxitin (73). The SME (*S. marcescens* enzyme) enzymes have been found in *S. marcescens* and include three variants that differ by one to two amino acids (SME-1 to SME-2) (73, 100). The IMI (imipenem-hydrolyzing enzyme) and NMC-A (not metalloenzyme carbapenemase A) have 97% amino acid identity and are related to SME-1 by ~70% amino acid identity (85). These are both rare and have been found in *Enterobacter* spp.

The resistance profile is unique for the molecular class A chromosomal carbapenemases. They are resistant to carbapenems, penicillins, and aztreonam but remain susceptible to extended-spectrum cephalosporins (73). All are poorly inhibited by sulbactam, and clavulanic acid inhibition varies. IMI and NMC-A enzymes are inhibited by clavulanic acid and SME enzymes are not. Substrate and inhibition profiles of the carbapenemase genes are shown in Table 4.

In terms of epidemiology, being chromosomally encoded enzymes, they have not disseminated well globally. The location on the chromosome and lack of association with mobile genetic elements have been thought to account for their limited distribution (73, 85,

100). SME enzymes have been isolated in the U.K. and the U.S.A., IMI enzymes have been isolated from U.S.A. and China, and NMC-A enzymes have been isolated from France, Argentina, and U.S.A.

2.1.2. Plasmid-Encoded: KPC and GES

The plasmid-encoded molecular class A carbapenemases include GES (Guiana extended-spectrum) and KPC (*K. pneumoniae* carbapenemase) enzymes. To date, there are 17 variants of GES (GES-1 to GES-17) and 13 variants of KPC (KPC-1 to KPC-13) (48, 72). The first GES enzyme was isolated in 1998 from an infant with a *K. pneumoniae* infection in French Guiana (73, 85, 100). GES enzymes are found within integron structures located on plasmids. Originally this family of enzymes were termed ESBLs due to their broad spectrum of activity against extended-spectrum cephalosporins (85). This was expanded in 2001 with the report of GES-2 in a clinical isolate of *P. aeruginosa* from South Africa. Up to now, these enzymes have been mainly found in *P. aeruginosa* but have also been found within the Enterobacteriaceae family. Although plasmid-borne, GES enzymes have not spread rapidly but are more frequently associated with single occurrences and small outbreaks associated with *P. aeruginosa* and *K. pneumoniae* (101). However, they have been found from different geographical locations including Greece, France, Portugal, South Africa, French Guiana, Brazil, Argentina, Korea, and Japan (85, 101).

GES enzymes are active against penicillins, cephalosporins, carbapenems (although weak), and aztreonam, but may be inhibited by commonly used β-lactamase inhibitors like clavulanic acid and tazobactam but at a lower level (73, 85, 101). Table 4 shows substrate/inhibition profiles. Among the class A plasmid-encoded carbapenemases, KPC

TABLE 4: Substrate and inhibition profiles of the carbapenemases. Modified from (15, 46, 73, 85)**.**

			Hydrolysis profile[a]					Inhibiton Profile[b]	
Molecular class	**Functional group**	**Enzyme**	**Penicillins**	**Early cephalosporins**	**Extended-spectrum cephalosporins**	**Aztreonam**	**Carbapenems**	**EDTA**	**CLA[c]**
A	**2f**	**NMC**	+	+	±	+	+	-	+
		IMI	+	+	±	+	+	-	+
		SME	+	+	±	+	+	-	+
		KPC	+	+	+	+	+	-	+
		GES	+	+	+	-	±	-	±
B	**3a**	**IMP**	+	+	+	-	+	+	-
		VIM	+	+	+	-	+	+	-
		GIM	+	+	+	-	+	+	-
		SPM	+	+	+	-	+	+	-
		NDM	+	+	+	-	+	+	-
D	**2d, 2de, 2df**	**OXA**	+	+	±	-	±	-	±

[a]Symbols: +, strong hydrolysis; ±, weak hydrolysis; -, no measurable hydrolysis.
[b]Symbols: +, reported inhibition; ±, variable inhibition; -, no inhibition reported.
[c]CLA, clavulanic acid.

enzymes pose the greatest threat.

2.2. *Klebsiella pneumoniae* Carbapenemase (KPC): Clinical Significance

Infections caused by bacteria producing KPC are becoming an increasingly significant problem worldwide since they were first detected more than a decade ago (4). Even though KPCs do not represent the first or sole mechanism of carbapenem resistance, they are fascinating in the sense that they are not often detected by routine laboratory susceptibility screening and have an exceptional potential for dissemination (4, 93). They are the most common carbapenemases encountered within the Enterobacteriaceae family worldwide especially in areas such as the U.S.A., Israel, and Greece where an endemic situation has been created (68, 73, 108). The first KPC was isolated from a clinical isolate of *K. pneumoniae* in North Carolina in 1996 (5, 72, 85). Genetic analysis of the bla_{KPC} genes indicates that their mobility is mainly associated with the spread of strains, plasmids, and transposons. As previously mentioned, plasmids can carry multiple genes including multiple β-lactamases, aminoglycoside modifying enzymes, resistance genes for fluoroquinolones (*qnr*, *aac(6')Ib-cr* and *qepA*) and trimethoprim-sulfamethoxazole, and virulence genes (68). Since the first report, many Enterobacteriaceae and non-Enterobacteriaceae have been shown to produce the KPC enzyme which has been a major concern in terms of infection control (Table 5). In addition to the infection control challenges, these organisms also present clinicians with serious treatment challenges (4).

The susceptibility patterns of KPC producers can vary. Isolates producing KPC can confer resistance to all β-lactam agents including penicillins, early and late generation cephalosporins, cephamycins, aztreonam, carbapenems and β-lactam/β-lactamase inhibitor combinations (73).

TABLE 5: Organisms that have been shown to produce KPC enzymes. Modified from (4, 22)**.**

Enterobacteriaceae	**Non-Enterobacteriaceae**
Citrobacter freundii	*Pseudomonas aeruginosa*
Escherichia coli	*Pseudomonas putida*
Enterobacter spp.	*Acinetobacter* spp.
Klebsiella pneumoniae	
Klebsiella oxytoca	
Proteus mirabilis	
Raoultella planticola	
Raoultella ornitholytica	
Salmonella enteric	
Serratia marcescens	

2.2.1. Epidemiology

Since their first isolation in 1996, KPC enzymes have spread worldwide (Figure 3). Most cases are sporadic although some regions such as north-eastern U.S.A. (New York, Pennsylvania, and New Jersey), Greece, and Israel are in endemic situations (68, 72, 83). From the 13 KPC subtypes, KPC-2 and KPC-3 seem to make up the vast majority and are distributed worldwide among Enterobacteriaceae, *P. aeruginosa*, and *Acinteobacter* spp. Based on reports in the literature and finding from isolates sent to Centers for Disease Control and Prevention (CDC) for reference testing, KPC enzymes seem to occur most commonly in *K. pneumoniae*, but is in no way limited to this organism as shown in Table 5 (73). Data regarding nosocomial infections reported to the CDC showed that the overall prevalence of carbapenem resistance among *K. pneumoniae* isolates rose from <1% in 2000 to 8% in 2007 (4). To date, KPC-producing bacteria have been isolated from at least 33 different states as well as reports in Brazil, China, Colombia, Norway, U.K., India, Sweden, and more recently in Italy and Finland as well as reports in Canada (4, 60).

A closer look at the molecular epidemiology of KPC-producing isolates demonstrates that a major clone is primarily responsible for dissemination of the bla_{KPC} gene (4, 44, 49, 72, 86). A look at all KPC-producing *K. pneumoniae* sent to CDC from 1996-2008 by pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) from 18 states as well as India and Israel shows a novel MLST type, ST258. Another sequence type, ST14, was found to predominate in the Midwest (4, 72). Other studies show that the global dissemination of the bla_{KPC} gene may be more complicated that the expansion of a finite number of clones and may be the result of horizontal gene transfer rather than clonal spread (27, 49, 84).

FIGURE 3: Geographic distribution of KPC worldwide (68)**.**

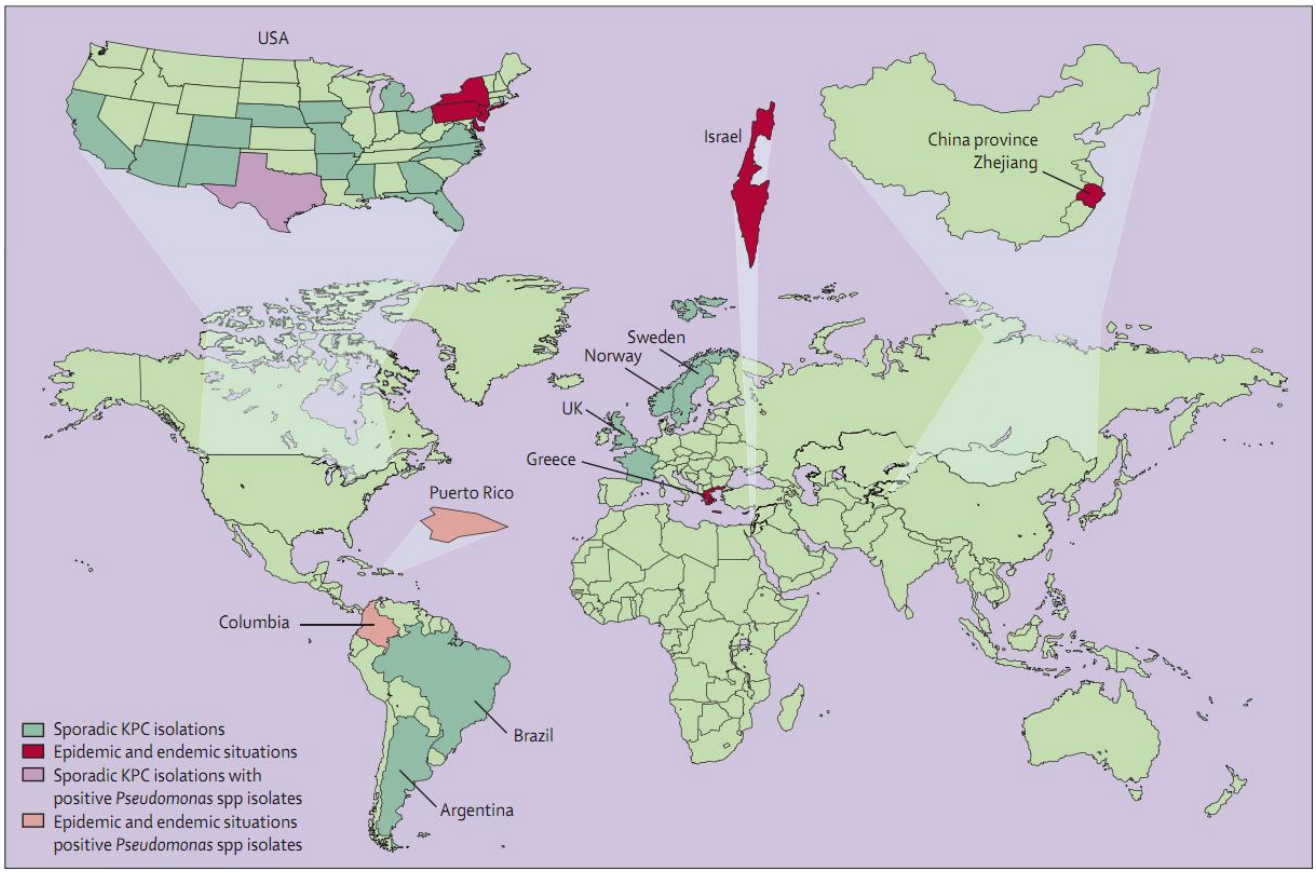
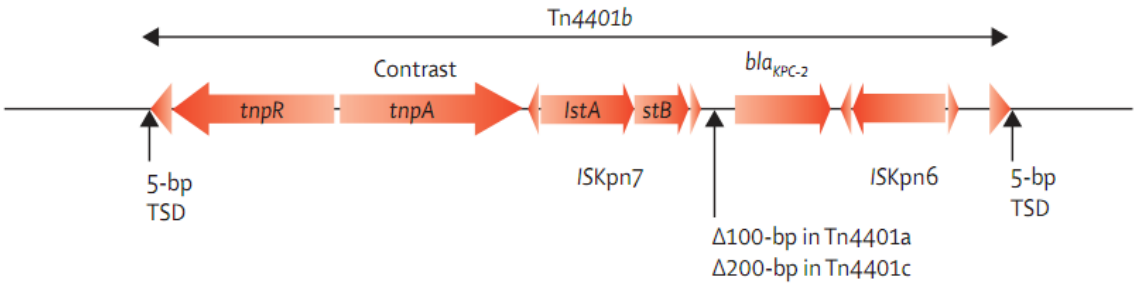


FIGURE 4: Schematic representation of Tn*4401* structures on naturally occurring plasmids (68)**.**



2.2.2. Mobilization and Spread

The *bla*$_{KPC}$ genes are found on plasmids, although there has been a single case where it was found on the chromosome of *P. aeruginosa* (68, 72, 100). They have been shown to be associated with a roughly 10 kb Tn*3*-type transposon, Tn*4401* with 3 isoforms described (Figure 4). The isoforms differ by deletions present upstream of *bla*$_{KPC}$. The main components of the transposon are the transposase gene (*tnpA*), resolvase gene (*tnpR*) and two unrelated insertion sequences (IS*Kpn7* and IS*Kpn6*).

2.3. Molecular Class D OXA Carbapenemases

OXA (oxacillin-hydrolyzing) enzymes are class D serine β-lactamases. The first OXA enzyme with carbapenemase activity was found in an isolate of *A. baumannii* in 1985 from a patient in Scotland (5, 85, 96). This enzyme was called ARI-1 which was later renamed OXA-23. To date, there are over 200 types of OXA enzymes reports and most are unable to hydrolyze the extended-spectrum cephalosporins (48, 72). A small number can confer resistance to extended-spectrum cephalosporins and low levels of resistance to the carbapenems. A list of carbapenem-hydrolyzing, class D enzymes are shown in Table 6 along with year of first isolation and organism, geographic distribution, and genetic location. Of these, OXA-48 seems to be of major concern within the Enterobacteriaceae family, the others are mainly restricted to *A. baumannii* (72, 85, 97). OXA-48 is mainly found in *K. pneumoniae* and has been reported from Turkey, China, India, and the U.K. (106). In terms of resistance, isolates producing OXA-48 are resistant against penicillins, cephalosporins, aztreonam, β-lactam/β-lactamase inhibitor combinations, and carbapenems.

TABLE 6: Carbapenem-hydrolyzing class D β-lactamases. Modified from (72)**.**

Enzyme group	First isolated	Organism	Geographic location	Genetic location
OXA-23/27	1985	*A. baumannii, P. mirabilis*	Europe, U.S.A., Middle East, Asia, Australia	Plasmid, chromosomal
OXA-24/40	1997	*A. baumannii*	Europe and U.S.A.	Plasmid, chromosomal
OXA-48	2001	*K. pneumoniae,* Enterobacteriaceae	Middle East, Europe, Argentina, India	Plasmid
OXA-51/66/69	1993	*A. baumannii*	Worldwide	Chromosomal
OXA-58	2003	*A. baumannii*	Europe, U.S.A., Middle East, South America	Plasmid
OXA-143	2004	*A. baumannii*	Brazil	Plasmid

2.4. Molecular Class B Metallo-β-Lactamases

Molecular class B metallo-β-lactamases (MBLs) are a group of enzymes that require a metal ion (Zn^{2+}) for β-lactam hydrolysis (72, 73, 85, 97). Therefore unlike serine β-lactamases, they are not inhibited by common inhibitors but can be inhibited by chelating agents like EDTA. Most MBLs can hydrolyze all β-lactams but not aztreonam. The first MBLs discovered were chromosomal enzymes from *Bacillus cereus, Aeromonas* spp., and *Stenotrophomonas maltophilia*. Of greater importance are the acquired or transferable families of MBLs which include VIM, IMP, GIM, SIM, SPM, and NDM which are located within gene cassettes as part of integron structures (98). These integron structures may then associate with transposons and plasmids which then can be easily transferred between bacteria. IMP and VIM carbapenemases pose the greatest clinical threat, and more recently NDM (5, 45, 63, 106). To date, 30 IMP and VIM variants exist (IMP-1 to IMP-30 and VIM-1 to VIM-30) (48). Both are mainly found in *P. aeruginosa* but have also disseminated worldwide throughout the Enterobacteriaceae family and amongst the *Acinetobacter* spp (72, 85, 98). Although IMP and VIM types are most common, more recent attention has been given to NDM-type MBLs.

2.4.1. New Delhi Metallo-β-Lactamase (NDM)

The New Delhi Metallo-β-lactamase (NDM-1) is a new molecular class B enzyme that was recently characterized from a *K. pneumoniae* isolate from a patient in Sweden who seems to have imported it from India (63, 72). To date, there are 5 NDM variants (NDM-1 to NDM-5) (48). Particularly NDM-1 is endemic to India but due to international travel, it is emerging as an important clinical threat worldwide (72). It's been found in, but not limited to, U.K., Austria, Belgium, Denmark, the Nordic countries, Germany, Italy,

The Netherlands, Slovenia, Spain, Australia, U.S.A., Canada, Japan, Taiwan, Singapore, China, Kenya, Oman, Israel, and Turkey. NDM-1 has been recovered from *K. pneumoniae, K. oxytoca, E. coli, C. Freundii, Morganella morganii, Providencia* spp, *Proteus* spp., *E. cloacae* and *A. baumannii.* It has been suggested that NDM-1 is the new CTX-M-15 and will soon become global (97). It has even been recently shown to be associated with the highly successful, virulent clone, ST131 (76). It may even soon surpass the success of CTX-M-15 as plasmids carrying NDM also carry significantly more resistance determinants (106).

2.5. Treatment for Carbapenemase Producing Bacteria

Treatment options remain very limited for infections caused by organisms producing carbapenemases. Often carriage of concomitant resistance genes leads to decreased susceptibility to other classes of agents including fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole. The best currently available options seem to be colistin (polymyxin E) and tigecycline (glycylcycline) (39, 72, 87). Caution must be taken with these agents as colistin has neurotoxic and nephrotoxic effects and tigecycline demonstrates low serum and urine concentrations (39, 68).

A few agents currently in development have also shown good *in vitro* activity against carbapenemase producing organisms. NXL-104 (Avibactam), a novel non-β-lactam β-lactamase inhibitor in phase III has shown excellent activity against β-lactamase producing Enterobacteriaceae, including KPC-types and OXA-48 (47, 54, 64). ACHN-490, a new aminoglycoside, and NAB739 and NAB7061 which are experimental polymyxin derivatives that are potentially less nephrotoxic than colistin, may be a viable options along with the novel monobactam, BAL30072 (72). Two other agents, BAL30376

and CP3242 are inhibitors of MBLs and may provide an option for organisms producing MBLs (87).

3. Outer Membrane Proteins

The outer membrane of Gram-negatives is the first line of defence against toxic compounds (71). It consists of a lipid bilayer that acts as an impermeable barrier for low molecular weight solutes (56). Influx of nutrients and antibiotics into the periplasmic space of the bacteria is facilitated by porins which are water filled channels that span the outer membrane and allow the penetration of hydrophilic molecules (43, 71). Some porins are substrate specific whereas others are nonspecific diffusion proteins. Examples of nonspecific diffusion proteins include OmpF and OmpC of *E. coli* and the homologous OmpK35 and OmpK36 of *K. pneumoniae* which are trimeric proteins (38, 58). Clinical studies have shown these diffusion porins of many Enterobacteriaceae serve as a major path for β-lactam antibiotics (43). It's no surprise then that modification of these porins can lead to drug resistance. Decrease in porin expression, mutations that result in premature termination and truncated proteins, or site specific point mutations in key regions like loop 3 can all lead to decreased uptake of the antimicrobial and hence drug resistance (31, 43, 56, 67, 71).

4. CLSI Breakpoints for *E. coli/K. pneumoniae*

Based on pharmacokinetic/pharmacodynamic properties, limited clinical data, and MIC distributions for recently described carbapenemase producing isolates, CLSI recently revised the interpretive criteria for carbapenems which were first published in June 2010 (25). For ertapenem, susceptibility values went from ≤1 μg/ml to ≤0.25 μg/ml. For imipenem and meropenem, values went from ≤4 μg/ml to ≤1 μg/ml. Doripenem values of

≤1 μg/ml were also assigned. These were changed to improve the ability of clinical microbiology laboratories to detect carbapenemase producing organisms by routine susceptibility testing without the use of additional confirmatory tests, as well as to give clinicians a meaningful result to guide therapy (28). However, even though there is a decrease in susceptibility, the specificity goes down. A higher percentage of isolates will be reported as resistant and this may decrease the potential benefit that patients may get from treatment with a carbapenem. However, this is offset by the lower proportion of isolates that will be reported as susceptible to carbapenems despite the presence of a carbapenemase. With the new breakpoints, fewer patients will be treated with carbapenems, a therapeutic approach not considered appropriate by some experts. This highlights the importance of confirmatory tests to detect these important resistance mechanisms in terms of spread and patient safety. For the purpose of this study, we screened any *E. coli* or *K. pneumoniae* with an ertapenem MIC of ≥0.12 μg/ml (reduced susceptibility to carbapenems [CRS]) as a potential carbapenemase producer and performed the additional confirmatory tests to confirm if carbapenemase production was present. Throughout this thesis, the term reduced susceptibility to carbapenems (CRS) applies to any isolate with an ertapenem MIC of 0.12 and 0.25 μg/ml, while carbapenem-resistant was defined as ertapenem MIC ≥0.5 μg/ml.

5. Hypotheses

1. Reduced susceptible and carbapenem-resistant *E. coli* and *K. pneumoniae* are emerging in Canada.

2. Reduced susceptible and carbapenem-resistant *E. coli* and *K. pneumoniae* in Canada are frequently multidrug resistant.

3. Reduced susceptible and carbapenem-resistant *E. coli* and *K. pneumoniae* in Canada arise through carbapenemase production as well as ESBL and/or AmpC production with porin alteration.

6. Scientific Rationale and Objectives

E. coli and *K. pneumoniae* are among the most prevalent organisms causing infections in Canada. Their frequent association with multi-drug resistance (MDR-defined as concomitant resistance to ≥ 3 different antimicrobial classes) has challenged traditional treatment options which include the use of β-lactam antibiotics (penicillins and cephalosporins). The major mechanism of β-lactam resistance in these organisms is through production of extended-spectrum β-lactamases and/or AmpC β-lactamases. In the last two decades, carbapenems have been considered the last line of defence against MDR ESBL and AmpC producing Enterobacteriaceae. Although, still rare, carbapenem resistance in Enterobacteriaceae (defined as meropenem MIC ≥ 4ug/ml) has emerged in Canada. Among *E. coli* and *K. pneumoniae*, the most common carbapenem-resistant mechanism is by *Klebsiella pneumoniae* carbapenemase (KPC) production, although resistance is not limited to this mechanism solely. Other methods of resistance include ESBL and/or AmpC production coupled with outer membrane porin (OMP) alterations. Altered porin expression can be the result of substitutions at key positions, insertions, deletions, frameshift mutations, nonsense mutations, as well as mutations within the promoter, causing down-regulation of transcription. In particular, the alteration of OmpF and OmpC and OmpK35 and OmpK36 in ESBL- and AmpC- producing *E. coli* and *K. pneumoniae*, respectively, has been shown to confer resistance to carbapenems. Although carbapenem-resistant Enterobacteriaceae are rare in Canada, we interested in *E. coli* and *K. pneumoniae* that demonstrate reduced susceptibility to carbapenems (CRS) with an ertapenem MIC of 0.12 and 0.25 μg/ml. Although these are not "carbapenem-resistant" strains they are not wild-type carbapenem susceptible strains, thus are designated as "carbapenem reduced susceptible-CRS" *E. coli* and *K. pneumoniae.*

In this study, all bacterial isolates were obtained from the Canadian Ward Surveillance Study (CANWARD) which is a national surveillance study looking at antimicrobial resistance in Canada. This allows for us to get the overall picture of what's going on in Canada as a whole. All isolates first underwent *in vitro* susceptibility testing to determine prevalence and antimicrobial resistance patterns. Any *E. coli* or *K. pneumoniae* with an ertapenem MIC of ≥0.12 μg/ml were selected for further analysis. Isolates with an MIC of 0.12 and 0.25 μg/ml were termed CRS; those ≥0.5 μg/ml were termed CIR (carbapenem intermediate/resistant). The term multidrug resistance was used to describe isolates with concomitant resistance to ≥3 different classes of antimicrobials.

To understand the epidemiology of these isolates, genotypic characterization was done. ESBL, AmpC, and carbapenemase genes underwent PCR and sequencing for the most common ESBL, AmpC, and carbapenemase genes, along with AmpC promoter/attenuator regions. This allowed for us to determine the roles these resistant determinates play in carbapenem resistance when combined with porin alteration.

To determine the role porins play in carbapenem reduced susceptibility, PCR, sequencing, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out for the major outer membrane porins of *E. coli* and *K. pneumoniae*.

To determine the genetic relatedness of CRS/CIR *E. coli* and *K. pneumoniae*, pulse-field gel electrophoresis was carried out. This would allow us to determine how these organisms are spreading in Canada.

7. Materials and Methods

7.1. Bacterial Isolates: Canadian WARD (CANWARD) Surveillance Study

Bacterial isolates were collected from the Canadian Ward (CANWARD) National Surveillance Study which is an ongoing studying (based at the Health Sciences Centre (HSC) in Winnipeg, Manitoba, Canada) assessing antimicrobial resistance in Canadian hospitals (109). For this thesis, isolates obtained were from the first four years of the study; from January 2007 through November 2010, inclusive. Participating centres (Table 7) submitted pathogens from patients attending hospital clinics, emergency rooms, medical and surgical wards, and intensive care units. Annually, each centre was asked to submit pathogens (consecutive, one per patient/infection site) from blood, respiratory, urine, and wound/IV infections. All isolates were identified at the originating centre using routine procedures performed at each site. Isolates were shipped to HSC on Amies charcoal swabs where they were subcultured onto blood agar and stocked in skim milk at -80ºC.

7.1.1. Selection of Wild Type Isolates

Wild type isolates (ceftazidime, ceftriaxone and ertapenem susceptible) were selected to compare carbapenem MICs with carbapenem-resistant and reduced susceptible isolates. This included 50 *E. coli* and 50 *K. pneumonia* randomly selected isolates. To eliminate any bias, specimens were selected to include all types of infection sites, age, ward, and geographic location.

7.1.2. Selection of Isolates with Reduced Susceptibility to Carbapenems

Isolates defined as having reduced susceptibility to the carbapenems (CRS) were any *E. coli* or *K. pneumoniae* with an ertapenem MIC of 0.12 or 0.25 μg/ml. MIC values of ≥0.5 μg/ml were considered carbapenem intermediate/resistant (CIR). Repeat MICs were done to confirm.

TABLE 7: Centers participating in the CANWARD study.

Centre	City	2007	2008	2009	2010
Children's Hospital of Eastern Ontario	Ottawa, ON	x	x	x	x
CHRTR Pavillion Sainte-Marie	Trois-Rivières, QC			x	x
Health Sciences Centre	Winnipeg, MB	x	x	x	x
Hôpital Maisonneuce-Rosemont	Montreal, QC	x	x	x	x
London Health Sciences Centre	London, ON	x	x	x	x
Montreal General Hospital	Montreal, QC	x	x	x	x
Mount Sinai Hospital	Toronto, ON	x	x	x	x
Queen Elizabeth II Health Sciences Centre	Halifax, NS	x	x	x	x
Royal University Hospital	Saskatoon, SK	x	x	x	x
Royal Victoria Hospital	Montreal, QC	x		x	x
South East Health Care Corporation	Moncton, NB			x	x
St. Joseph's Hospital	Hamilton, ON	x			
St. Michaels' Hospital	Toronto, ON			x	x
The Ottawa Hospital	Ottawa, ON			x	
University of Alberta Hospital	Edmonton, AB	x	x	x	x
Vancouver General Hospital	Vancouver, BC	x	x	x	x

7.2. Antimicrobial Susceptibility Testing

7.2.1. Preparation of Antimicrobial Stock Solutions

Antimicrobial stock solutions were prepared from powder according to CLSI guidelines (23). Antimicrobials tested include: ceftobiprole, ceftaroline, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, cefazolin, ceftriaxone, ceftazidime, cefepime, meropenem, doripenem, ertapenem, tigecycline, levofloxacin, ciprofloxacin, moxifloxacin, colistin, amikacin, gentamicin, nitrofurantoin, and trimethoprim-sulfamethoxazole. Quality control strains (*Staphylococcus aureus* ATCC® 29213, *Enterococcus faecalis* ATCC® 29212, *E. coli* ATCC® 25922, and *Pseudomonas aeruginosa* ATCC® 27853) were used to evaluate the activity of each antimicrobial as a control.

7.2.2. Broth Microdilution

Following two subcultures from frozen stock on Trypticase soy agar plates with 5% sheeps blood (TSA-5%SB), the *in vitro* activity of various antimicrobials was determined in duplicate using 96-well microtitre plates in accordance with CLSI guidelines (23). Food and Drug Administration interpretation breakpoints were used for tigecycline (S: ≤2 μg/ml, I: 4 μg/ml, R: ≥8 μg/ml) and colistin (polymyxin E) [S: ≤2 μg/ml, R: ≥4 μg/ml]. Health Canada interpretation breakpoints were used for ceftobiprole (S: ≤1 μg/ml, I: 2 μg/ml, R: ≥4 μg/ml).

7.3. Phenotypic Identification of Carbapenemase Producers: Modified Hodge Test

For this thesis, any *E. coli* or *K. pneumoniae* isolate with and ertapenem MIC of ≥0.12 μg/ml was tested for carbapenemase production using the Modified Hodge Test (MHT) with 10 μg ertapenem disks according to CLSI guidelines (25). The positive and

negative control strains used were *K. pneumoniae* ATCC® BAA-1705 and *K. pneumoniae* ATCC® BAA-1706, respectively.

7.4. Genotypic Detection Methods for ESBL, AmpC and Carbapenemase Genes

7.4.1. Lysate Preparation

All isolates underwent a DNA extraction procedure involving boiling. Following two subcultures from frozen stock on TSA-5%SB, a few colonies were suspended in 100 µl of sterile distilled water using a sterile loop and vortexed. Each sample was then boiled on a dry bath at 90°C for 20 minutes to allow for DNA extraction. Lysates were then stored at -20°C.

7.4.2. PCR of ESBL Genes

Universal primer sets were used to PCR only the most common ESBL variants as previously described. These included $bla_{\text{CTX-M}}$ (8), bla_{SHV} (69), bla_{TEM} (89), and $bla_{\text{OXA-1-type}}$ (91). Isolates positive for $bla_{\text{CTX-M}}$ using the universal primer set (CTX-M-U1 and CTX-M-U2) were subject to three additional PCR reactions to specify the group (CTX-M-1, CTX-M-2, and CTX-M-9). PCR reactions were carried out using 10 mM dNTP mix, 25 mM $MgCl_2$, 5X colorless GoTaq® Flexi Buffer, and GoTaq® Flexi DNA polymerase (Promega, Madison, WI). Table 8 shows a list of all the primer used for screening and sequencing ESBL genes along with positive controls used.

7.4.3. PCR of AmpC Genes

E. coli isolates were screened for both plasmid-mediated AmpC β-lactamases and chromosomal mutations within the promoter region of the *ampC* gene. Isolates were first screened for the most common AmpC variants; DHA, ENT, CIT, and FOX; as previously described (61). All CIT positive isolates were further tested for the presence of $bla_{\text{CMY-2}}$

gene using CMY-2-1 and CMY-2-2 primers. Table 9 shows a list of all the primers used for screening and sequencing. Positive controls were provided by the National Microbiology Laboratory (NML): *K. pneumoniae* N07-1535 (DHA positive), *K. pneumoniae* ESBL-99 (FOX positive), *E. coli* N02-080 (CIT/CMY-2 positive), and *E. cloacae* (ENT positive). Isolates negative for all plasmid-mediated AmpC β-lactamases were then tested for mutations within the promoter region of the chromosomal *ampC* gene using the AmpC1 and AmpC2 primers shown in Table 9. The promoter sequences were then compared to the *E. coli* K-12 promoter sequence: GeneBank accession number U00096. Any mutations were noted as previously described (21). PCR reactions were carried out using 10 mM dNTP mix, 25 mM $MgCl_2$, 5X colorless GoTaq® Flexi Buffer, and GoTaq® Flexi DNA polymerase (Promega, Madison, WI).

TABLE 8: List of primers used for PCR and sequencing of ESBL and KPC genes.

ESBL genes	Primers	Primer sequence (5' → 3')	Product size (bp)	Positive controls	Reference
TEM	TEM-1 TEM-2 TEM-B TEM-C TEM-D TEM-F	ATAAAATTCTTGAAGAC TTACCAATGCTTAATCA AAAACTCTCAAGGATCTT AAAGATGCTGAAGATCA TTTGGTATGGCTTCATTC TTTTTTGCACAACATGGG	Universal Primer Set TEM-1/2: 1079	62175	(89)
SHV	SHV-up SHV-lo	CGCCGGGTTATTCTTATTTGTCGC TCTTTCCGATGCCGCCGCCAGTCA	1016	80940	(69)
CTX-M	CTX-M-U1 CTX-M-U2	ATGTGCAGYACCAGTAARGTKATGG-C TGGGRRAARTARGTSACCAGAAYCA-GCGG	593	59096	(8)
CTX-M-1 group	CTX-M-1-A CTX-M-1-B CTX-M-1-C CTX-M-1-D	TGGTTAAAAAATCACTGCG ATTACAAACCGTCGGTGAC ATAACGRGGCGATGAATAAG ATTCATCGCCACGTTATCG	CTX-M-1-A/B: 876	59069	(8)
CTX-M-2 group	TOHO-1-1 TOHO-1-2	ACTCAGAGCATTCGCCGCTCA TTATTGCATCAGAAACCGTG	879	64539	(8)
CTX-M-9 group	TOHO-2-1 TOHO-2-2 TOHO-2-3 TOHO-2-4	ATGGTGACAAAGAGAGTGCAACG ACAGCCCTTCGGCGATGATTC CGATCGGCGATGAGACGTTT ACGTCTCATCGCCCGATCGC	TOHO-2-1/2: 837	62175	(8)
OXA-1	OXA1.F OXA1.R	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	813	77713	(91)
KPC	KPC-F KPC-R KPC1 KPC2 ISKpn7-3781L ISKpn6-4714	TGTCACTGTATCGCCGTC CTCAGTGCTCTACAGAAAACC ATGTCACTGTATCGCCGTC AATCCCTCGAGCGCGAGT GCTTTCTTGCTGCCGCTGTG GAAGATGCCAAGGTCAATGC	KPC-F/R: 1000bp	BAA-1705	(65)

TABLE 9: List of primers used for PCR and sequencing of AmpC genes.

AmpC genes	Primers	Primer sequence (5 → 3')	Product size (bp)	Positive controls	Reference
DHA	DHA-1 DHA-2	TTCTGCCGCTGATAATGTCGC GGCTTTGACTCTTTCGGTATTC	1047	N07-1535	(61)
ENT	ENT-A ENT-B	TGTGGACGGCATTATCCAG AAAGCGCGTAACCGGATTG	877	N03-0087	(61)
FOX	FOX-A FOX-B	AGTAAAACCTTCACCTTCACCG ATGCGCCTCTTCCGCTTTC	405	ESBL-99	(61)
CIT	CIT-A CIT-B	ATGCAGGAGCAGGCTATTC TGGAGCGTTTTCTCCTGAAC	689	N02-0080	(61)
CMY-2	CMY-2-1 CMY-2-2 CMY-2-4 CMY-2-5	ACACTGATTGCGTCTGACG AATATCCTGGGCCTCATCG TGCAACCATTAAAACTGGC TTGCTTTTAATTACGGAAC	-	-	unpublished
AmpC promoter	AmpC1 AmpC2	AATGGG-TTTTCTACGGTVTG GGGCAGCAAATGTGGAGCAA	191	-	(21)

7.4.4. PCR of Carbapenemase Genes

All isolates, regardless of MHT results, were subject to KPC PCR as previously described (102). Universal primers, KPC-F and KPC-R were used to amplify the KPC gene. The positive and negative control strains used were *K. pneumoniae* ATCC® BAA-1705 and *K. pneumoniae* ATCC® BAA-1706, respectively. The list of primers used for screening and sequencing are shown in Table 8. Isolates with a positive MHT results were subject to a multiplex PCR, as previously described (60), which included primers for NDM, KPC, IMP, VIM, GES, and OXA-48. KIVNGO positive controls were used from NML.

7.4.5. Agarose Gel Electrophoresis

All PCR products were analyzed by electrophoresis using 1.5% agarose gels. Gels were made by bringing 1.5 g of agarose (Invitrogen, Carlsbad, CA) to a boil in 100 ml of 0.5X Tris-Borate-EDTA buffer (TBE; 0.045M Tris-borate, 0.0001M EDTA [pH 8.3± 0.3]). Before pouring the gel into a casting tray with a comb, 1 µl of ethidium bromide was added to the solution. The gel was allowed to solidify for ~30 minutes before it was put into the electrophoresis chamber containing 0.5X TBE. Approximately 10 µl of each sample was loaded with 2 µl of Ficoll dye. A 123bp DNA ladder (Invitrogen, Carlsbad, CA) was used as the molecular standard for size comparison. Each gel was run for ~50 minutes at 110 Volts/cm and visualized using the AlphaImager HP (Alpha Innotech, Santa Clara, CA).

7.5. Porin Analysis

7.5.1. Lysate Preparation

A glass bead DNA extraction procedure was used for all isolates. Following two subcultures from frozen stock on TSA-5%SB, 3-5 colonies were emulsified in 600 µl of neutralization buffer (30mM Tris [pH 8.4] and 2mM EDTA [pH 9]) containing 50 µl of glass beads using a sterile loop and vortexed. The samples were then heated for 2 minutes at 95-100°C in a heating block then processed on the Disruptor Genie® for 2 minutes to allow for cell lysis and DNA extraction to occur. Before samples were used for PCR, each tube was centrifuged at 3000 rpm for 1 minute to allow glass beads to collect at the bottom.

7.5.2. PCR of Porin Genes

Outer membrane porins (OMPs) were amplified by PCR as previously described (31). Primers used to amplify the two major *E. coli* OMPs were EcOmpFA and EcOmpFB (OmpF porin) and EcOmpCA and EcOmpCB (OmpC porin). To amplify the two major *K. pneumoniae* OMPs, OmpK35-F and OmpK35-R (OmpK35 porin) and OmpK36-F and OmpK36-R (OmpK36 porin) were used. The list of primers used are shown in Table 10. PCR products were run on an agarose gel as described in section 7.4.5. For the PCR reactions, a 2X master mix was prepared by combining 200 µl of 10X PCR Buffer (Applied Biosystems, Carlsbad, CA), 240µl of 25mM $MgCl_2$, 16µl of 100mM deoxynucleoside triphosphates (dATP, dCTP, dTTP, and dGTP) (Applied Biosystems, Carlsbad, CA), and 544µl of double distilled water (ddH_2O). Ampli-Taq Gold (Applied Biosystems, Carlsbad, CA) was used for all reactions.

7.5.3. Porin Extraction: Cell Preparation from Culture

Each isolate was first inoculated into 25 ml of Nutrient Broth (Fisher Scientific, Ottawa, ON) and incubated at 37°C with shaking for 18-20 hours. Cells were then spun down at 6000 rpm at 4°C for 15 minutes using the Avnati^{TM} JA-25 with the JA-12 rotor (Beckman Coulter, Mississauga, ON). The supernatant was aspirated and discarded while the cells were kept on ice. The pellet was resuspended in 2 ml of 10mM Tris-HCl (pH 7.2) and then sonicated (30 seconds sonication and 30 seconds rest, 4X) using the Virsonic Ultrasonic Cell Disruptor 100 (VirTis, Warminster, PA). After sonication, the supernatant was transferred into two 1.5 ml tubes (1 ml into each tube) and spun down at 6000 rpm for 10 minutes. The supernatant was transferred into new tubes and the pellet was discarded. One hundred microliters of 20% sarcosyl was added to each tube and incubated at room temperature for 30 minutes. After 30 minutes, the supernatant was transferred into a Quick-Seal® 5.1 ml ultracentrifuge tube (Beckman Coulter, Mississauga, ON) and filled to the top with 10mM Tris-HCl (pH 7.2). Samples were ultracentrifuged at 90000 xg at 4°C for 1 hour using the Optima^{TM} XL-100K Ultracentrifuge with the NVT-100 rotor (Beckman Coulter, Mississauga, ON). The supernatant was aspirated and the pellet was resuspended in 100 µl of 20mM Tris (pH 7). Samples were stored at -20°C.

TABLE 10: List of primers used to amplify porins of *E. coli* and *K. pneumoniae*.

OMP gene	Primers	Primer sequence (5' → 3')	Product size (bp)	Reference
OmpF	EcOmpFA	CAGGTACTGCAAACGCTGC	-	unpublished
	EcOmpFB	GTCAACATAGGTGGACATG		
OmpC	EcOmpCA	GTTAAAGTACTGTCCCTCCTG	-	unpublished
	EcOmpCB	GAACTGGTAAACCAGACCCAG		
OmpK35	OmpK35-F	GCACGAAACAGATCGGCCAG	1080	(31)
	OmpK35-R	TTACGTCACCGGCGTGCAGAA		
	OmpK35x1	GCGCAATATTCTGGCAGTGGTGAT		
	OmpK35x2	TGACGGCCGCATAGATGTTGTTAG		
	OmpK35x3	CGACGTCGAAGCGGCAACC		
	OmpK35x4	CTCACGAATACAGAAAAGCAGGAC		
OmpK36	OmpK36-F	GACCCGCCAGAAGGTGCCCA	1165	(31)
	OmpK36-R	TGATGTTGCCGGGGATCAGGGA		
	OmpK36x1	TGGTAGCAGGCGCAGCAAATG		
	OmpK36x2	CGCTGCCGTTTTTACCCTGATACT		
	OmpK36xA	TGCAGCACAATGAAATAGCC		
	OmpK36xB	CCCTGATACTGCAGAGCAAA		

7.5.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

For SDS-PAGE, 12.5% running gels and 4.5-5% stacking gels were made. The components of the running gel include: 2.24 ml of Tris-HCl (pH 8.8), 2.5 ml of acrylamide/bis-acrylamide, 30 µl of 10% SDS, 20 µl of 10% ammonium persulfate (APS), 10 µl of tetramethylethylenediamine (TEMED), and 1.44 g of urea. The components of the stacking gel include: 1.4 ml of water, 250 µl of Tris-HCl (pH 6.8), 330 µl of acrylamide/bis-acrylamide, 10 µl of 10% SDS, 10 µl of 10% APS, and 10 µl of TEMED. Note that APS is a polymerization initiator used with TEMED for polyacrylamide gel formation. These two components are added last, just before pouring the solution between the glass plates. First the running gel components were mixed except for APS and TEMED. From this, 1 ml was transferred into a 1.5 ml tube and 10 µl of APS and 10 µl of TEMED was added to this. This was poured between the glass plates to the bottom and allowed to solidify for ~5 minutes to seal the bottom of the gel. APS and TEMED was added to the remaining running gel solution and poured between the glass plates leaving about an inch at the top for the stacking gel. Immediately, 70% ethanol was poured on top to cover the gel (this seals the running gel and allows it to polymerize in a uniformly straight line). This was left for ~30 minutes to allow polymerization. After 30 minutes, the ethanol was poured off and a comb was placed in between the glass plates. All the components of the stacking gel were mixed and immediately poured between the glass plates to the very top and allowed to solidify for ~20 minutes. Once the gel was solidified, the comb was removed and the gel was loaded into the Mini Trans-Blot® Cell (Bio-Rad, Hercules, CA) containing 1X running buffer (Trizma-base, glycine and SDS). Before loading the samples into the wells they were prepared. Forty microliters of sample was

mixed with 10 µl of 5X sample buffer (0.56mM Tris-HCl [pH 7.0], 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-beta-mercaptoethanol, and 1 ml 0.05% bromophenol blue) and boiled for 5 minutes. About 30 µl of sample was loaded into the wells with the wells on each end loaded with EZ-Run Rec Protein Ladder (Fisher Scientific, Ottawa, ON). The gel was run at 200V for 60 minutes. Once the run was complete, the gel was placed into a container and washed with sterile distilled water for 5 minutes (process done 3 times) then stained with Coomassie Blue G250 Stain (Bio-Rad, Hercules, CA) for 60 minutes. After 60 minutes the stain was poured off and the gel was destained for 30 minutes with boiling sterile distilled water. The gel was then vacuum dried onto blot paper using the HydroTech Vacuum Pump with the Model 583 Gel Dryer (Bio-Rad, Hercules, CA).

7.6. PCR Product Purification

PCR products were purified using Ultracel® YM-100 membranes (Millipore, Billerica, MA). First ~160 µl of sterile water was added to the remaining 40 µl of PCR product (10 µl was used for the initial gel electrophoresis) to bring the volume up to 200 µl which was then added to the YM-100 quick spin columns and spun at 500 xg for 10 minutes. The columns were inverted into new tubes and 25 µl of sterile water was added and spun at 1000 xg for 5 minutes to elute the DNA. The concentration of purified product was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted with sterile water to 50 ng/ml before samples were sequenced.

7.7. DNA Sequencing

All DNA sequencing reactions were done using Applied Biosystems BigDye Terminator Version 3.1 chemistry. MJ Research instruments were used for thermal cycling

and purification was done using Agencourt CleanSeq magnetic bead technology (Beckman Coulter, Mississauga, ON). The sequencing reactions were run on the ABI 3730XL DNA Sequencer (Applied Biosystems, Carlsbad, CA).

7.8. Sequence Analysis

All sequence analysis was done using Lasergene® Core Suite software (DNASTAR, Madison, WI). SeqMan Pro was used for contig assembly and analysis. EditSeq was used to translate the DNA sequence into a protein sequence. MegAlign was used to align all the protein and DNA sequences for final analysis. A BLAST search was done for ESBL, AmpC, and KPC genes to determine the specific variants. Porin sequences of *E. coli* (OmpF and OmpC) and *K. pneumoniae* (OmpK35 and OmpK36) were compared to wild type OMP sequences using *E. coli* ATCC® 25922 and *K. pneumoniae* ATCC® 13883. Any mutations leading to a modified protein were noted.

7.9. Genetic Relationships

7.9.1. Pulse Field Gel Electrophoresis (PFGE)

To determine the genetic relationships among *E. coli* and *K. pneumoniae*, PFGE was performed using the following protocol. Following two subcultures from frozen stock on TSA-5%SB, each isolate was suspended in 2 ml of Cell Suspension Buffer (100mM Tris-HCl [pH 8.0] and 100mM EDTA [pH 8.0]) which was adjusted to an absorbance of 1.3-1.4 at 610 nm. One hundred microliters of the adjusted cell suspension was mixed with 100 µl of 1.6% Certified Low-Melt Agarose (Bio-Rad, Hercules, CA) which was first melted on a hot plate. Immediately, ~100 µl of the mixture was dispensed into disposable plug molds (Bio-Rad, Hercules, CA) and allowed to solidify at room temperature for 15 minutes. Next was cell lysis which was carried out by adding the solidified plug to 1 ml of

Cell Lysis Buffer (50mM Tris-HCl [pH 8.0], 50mM EDTA [pH 8.0], and 1% sarcosyl) with 10 µl of Proteinase K (25 mg/ml) an incubated in 50°C water bath for 2 hours. The plugs were then washed twice with 1 ml sterile distilled water followed by four washes with 1 ml TE buffer (10mM Tris-HCl [pH 8.0] and 1mM EDTA [pH 9.0]) with each wash incubated in a 50°C water bath for 15 minutes in between. The plugs were stored in TE buffer at 4°C until ready for restriction endonuclease digestion. Restriction digests were set up as 200 µl reactions. Half of each plug (cut with sterile scalpel) were added to the restriction digest mix which included 176 µl sterile distilled water, 20 µl NEBuffer 4 (New England BioLabs, Pickering, ON), and 4 µl of *Xba*I (New England BioLabs, Pickering, ON) and incubated for 4 hours in a 37°C water bath after mixing. After digestion, each sample was run on a 1% Pulse-Field Certified agarose gel (Bio-Rad, Hercules, CA) which was made by dissolving the agarose in 0.5X TBE. Before loading the samples, the digest mixture was aspirated and the plugs were incubated at room temperature in 200 µl of 0.5X TBE for 5 minutes. The TBE was then aspirated and the plugs were melted in a 69°C dry bath for 5 minutes. Thirty-five microliters of melted plug was loaded into each well of the gel and allowed to solidify for 5 minutes. Using a sterile scalpel and spatula, a 0.5-1 mm thick section of lambda ladder (New England BioLabs, Pickering, ON) was loaded into the first and last wells as a molecular size standard. The gel was then placed in the CHEF DRIII electrophoresis chamber (Bio-Rad, Hercules, CA) containing 2000ml of 0.5X TBE with 2 ml of 100mM thiourea. The power supply was set to 75-80 and cooling module was set to 14°C. The following parameters were used for the run: initial switch time was 2.2 seconds, final switch time was 54.2 seconds, voltage was set to 6 V/cm, 200V, included angle was 120 and the run time was 19 hours. Once the run was complete, the gel was

stained with SYBR® Green (Molecular Probes, Eugene, OR) for 50 minutes in the dark and destained with sterile distilled water if necessary. The gel was then visualized with the AlphaImager HP (Alpha Innotech, Santa Clara, CA) and a picture was saved.

7.9.2. Pattern Analysis

The saved picture of the PFGE gel was then analyzed using Bionumerics version 3.5 (Applied Maths, Austin, TX). The unweighted pair-group method was used for cluster analysis and to produce the dendrogram. Genetic similarity (%) was calculated based on the Dice coefficient. Isolates with greater than 80% similarity were considered to be genetically related.

7.10. Statistical Analysis

Statistical analysis was done using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/index.cfm). Values not statistically significant will be denoted NS. P values > 0.05 are considered NS.

8. Results

8.1. Prevalence of CRS and CIR Isolates in Canadian Hospitals from 2007 to 2010

A total of 23,243 clinically significant organisms were collected from January 2007 to November 2010, inclusive, as part of the CANWARD National Surveillance Study. *E. coli* and *K. pneumoniae* ranked first and fifth in terms of numbers of organisms submitted and made up 20.7% (4807/23,243) and 6.1% (1419/23,243) of all isolates, respectively. Out of the 4807 *E. coli*, 0.83% (40/4807) had an ertapenem MIC of 0.12 or 0.25 μg/ml and were categorized as carbapenem reduced susceptible (CRS); 0.35% (17/4807) had an ertapenem MIC ≥0.5 μg/ml and were categorized carbapenem intermediate/resistance (CIR). Out of the 1419 *K. pneumoniae*, 0.85% (12/1419) were categorized as CRS; 0.78% (11/1419) were categorized as CIR.

When looking at the increase from 2007 to 2010 of CRS and CIR isolates, no observable trend was present when the two cohorts were separated. However there was a doubling in prevalence for *K. pneumoniae* (2007: 1.1%, 2008: 1.3%, 2009: 2.5%, and 2010: 2.6%; P value=NS) when looking at all isolates with MICs ≥0.12 μg/ml. When looking at all *E. coli* isolates with MICs ≥0.12 μg/ml, the prevalence from year to year was relatively stable (2007: 1.3%, 2008: 2.0%, 2009: 1.4%, and 2010: 1.2%; P value=NS). This is summarized in Table 11.

TABLE 11: The national prevalence rates of CRS/CIR *E. coli* and *K. pneumoniae* from CANWARD 2007 to 2010.

	#/total (%)	
Study year	**CRS/CIR *E. coli***	**CRS/CIR *K. pneumoniae***
2007	7/524 (1.3)	2/188 (1.1)
2008	23/1132 (2.0)	4/314 (1.3)
2009	15/1097 (1.4)	9/357 (2.5)
2010	12/1018 (1.2)	8/307 (2.6)

8.2. Patient Demographics

8.2.1. CRS-*E. coli* and CRS-*K. pneumoniae*

Patient demographics for all CRS and CIR isolates are summarized in Table 12. The prevalence of CRS-*E. coli* (CRS-EC) was even among males (0.76%) and females (0.88%) although it was slightly higher in females (P value=NS). In contrast, CRS-*K. pneumoniae* (CRS-KP) were more common among males (1.04%) than in females (0.61%) (P value=NS). Both CRS- EC and KP infections were seen more commonly in patients with ≥18 years of age (P value=NS). Infections with CRS-EC were most likely seen in BC/AB (1.47%) than SK/MB (0.62%), ONT (0.91%), and QC/Mar (0.59%) (P value=0.03). In contrast, infections with CRS-KP were most likely seen in ONT (1.74%), than BC/AB (0%), SK/MB (0%), and QC/Mar (0.77%) (P value=0.02). CRS-EC were most commonly isolated from ICUs (2.36%) followed by surgical wards (1.17%), medical wards (1.09%), outpatient clinics (0.60%), and ERs (0.33%) (P value=0.002). CRS-KP were most commonly isolated from medical wards (1.25%) followed by ICUs (1.12%), surgical wards (0.70%), outpatient clinics (0.62%), and ERs (0.27%) (P value=NS). The most common source of isolation of CRS-EC was the respiratory tract (1.34%) followed by wound (1.16%), urine (0.82%), and blood (0.76%) (P value=NS). The most common source of isolation of CRS-KP was from wounds (1.45%) followed by blood (1.05%), urine (0.91%), and respiratory (0%) (P value=NS).

8.2.2. CIR-*E. coli* and CIR-*K. pneumoniae*

Infections with CIR-EC were slightly more prevalent in males (0.49%) than females (0.27%) (P value=NS). Infections with CIR-KP were even among males (0.78%) and females (0.77%). In contrast to CRS isolates, CIR isolates are more common among

TABLE 12: Demographics, hospital ward and specimen source types from patients with CRS-EC, CIR-EC, CRS-KP, and CIR-KP infections in Canadian hospitals (2007 to 2010).

		#CRS/#Total (%)		#CIR/#Total (%)		# (%) of patients			
Parameter	**Value**	**CRS-EC *n*=40/*n*=4807 (0.83)**	**CRS-KP *n*=12/*n*=1419 (0.85)**	**CIR-EC *n*=17/*n*=4807 (0.35)**	**CIR-KP *n*=11/*n*=1419 (0.78)**	**CRS-EC *n*=40**	**CRS-KP *n*=12**	**CIR-EC *n*=17**	**CIR-KP *n*=11**
Sex	**M**	14/1845 (0.76)	8/767 (1.04)	9/1845 (0.49)	6/767 (0.78)	14 (35)	8 (66.7)	9 (52.9)	6 (54.5)
	F	26/2962 (0.88)	4/652 (0.61)	8/2962 (0.27)	5/652 (0.77)	26 (65)	4 (33.3)	8 (47.1)	5 (45.5)
Age	**≤17**	2/513 (0.39)	1/140 (0.71)	3/513 (0.58)	2/140 (1.43)	2 (5)	1 (8.3)	3 (17.6)	2 (18.2)
	18-65	20/2076 (0.96)	6/606 (0.99)	7/2076 (0.34)	5/606 (0.83)	20 (50)	6 (50)	7 (41.2)	5 (45.5)
	≥66	18/2218 (0.81)	5/673 (0.74)	7/2218 (0.32)	4/673 (0.59)	18 (45)	5 (41.7)	7 (41.2)	4 (36.4)
Patient region[a]	**BC/AB**	12/814 (1.47)	0/241 (0)	8/814 (0.98)	1/241 (0.41)	12 (30)	0 (0)	8 (47.1)	1 (9.1)
	SK/MB	5/804 (0.62)	0/197 (0)	1/804 (0.12)	0/197 (0)	5 (12.5)	0 (0)	1 (5.9)	0 (0)
	ONT	12/1322 (0.91)	8/459 (1.74)	4/1322 (0.30)	10/459 (2.18)	12 (30)	8 (66.7)	4 (23.5)	10 (90.0)
	QC/Mar	11/1867 (0.59)	4/522 (0.77)	4/1867 (0.21)	0/522 (0)	11 (27.5)	4 (33.3)	4 (23.5)	0 (0)
Hospital ward[b]	**Medical**	15/1381 (1.09)	6/480 (1.25)	7/1381 (0.51)	6/480 (1.25)	15 (37.5)	6 (50)	7 (41.2)	6 (54.5)
	Surgical	4/341 (1.17)	1/143 (0.70)	0/341 (0)	0/143 (0)	4 (10)	1 (8.3)	0 (0)	0 (0)
	ICU	10/424 (2.36)	3/267 (1.12)	6/424 (1.42)	5/267 (1.87)	10 (25)	3 (25)	6 (35.3)	5 (45.5)
	ER	6/1831 (0.33)	1/367 (0.27)	2/1831 (0.11)	0/367 (0)	6 (15)	1 (8.3)	2 (11.8)	0 (0)
	Clinic	5/830 (0.60)	1/162 (0.62)	2/830 (0.24)	0/162 (0)	5 (12.5)	1 (8.3)	2 (11.8)	0 (0)
Specimen source[c]	**Urine**	16/1953 (0.82)	3/330 (0.91)	3/1953 (0.15)	1/330 (0.30)	16 (40)	3 (25)	3 (17.6)	1 (9.1)
	Blood	18/2382 (0.76)	8/763 (1.05)	8/2382 (0.34)	5/763 (0.66)	18 (45)	8 (66.7)	8 (47.1)	5 (45.5)
	Wound	2/173 (1.16)	1/69 (1.45)	2/173 (1.16)	1/69 (1.45)	2 (5)	1 (8.3)	2 (11.8)	1 (9.1)
	Resp.	4/299 (1.34)	0/257 (0)	4/299 (1.34)	4/257 (1.56)	4 (10)	0 (0)	4 (23.5)	4 (36.4)

[a]BC/AB, British Columbia and Alberta; SK/MB, Saskatchewan and Manitoba; ONT, Ontario; QC/Mar, Québec and Maritimes.
[b]ICU, intensive care unit; ER, Emergency room; clinic, Outpatient clinic.
[c]Resp., respiratory.

patients that are ≤17 years of age (P value=NS). Infections with CIR-EC were most likely seen in BC/AB (0.98%) than SK/MB (0.12%), ONT (0.30%), and QC/Mar (0.21%) (P value=0.004). Infections with CIR-KP were most likely seen in ONT (2.18%) than BC/AB (0.41%), SK/MB (0%), and QC/Mar (0%) (P value=0.0001). CIR-EC isolates were most commonly isolated from ICUs (1.42%) followed by medical wards (0.51%), clinics (0.24%), and ERs (0.11%) (P value=0.002). No CIR-EC were isolated from surgical wards. CIR-KP isolates were also most commonly isolated from ICUs (1.87%) followed by medical wards (1.25%) (P value=NS). No CIR-KP were isolated from surgical wards, ERs, and clinics. The most common specimen source of CIR-EC was the respiratory tract (1.34%) followed by wound (1.16%), blood (0.34%), and urine (0.15%) (P value=0.02). The most common specimen source of CIR-KP was the respiratory tract (1.56%) followed by wound (1.45%), blood (0.66%), and urine (0.30%) (P value=NS).

8.3. Antimicrobial Susceptibilities

8.3.1. CRS-*E. coli* and CRS-*K. pneumoniae*

Antimicrobial susceptibilities among CRS isolates are summarized in Table 13. Among CRS isolates, resistance rates were relatively high for cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole. MDR was seen in 21/40 (52.5%) and 5/12 (41.7%) of CRS-EC and CRS-KP isolates, respectively. The most active agents against CRS- EC were tigecycline (100% susceptible), amikacin (95% susceptible), and colistin (97.5% susceptible), and the carbapenems. Piperacillin-tazobactam remained relatively active (77.5% susceptible). The most active agents against CRS-KP were amikacin (91.7% susceptible), gentamicin (83.3% susceptible), and colistin (100% susceptible).

TABLE 13: Antimicrobial susceptibilities of CRS-EC and CRS-KP from Canadian hospitals (2007-2010).

	CRS-EC (*n* = 40)					CRS-KP (*n* = 12)				
Drug*	**MIC_{50}**	**MIC_{90}**	**% Susceptible**	**% Intermediate**	**% Resistant**	**MIC_{50}**	**MIC_{90}**	**% Susceptible**	**% Intermediate**	**% Resistant**
Cefazolin	>128	>128	0	0	100	64	>128	0	8.3	91.7
Ceftazidime	32	>32	22.5	2.5	75	16	>32	25	0	75
Ceftriaxone	32	>64	15	0	85	0.5	>64	58.3	0	41.7
Cefepime[a]	4	>32	52.9	5.9	41.2	8	32	57.1	14.3	28.6
Cefoxitin	32	>32	22.5	15	62.5	8	>32	66.7	8.3	25
Doripenem	≤0.12	≤0.12	100	0	0	≤0.12	≤0.12	100	0	0
Ertapenem	0.12	0.25	100	0	0	0.25	0.25	100	0	0
Meropenem	≤0.12	≤0.12	100	0	0	≤0.12	0.25	100	0	0
AMC	16	>32	42.5	32.5	25	8	32	58.3	25	16.7
TZP	8	64	77.5	17.5	5	16	>512	66.7	8.3	25
Ciprofloxacin	>16	>16	32.5	0	67.5	8	>16	33.3	8.3	58.3
Levofloxacin[b]	16	>32	23.5	0	76.5	8	>32	28.6	14.3	57.1
SXT	0.5	>8	55	-	45	4	>8	41.7	-	58.3
Tigecycline	0.5	1	100	0	0	1	8	66.7	16.7	16.7
Amikacin	2	8	95	2.5	2.5	1	16	91.7	0	8.3
Gentamicin	1	>32	57.5	0	42.5	0.5	>32	83.3	0	16.7
Colistin	0.5	1	97.5	-	2.5	0.5	1	100	-	0

*Drug concentrations in μg/ml; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole.

[a]Cefepime *n*=34 for CRS-EC; *n*=7 for CRS-KP

[b]Levofloxacin *n*=34 for CRS-EC; *n*=7 for CRS-KP

Tigecycline was less active against CRS-KP with only 66.7% remaining susceptible. Carbapenems were still highly active.

8.3.2. CIR-*E. coli* and CIR-*K. pneumoniae*

Antimicrobial susceptibilities among CIR isolates are summarized in Table 14. CIR isolates are those that are intermediate or resistant to ertapenem. MDR was seen in 11/17 (64.7%) and 9/11 (81.8%) of CIR-EC and CIR-KP, respectively. Doripenem and meropenem still remained active with 100% and 90.9% of CIR-EC and CIR-KP isolates still susceptible. Resistance to third generation cephalosporins was very high at >90% of isolates being resistant. The most active agents against CIR-EC were tigecycline (94.1% susceptible), amikacin (82.4%), and colistin (100% susceptible). The most active agents against CIR-KP were amikacin and colistin, both with 90.9% of isolates still remaining susceptible.

8.3.3. Wild Type *E. coli* and *K. pneumoniae*

As a control, 50 wild type *E. coli* and 50 wild type *K. pneumoniae* MICs were compared to CRS and CIR isolates. Both wild type *E. coli* and *K. pneumoniae* had an average ertapenem MIC of 0.008 μg/ml. CRS- *E. coli* and *K. pneumoniae* were isolates with ertapenem MICs of 0.12 and 0.25 μg/ml; a 16-32x increase in MIC compared to wild type. When comparing wild type *E. coli* to CIR-*E. coli*, ertapenem MICs ranged from 0.5 to 4 μg/ml; a 64-512x increase in MIC compared to wild type. CIR-*K. pneumoniae* ertapenem MICs ranged from 0.5 to 16 μg/ml; a 64-2048x increase in MIC compared to wild type isolates.

TABLE 14: Antimicrobial susceptibilities of CIR-EC and CIR-KP from Canadian hospitals (2007-2010).

	CIR-EC (*n* = 17)					CIR-KP (*n* = 11)				
Drug*	**MIC_{50}**	**MIC_{90}**	**% Susceptible**	**% Intermediate**	**% Resistant**	**MIC_{50}**	**MIC_{90}**	**% Susceptible**	**% Intermediate**	**% Resistant**
Cefazolin	>128	>128	0	0	100	>128	>128	0	0	100
Ceftazidime	>32	>32	0	5.9	94.1	>32	>32	9.1	0	90.9
Ceftriaxone	>64	>64	0	0	100	>64	>64	9.1	0	90.9
Cefepime[a]	32	>32	36.4	9.1	54.5	32	>32	12.5	25	62.5
Cefoxitin	32	>32	0	29.4	70.6	8	>32	54.5	18.2	27.3
Doripenem	≤0.12	0.5	100	0	0	≤0.12	≤0.12	90.9	0	9.1
Ertapenem	1	2	0	41.2	58.8	0.5	1	0	72.7	27.3
Meropenem	≤0.12	1	100	0	0	≤0.12	0.25	90.9	0	9.1
AMC	32	>32	17.6	23.5	58.8	16	32	27.3	54.5	18.2
TZP	32	64	47.1	35.3	17.6	16	64	36.4	54.5	9.1
Ciprofloxacin	>16	>16	17.6	5.9	76.5	>16	>16	9.1	0	90.9
Levofloxacin[b]	16	>32	27.3	0	72.7	16	>32	12.5	0	87.5
SXT	2	>8	52.9	-	47.1	4	>8	45.5	-	54.5
Tigecycline	0.5	1	94.1	5.9	0	1	4	63.6	36.4	0
Amikacin	8	32	82.4	11.8	5.9	2	16	90.9	9.1	0
Gentamicin	2	>32	64.7	0	35.3	16	>32	36.4	9.1	54.5
Colistin	0.5	0.5	100	-	0	0.5	0.5	90.9	-	9.1

*Drug concentrations in μg/ml; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole.

[a]Cefepime *n*=11 for CIR-EC; *n*=8 for CIR-KP

[b]Levofloxacin *n*=11 for CIR-EC; *n*=8 for CIR-KP

8.4. Molecular Characterization of CRS- *E. coli* and *K. pneumoniae*

The molecular mechanisms of resistance for CRS isolates are outlined in Tables 15 and 16 and Figure 5. Among CRS-EC isolates, the most common mechanism of resistance was AmpC P/A (promoter/attenuator mutations) or CMY-2 at 43% followed by CTX-M-15 production at 37%. Five percent of CRS-EC isolates were positive for CTX-M-14 and 15% had either an ESBL other than CTX-M- 14 or 15 or an unknown mechanism which may be attributed to porin mutations. Among CRS-KP isolates, 67% had an unknown mechanism of resistance which may be attributed to porin mutations. Twenty-five percent of CRS-KP isolates were positive for CTX-M-15 followed by 8% positive for CTX-M-14.

8.5. Molecular Characterization of CIR- *E. coli* and *K. pneumoniae*

The molecular mechanisms of resistance for CIR isolates are outlined in Tables 15 and 16 and Figure 6. Among CIR-EC the most common resistance mechanism was the production of CTX-M-15 at 41%. Twenty-nine percent had AmpC P/A or CMY-2 present. Twelve percent of CIR-EC isolates were producing CTX-M-15 and 1 isolate (6%) was producing KPC-3. The other 12% had an unknown mechanism of resistance. Among CIR-KP the most common resistance mechanism was through production of CTX-M-15 at 82%. One isolate (9%) was producing KPC-3 and 1 isolates (9%) had an unknown mechanism of resistance. Both KPC isolates mentioned above had positive MHT results. Two other isolates, 82940 and 92885, had a positive MHT result but no carbapenemase gene was identified by PCR.

TABLE 15: Molecular mechanisms of resistance among CRS and CIR *E. coli*.

Isolate #	Molecular mechanism*	MIC (µg/ml)[a]								
		ETP	MEM	DOR	CRO	CAZ	FEP	FOX	AMC	TZP
76576	CTX-M-15, OXA-1	0.12	≤0.06	≤0.06	>64	32	16	8	16	8
76789	CTX-M-15, OXA-1	0.12	≤0.06	≤0.06	>64	>32	>32	16	8	16
76819	CTX-M-15, OXA-1	0.25	≤0.06	≤0.06	>64	32	32	4	16	4
76838	CTX-M-15, TEM-1, OXA-1	0.12	≤0.06	≤0.06	>64	>32	>32	8	16	64
76897	CMY-2	0.12	≤0.06	≤0.06	16	32	4	>32	16	4
77298	CMY-2	0.5	≤0.06	≤0.06	>64	>32	16	>32	32	32
78399	CTX-M-3, TEM-1	0.12	≤0.06	≤0.06	>64	4	32	>32	4	8
78967	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	1	0.12	≤0.06	>64	>32	4	>32	32	256
79078	CTX-M-15, TEM-1	0.12	≤0.06	≤0.06	>64	>32	32	16	8	4
79170	CTX-M-15, OXA-1	0.25	≤0.06	≤0.06	>64	>32	>32	8	8	4
79234	CTX-M-15, OXA-1	0.12	≤0.06	≤0.06	>64	>32	>32	8	>32	16
79341	CMY-2	0.25	≤0.06	≤0.06	32	32	0.5	>32	16	8
79652	CMY-2	0.25	≤0.06	≤0.06	64	>32	0.5	>32	16	8
79692	-28 (G to A), +81 (G to A)	0.12	≤0.06	≤0.06	≤0.25	1	1	32	8	128
80057	Unknown	0.25	≤0.06	≤0.06	>64	>32	0.5	>32	8	16
80083	CTX-M-15, OXA-1	0.25	≤0.06	≤0.06	>64	>32	>32	32	8	32
80386	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	0.12	0.12	0.25	0.5	4	≤0.25	32	8	4
80517	CTX-M-15, TEM-1, OXA-1	0.12	≤0.06	≤0.06	>64	>32	>32	8	8	8
80835	Unknown	0.5	≤0.06	≤0.06	64	>32	1	>32	8	32
80851	CMY-2	0.12	≤0.06	≤0.06	4	4	≤0.25	32	8	≤1
80960	CTX-M-15, TEM-1	1	≤0.06	≤0.06	>64	>32	>32	16	8	4
81146	CTX-M-15	0.25	≤0.06	≤0.06	64	>32	>32	8	8	8
81687	CTX-M-15, OXA-1	0.25	0.12	0.12	>64	>32	>32	16	8	128

81960	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	0.12	≤0.06	≤0.06	≤0.25	≤0.5	0.5	>32	16	64
82395	CTX-M-14, TEM-1	0.12	≤0.06	≤0.06	64	2	>32	32	4	4
82929	CTX-M-15, TEM-1, OXA-1	0.5	≤0.06	≤0.06	>64	>32	>32	>32	8	16
82940	+70 (C to T)	1	0.12	≤0.06	>64	>32	4	>32	32	64
83154	CMY-2	0.12	≤0.06	≤0.06	32	32	1	>32	8	8
83204	CMY-2	0.25	≤0.06	≤0.06	32	>32	≤0.25	>32	16	4
84251	CMY-2	0.12	≤0.06	≤0.06	16	32	≤0.25	>32	8	8
83724	CMY-2	0.25	≤0.06	≤0.06	64	>32	2	>32	>32	32
84664	CTX-M-15, OXA-1	0.5	≤0.06	≤0.06	>64	>32	32	32	16	16
84814	CMY-2	0.12	≤0.06	≤0.06	16	32	1	>32	32	16
84816	CMY-2	0.25	≤0.06	≤0.06	8	>32	0.5	>32	32	64
85332	CTX-M-14, TEM-1	0.12	≤0.06	≤0.06	32	2	4	32	16	8
86230	Unknown	0.12	≤0.06	≤0.06	≤0.25	1	≤0.25	16	16	32
86609	CTX-M-15	0.12	≤0.06	≤0.06	>64	>32	32	8	8	2
86934	Unknown	0.12	≤0.06	≤0.06	≤0.25	1	1	>32	8	8
87164	CTX-M-15, TEM-1	0.5	≤0.06	≤0.06	>64	>32	64	16	16	4
87399	CMY-2	0.12	≤0.06	≤0.06	16	16	≤0.25	32	32	4
88273	CTX-M-15, TEM-1, OXA-1	4	1	0.5	>64	16	32	32	32	8
88937	CMY-2	0.5	≤0.06	≤0.06	64	>32	1	32	32	64
89386	CTX-M-15, OXA-1	0.12	≤0.06	≤0.06	>64	>32	>64	16	16	64
89439	CTX-M-15, OXA-1	1	0.12	≤0.06	>64	>32	>64	>32	16	64
89722	CTX-M-15, OXA-1	0.12	≤0.06	≤0.06	>64	32	16	8	16	4
90087	CTX-M-15, OXA-1	0.25	≤0.06	≤0.06	>64	>32	-	16	16	16
90484	-18 (G to A), -1 (C to A), +58 (C to T)	0.12	≤0.06	≤0.06	0.5	8	-	>32	>32	≤1
90789	KPC-3, TEM-1	2	1	1	32	>32	-	16	>32	128
91191	CTX-M-14	1	≤0.06	≤0.06	>64	8	-	16	16	4
92756	CTX-M-14, TEM-1	0.5	≤0.06	≤0.06	>64	32	-	32	32	4
92885	Unknown	0.25	≤0.06	≤0.06	16	32	-	>32	32	4

92969	CTX-M-15, OXA-1	2	0.12	0.12	>64	>32	-	16	32	16
92995	CMY-2	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	8
93871	Unknown	2	0.12	0.12	>64	>32	-	>32	>32	128
93960	CMY-2	1	0.12	≤0.06	>64	>32	-	>32	>32	64
93983	CMY-2	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	2
95090	Unknown	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	8

Note: CIR isolates are highlighted.

-, not tested.

*, chromosomal AmpC promoter mutations are indicated with the location and nucleotide change [example: +22 (C to T)].

[a]ETP, ertapenem; MEM, meropenem; DOR, doripenem; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam.

TABLE 16: Molecular mechanisms of resistance among CRS and CIR *K. pneumoniae*.

Isolate #	Molecular mechanism	MIC (µg/ml)[a]								
		ETP	MEM	DOR	CRO	CAZ	FEP	FOX	AMC	TZP
77575	CTX-M-2, SHV-11	0.12	≤0.06	≤0.06	>64	16	8	8	8	4
77808	SHV-1	0.25	≤0.06	≤0.06	1	32	2	8	16	>512
80008	CTX-M-15, SHV-11, TEM-1, OXA-1	0.25	≤0.06	≤0.06	>64	>32	16	8	8	32
80026	CTX-M-15, SHV-12, TEM-1	0.25	≤0.06	≤0.06	>64	>32	32	8	8	16
81261	CTX-M-15, SHV-11, TEM-1, OXA-1	0.25	≤0.06	0.12	>64	>32	32	4	8	16
83848	Unknown	1	0.25	0.12	0.5	1	1	>32	4	16
86236	Unknown	0.25	0.25	0.12	0.5	1	1	>32	8	16
86717	CTX-M-15, OXA-1, SHV-1	0.5	≤0.06	≤0.06	>64	>32	64	8	16	32
86781	CTX-M-15, SHV-1	0.5	≤0.06	≤0.06	>64	>32	64	8	8	8
87702	KPC-3	16	4	4	>64	>32	16	>32	>32	512
88117	CTX-M-15, SHV-1, TEM-1, OXA-1	0.5	≤0.06	≤0.06	>64	>32	64	8	16	16
88378	CTX-M-15, SHV-11	0.5	≤0.06	≤0.06	>64	>32	16	16	8	8
88648	CTX-M-15, SHV-1, OXA-1	0.5	≤0.06	≤0.06	>64	>32	64	8	16	32
88846	CTX-M-15, OXA-1	0.5	≤0.06	≤0.06	>64	>32	32	16	16	64
89263	SHV-31, TEM-1	0.12	≤0.06	≤0.06	≤0.25	16	≤0.25	8	16	256
90500	CTX-M-15, SHV-1, OXA-1	0.5	≤0.06	≤0.06	>64	>32	-	8	16	32
91246	CTX-M-15, OXA-1	1	0.12	0.12	>64	>32	-	>32	32	32
92409	CTX-M-15, SHV-1, OXA-1	0.5	≤0.06	≤0.06	>64	>32	-	8	16	32
92737	Unknown	0.12	≤0.06	≤0.06	0.5	2	-	>32	8	8
93188	Unknown	0.12	≤0.06	≤0.06	≤0.25	16	-	8	32	>512
93739	SHV-1, TEM-1	0.25	≤0.06	0.12	0.5	>32	-	16	32	16
92881	CTX-M-14, SHV-1	0.25	≤0.06	≤0.06	>64	16	-	8	16	16
95396	unknown	0.12	≤0.06	≤0.06	≤0.25	2	-	>32	8	16

Note: CIR isolates are highlighted.
-, not tested.

[a]ETP, ertapenem; MEM, meropenem; DOR, doripenem; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam.

FIGURE 5: Resistance mechanisms among CRS isolates. AmpC P/A represent isolates with promoter and/or attenuator mutations of the *ampC* gene. The other/unknown group are isolates with an ESBL other than CTX-M- 15 or 14 or isolates with an unknown mechanism of resistance.

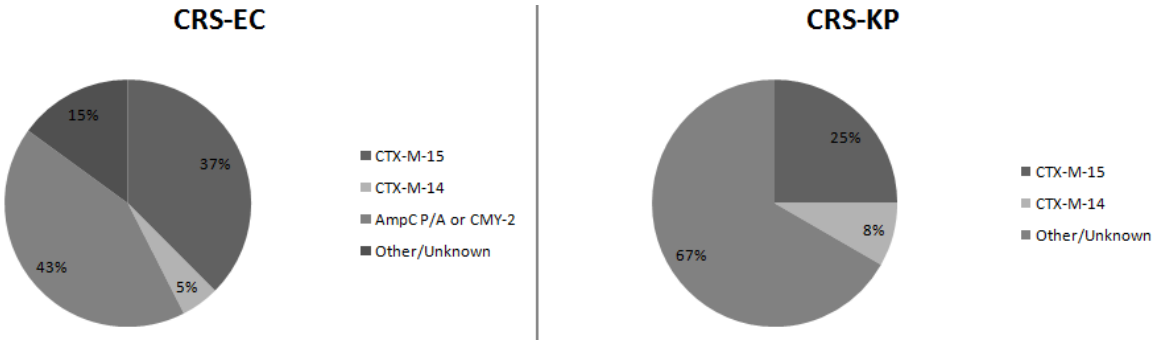
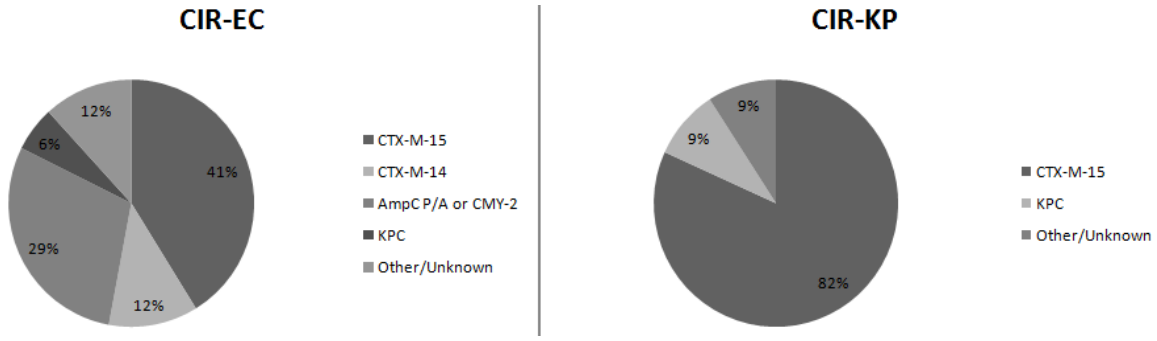


FIGURE 6: Resistance mechanisms among CIR isolates. AmpC P/A represent isolates with promoter and/or attenuator mutations of the *ampC* gene. The other/unknown group are isolates with an ESBL other than CTX-M- 15 or 14 or isolates with an unknown mechanism of resistance.



8.6. Porin Analysis

8.6.1. *E. coli*: OmpF and OmpC

Table 17 summarizes the porin profiles among all *E. coli* isolates. When considering the o*mpF* gene among *E. coli* isolates, 10/57 (17.5%) were shown to have mutations by sequencing. The most common mutations were insertions and deletions leading to premature termination of protein synthesis. One isolate (76819) had an adenine insertion, one isolate (93871) had a thymine deletion, one isolate (82940) had a GCGT deletion, and 4 isolates (82395, 84664, 85332, and 92756) had a GAAC insertion which all led to premature termination. Three isolates (80960, 84251, and 87164) were shown to have nonsense mutations. Not as many variations were seen in the o*mpC* gene. One isolate (81687) was shown to have a TTGGG deletion and one isolate (88273) was shown to have a CGAG insertion, both of which led to premature termination. One isolate (82969) was shown to have a nonsense mutation within the *ompC* gene. Two isolates (86230 and 90484) were shown to have a point mutation (adenine → guanine) causing an amino acid change from lysine to glutamic acid at a key residue shown to have an effect on antibiotic uptake into the cell (Figure 7). When the SDS-PAGE gels were examined, forty-two (73.7%) isolates had all three bands present (OmpA [always present], OmpF and OmpC).

8.6.2. *K. pneumoniae*: OmpK35 and OmpK36

Table 18 summarizes the porin profiles among all *K. pneumoniae* isolates. Sequence results of the *ompK35* gene revealed the most common mutations as being insertions or deletions. Two isolates (77575 and 87702) had a guanine insertion and 6 isolates (86717, 86781, 88117, 88648, 90500, and 92409) had a guanine deletion leading to premature termination. Two isolates (89263 and 92737) had a nonsense mutation. Two

isolates (93188 and 93739) were shown to have mutations right at the start codon, ATG→ATA. Sequence results of the *ompK36* gene showed one isolate (80008) with a cytosine insertion and one isolate (88846) with an adenine deletion, both of which lead to a premature stop codon. No other specific mutations were observed within the *ompK36* gene. In contrast to the *E. coli* isolates, SDS-PAGE showed only 1 (4.3%) isolate with 3 bands. One (4.3%) isolate had only 1 band present. Twenty-one (91.3%) isolates had only 2 bands present on the SDS-PAGE gels. A sample SDS-PAGE gel is shown in Figure 8.

FIGURE 7: Comparison by alignment of the deduced OmpF and OmpC sequences from *E. coli* (ATCC 25922) and the OmpK35 and OmpK36 sequences of *K. pneumoniae* (ATCC 13883). β-strands, external loops, periplasmic turns, and relevant residues are highlighted. Modified from (29)**.**

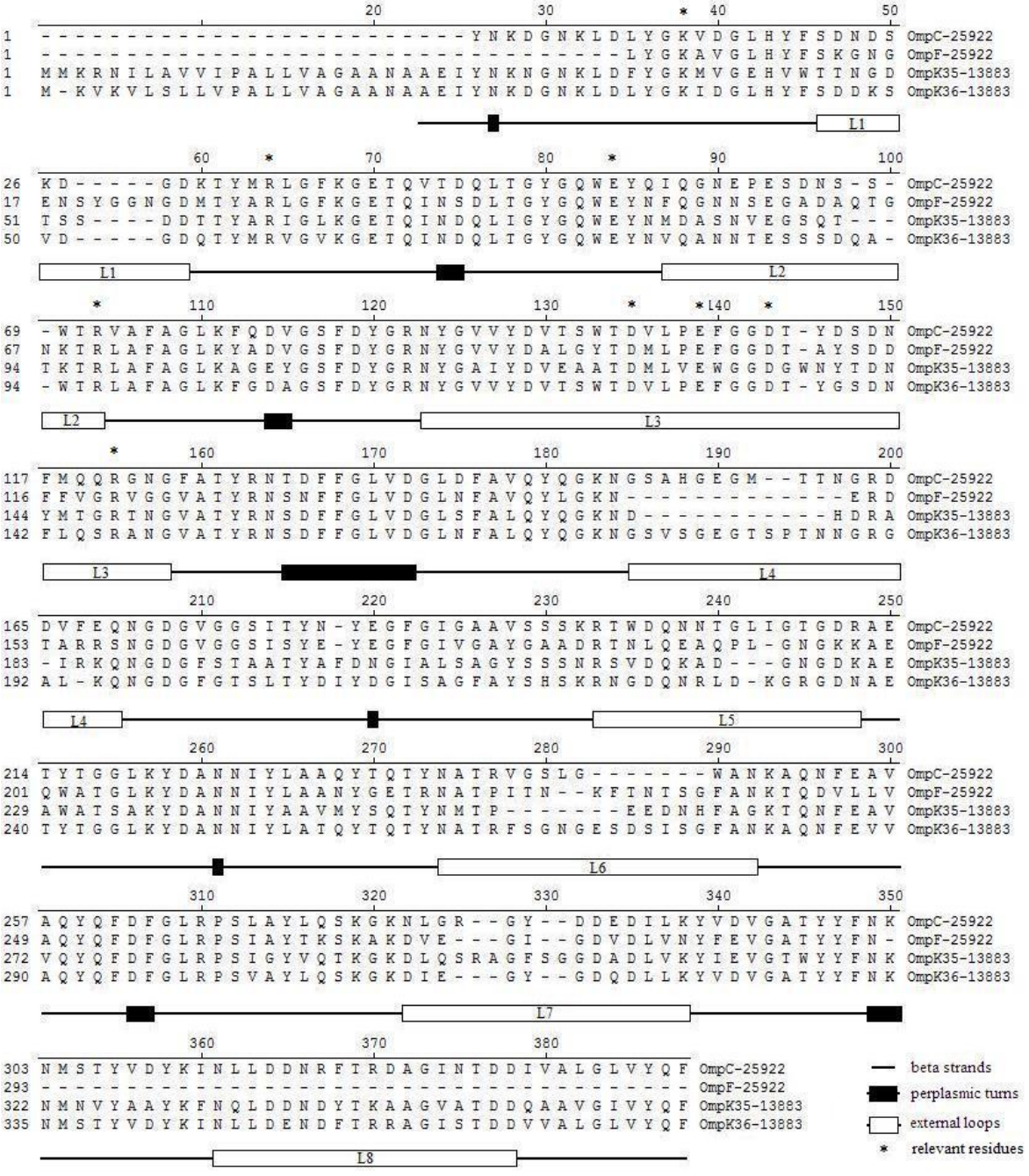


FIGURE 8: SDS-PAGE gel showing porin banding patterns in *E. coli* and *K. pneumoniae.*

Lane 1: EZ-Run Rec Protein Ladder
Lane 2: 25922 (E. coli + control)
Lane 3: 92995 (E. coli)
Lane 4: 93871 (E. coli)
Lane 5: 93960 (E. coli)
Lane 6: 93983 (E. coli)
Lane 7: 95090 (E. coli)
Lane 8: 13883 (K. pneumoniae + control)
Lane 9: 90500 (K. pneumoniae)
Lane 10: EZ-Run Rec Protein Ladder

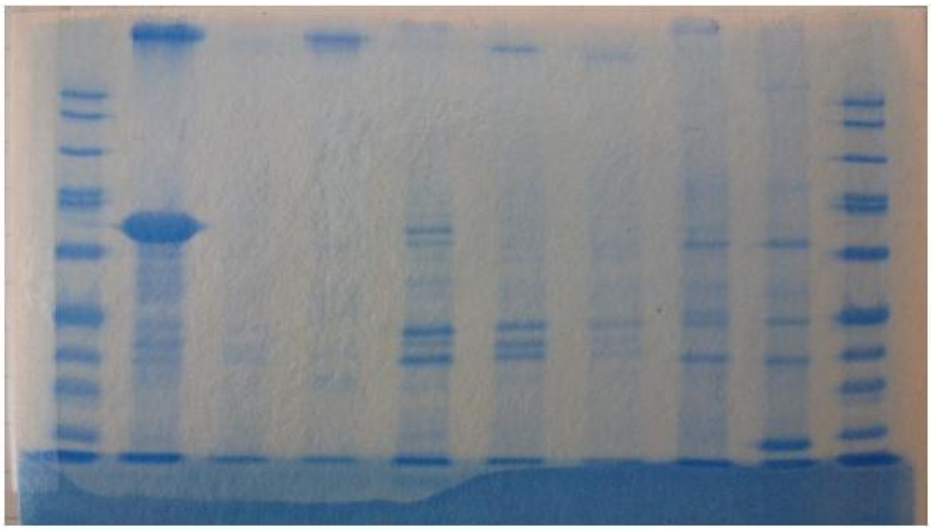


TABLE 17: Porin profiles of *Escherichia coli* with reduced susceptibility to the carbapenems-CRS (2007-2010).

Isolate #	ESBL	AmpC	MIC (µg/ml)[c]									# bands on SDS-PAGE[a]	OmpF (genetic lesion)[b]	OmpC (genetic lesion)[b]
			ETP	MEM	DOR	CRO	CAZ	FEP	FOX	AMC	TZP			
76576	YES	NO	0.12	≤0.06	≤0.06	>64	32	16	8	16	8	3	NEG	NEG
76789	YES	NO	0.12	≤0.06	≤0.06	>64	>32	>32	16	8	16	3	NEG	NEG
76819	YES	NO	0.25	≤0.06	≤0.06	>64	32	32	4	16	4	2	A ins→PS	NEG
76838	YES	NO	0.12	≤0.06	≤0.06	>64	>32	>32	8	16	64	3	NEG	NEG
76897	NO	YES	0.12	≤0.06	≤0.06	16	32	4	>32	16	4	3	NEG	NEG
77298	NO	YES	0.5	≤0.06	≤0.06	>64	>32	16	>32	32	32	3	NEG	NEG
78399	YES	NO	0.12	≤0.06	≤0.06	>64	4	32	>32	4	8	3	NEG	NEG
78967	NO	YES	1	0.12	≤0.06	>64	>32	4	>32	32	256	3	NEG	NEG
79078	YES	NO	0.12	≤0.06	≤0.06	>64	>32	32	16	8	4	3	NEG	NEG
79170	YES	NO	0.25	≤0.06	≤0.06	>64	>32	>32	8	8	4	2	NEG	NEG
79234	YES	NO	0.12	≤0.06	≤0.06	>64	>32	>32	8	>32	16	3	NEG	NEG
79341	NO	YES	0.25	≤0.06	≤0.06	32	32	0.5	>32	16	8	3	NEG	NEG
79652	NO	YES	0.25	≤0.06	≤0.06	64	>32	0.5	>32	16	8	3	NEG	NEG
79692	NO	YES	0.12	≤0.06	≤0.06	≤0.25	1	1	32	8	128	3	NEG	NEG
80057	YES	NO	0.25	≤0.06	≤0.06	>64	>32	0.5	>32	8	16	3	NEG	NEG
80083	YES	NO	0.25	≤0.06	≤0.06	>64	>32	>32	32	8	32	3	NEG	NEG
80386	NO	YES	0.12	0.12	0.25	0.5	4	≤0.25	32	8	4	3	NEG	NEG
80517	YES	NO	0.12	≤0.06	≤0.06	>64	>32	>32	8	8	8	3	NEG	NEG
80835	YES	NO	0.5	≤0.06	≤0.06	64	>32	1	>32	8	32	3	NEG	NEG
80851	NO	YES	0.12	≤0.06	≤0.06	4	4	≤0.25	32	8	≤1	3	NEG	NEG
80960	YES	NO	1	≤0.06	≤0.06	>64	>32	>32	16	8	4	3	NS mut	NEG
81146	YES	NO	0.25	≤0.06	≤0.06	64	>32	>32	8	8	8	3	NEG	N/A
81687	YES	NO	0.25	0.12	0.12	>64	>32	>32	16	8	128	1	NEG	TTGGG del→PS

81960	NO	YES	0.12	≤0.06	≤0.06	≤0.25	≤0.5	0.5	>32	16	64	2	NEG	NEG
82395	YES	NO	0.12	≤0.06	≤0.06	64	2	>32	32	4	4	2	GAAC ins→PS	NEG
82929	YES	NO	0.5	≤0.06	≤0.06	>64	>32	>32	>32	8	16	3	NEG	NEG
82940	NO	YES	1	0.12	≤0.06	>64	>32	4	>32	32	64	2	GCGT del→PS	NEG
83154	NO	YES	0.12	≤0.06	≤0.06	32	32	1	>32	8	8	3	NEG	NEG
83204	NO	YES	0.25	≤0.06	≤0.06	32	>32	≤0.25	>32	16	4	3	NEG	NEG
84251	NO	YES	0.12	≤0.06	≤0.06	16	32	≤0.25	>32	8	8	3	NS mut	NEG
83724	NO	YES	0.25	≤0.06	≤0.06	64	>32	2	>32	>32	32	3	NEG	NEG
84664	YES	NO	0.5	≤0.06	≤0.06	>64	>32	32	32	16	16	2	GAAC ins→PS	NEG
84814	NO	YES	0.12	≤0.06	≤0.06	16	32	1	>32	32	16	3	NEG	NEG
84816	NO	YES	0.25	≤0.06	≤0.06	8	>32	0.5	>32	32	64	3	NEG	NEG
85332	YES	NO	0.12	≤0.06	≤0.06	32	2	4	32	16	8	2	GAAC ins→PS	NEG
86230	NO	NO	0.12	≤0.06	≤0.06	≤0.25	1	≤0.25	16	16	32	3	NEG	A→G (RRPM)
86609	YES	NO	0.12	≤0.06	≤0.06	>64	>32	32	8	8	2	3	NEG	NEG
86934	NO	NO	0.12	≤0.06	≤0.06	≤0.25	1	1	>32	8	8	2	NEG	NEG
87164	YES	NO	0.5	≤0.06	≤0.06	>64	>32	64	16	16	4	3	NS mut	NEG
87399	NO	YES	0.12	≤0.06	≤0.06	16	16	≤0.25	32	32	4	3	NEG	NEG
88273	YES	NO	4	1	0.5	>64	16	32	32	32	8	2	NEG	CGAG ins→PS
88937	NO	YES	0.5	≤0.06	≤0.06	64	>32	1	32	32	64	3	NEG	NEG
89386	YES	NO	0.12	≤0.06	≤0.06	>64	>32	>64	16	16	64	3	NEG	NEG
89439	YES	NO	1	0.12	≤0.06	>64	>32	>64	>32	16	64	3	N/A	NEG
89722	YES	NO	0.12	≤0.06	≤0.06	>64	32	16	8	16	4	3	NEG	N/A
90087	YES	NO	0.25	≤0.06	≤0.06	>64	>32	-	16	16	16	2	NEG	NEG

90484	NO	YES	0.12	≤0.06	≤0.06	0.5	8	-	>32	>32	≤1	3	NEG	A→G (RRPM)
90789	NO*	NO	2	1	1	32	>32	-	16	>32	128	3	NEG	NEG
91191	YES	NO	1	≤0.06	≤0.06	>64	8	-	16	16	4	2	N/A	NEG
92756	YES	NO	0.5	≤0.06	≤0.06	>64	32	-	32	32	4	2	GAAC ins→PS	NEG
92885	NO	YES	0.25	≤0.06	≤0.06	16	32	-	>32	32	4	3	NEG	NEG
92969	YES	NO	2	0.12	0.12	>64	>32	-	16	32	16	2	NEG	NS mut
92995	NO	YES	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	8	3	NEG	NEG
93871	NO	YES	2	0.12	0.12	>64	>32	-	>32	>32	128	1	T del→PS	NEG
93960	NO	YES	1	0.12	≤0.06	>64	>32	-	>32	>32	64	3	NEG	NEG
93983	NO	YES	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	2	3	NEG	NEG
95090	YES	NO	0.12	≤0.06	≤0.06	16	>32	-	>32	>32	8	3	NEG	NEG

Note: CIR isolates are highlighted.

*, KPC-3.

-, not tested.

[a]1 band represents OmpA, OmpK35 and OmpK36 absent; 2 bands represents OmpA and one of either OmpK35 or OmpK36; 3 bands represents presence of all three proteins.

[b]A ins→PS, insertion of a adenine leading to a premature stop codon downstream; T del→PS, deletion of a thymine leading to a premature stop codon downstream; GAAC ins→PS, insertion of guanine-adenine-adenine-guanine leading to a premature stop codon downstream; GCGT del→PS, deletion of guanine-cytosine-guanine-thymine leading to a premature stop codon downstream; TTGGG del→PS, deletion of thymine-thymine-guanine-guanine-guanine leading to a premature stop codon downstream; CGAG ins→PS, insertion of cytosine-guanine-adenine-guanine leading to a premature stop codon downstream; A→G(RRPM), relevant residue point mutation; NS mut, nonsense mutation; N/A, did not amplify; NEG, no genetic lesion observed.

[c]ETP, ertapenem; MEM, meropenem; DOR, doripenem; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam.

TABLE 18: Porin profiles of *Klebsiella pneumoniae* with reduced susceptibility to the carbapenems-CRS (2007-2010).

Isolate #	ESBL	MIC (µg/ml)[c]									# bands on SDS-PAGE[a]	OmpK35 (genetic lesion)[b]	OmpK36 (genetic lesion)[b]
		ETP	MEM	DOR	CRO	CAZ	FEP	FOX	AMC	TZP			
77575	YES	0.12	≤0.06	≤0.06	>64	16	8	8	8	4	2	G ins→PS	NEG
77808	YES	0.25	≤0.06	≤0.06	1	32	2	8	16	>512	2	NEG	NEG
80008	YES	0.25	≤0.06	≤0.06	>64	>32	16	8	8	32	2	NEG	A del→PS
80026	YES	0.25	≤0.06	≤0.06	>64	>32	32	8	8	16	2	NEG	NEG
81261	YES	0.25	≤0.06	0.12	>64	>32	32	4	8	16	3	NEG	NEG
83848	NO	1	0.25	0.12	0.5	1	1	>32	4	16	1	NEG	N/A
86236	NO	0.25	0.25	0.12	0.5	1	1	>32	8	16	2	NEG	N/A
86717	YES	0.5	≤0.06	≤0.06	>64	>32	64	8	16	32	2	G del→PS	NEG
86781	YES	0.5	≤0.06	≤0.06	>64	>32	64	8	8	8	2	G del→PS	NEG
87702	NO*	16	4	4	>64	>32	16	>32	>32	512	2	G ins→PS	N/A
88117	YES	0.5	≤0.06	≤0.06	>64	>32	64	8	16	16	2	G del→PS	NEG
88378	YES	0.5	≤0.06	≤0.06	>64	>32	16	16	8	8	2	NEG	NEG
88648	YES	0.5	≤0.06	≤0.06	>64	>32	64	8	16	32	2	G del→PS	NEG
88846	YES	0.5	≤0.06	≤0.06	>64	>32	32	16	16	64	2	N/A	C ins→PS
89263	YES	0.12	≤0.06	≤0.06	≤0.25	16	≤0.25	8	16	256	2	NS mut	NEG
90500	YES	0.5	≤0.06	≤0.06	>64	>32	-	8	16	32	2	G del→PS	NEG
91246	YES	1	0.12	0.12	>64	>32	-	>32	32	32	2	UGS	NEG
92409	YES	0.5	≤0.06	≤0.06	>64	>32	-	8	16	32	2	G del→PS	UGS
92737	NO	0.12	≤0.06	≤0.06	0.5	2	-	>32	8	8	2	NS mut	UGS
93188	NO	0.12	≤0.06	≤0.06	≤0.25	16	-	8	32	>512	2	Start site ATG→ATA	UGS
93739	YES	0.25	≤0.06	0.12	0.5	>32	-	16	32	16	2	Start site ATG→ATA	NEG

92881	YES	0.25	≤0.06	≤0.06	>64	16	-	8	16	16	2	NEG	NEG
95396	NO	0.12	≤0.06	≤0.06	≤0.25	2	-	>32	8	16	2	N/A	NEG

Note: CIR isolates are highlighted.

*, KPC-3

-, not tested.

[a]1 band represents OmpA, OmpK35 and OmpK36 absent; 2 bands represents OmpA and one of either OmpK35 or OmpK36; 3 bands represents presence of all three proteins.

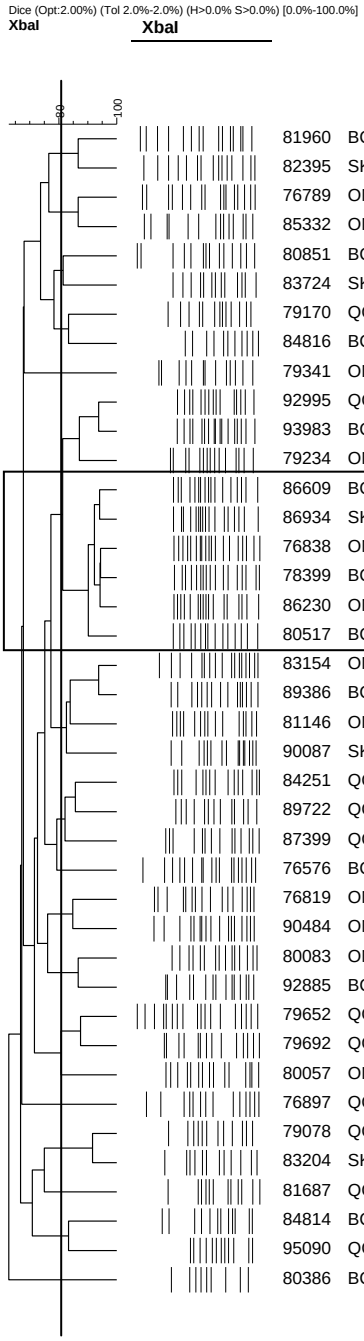
[b]G ins→PS, insertion of a guanine leading to a premature stop codon downstream; G del→ PS, deletion of a guanine leading to a premature stop codon downstream; NS mut, nonsense mutation; Start site ATG→ATA, mutation at start codon; UGS, unrecognizable gene sequence; A del→PS, deletion of adenine leading to a premature stop codon; C ins→PS, insertion of a cytosine leading to a premature stop codon; N/A, did not amplify; NEG, no genetic lesion observed.

[c]ETP, ertapenem; MEM, meropenem; DOR, doripenem; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam.

8.7. Genetic Relationships Among CRS and CIR Isolates

Pulsed-field gel electrophoresis was done to determine the genetic relationships among isolates. Whether looking at CRS- EC or KP or CIR- EC or KP, generally the isolates were genetically unrelated meaning they had less than 80% similarity, although small clusters of related isolates were observed. When looking at CRS-*E. coli* (Figure 9), a few small clusters were observed with one major cluster highlighted in Figure 9. However, isolates within these clusters were geographically distributed among British Columbia/Alberta, Saskatchewan/Manitoba, and Ontario with varying years of isolation. A few small clusters were also observed among CRS-*K. pneumoniae* (Figure 10) and CIR-*E. coli* (Figure 11) but again these isolates had varying geographic distribution and different time frames of isolation. Among the CIR-*K. pneumoniae* (Figure 12), one major cluster was observed as shown by the highlight with all isolates coming out of Ontario from 2009 and 2010.

FIGURE 9: Genetic relationships among CRS-*E. coli.* Isolate number, region, age, sex, genotype, and year of isolation are also included. The line represents 80% similarity.



81960	BC/AB	64	M	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	2008	
82395	SK/MB	54	M	CTX-M-14, TEM-1	2008	ST405
76789	ONT	44	M	CTX-M-15, OXA-1	2007	ST648
85332	ONT	45	F	CTX-M-14, TEM-1	2009	ST405
80851	BC/AB	26	F	CMY-2	2008	
83724	SK/MB	58	F	CMY-2	2009	
79170	QC/Marit.	54	F	CTX-M-15, OXA-1	2008	ST405
84816	BC/AB	81	F	CMY-2	2009	
79341	ONT	93	M	CMY-2	2008	
92995	QC/Marit.	47	F	CMY-2	2010	ST131
93983	BC/AB	1	F	CMY-2	2010	ST131
79234	ONT	85	F	CTX-M-15, OXA-1	2008	ST90
86609	BC/AB	49	F	CTX-M-15	2009	ST131
86934	SK/MB	47	M	Unknown	2009	
76838	ONT	55	M	CTX-M-15, TEM-1, OXA-1	2007	ST131
78399	BC/AB	73	F	CTX-M-3, TEM-1, OXA-1	2007	ST131
86230	ONT	72	F	Unknown	2009	
80517	BC/AB	70	F	CTX-M-15, TEM-1, OXA-1	2008	ST131
83154	ONT	84	F	CMY-2	2008	
89386	BC/AB	62	M	CTX-M-15, OXA-1	2009	ST131
81146	ONT	1	F	CTX-M-15	2008	ST131
90087	SK/MB	63	F	CTX-M-15, OXA-1	2010	
84251	QC/Marit.	80	F	CMY-2	2008	
89722	QC/Marit.	57	F	CTX-M-15, OXA-1	2009	ST648
87399	QC/Marit.	61	F	CMY-2	2009	
76576	BC/AB	64	F	CTX-M-15, OXA-1	2007	ST648
76819	ONT	80	F	CTX-M-15, OXA-1	2007	ST405
90484	ONT	88	M	-18 (G to A), -1 (C to A), +58 (C to T)	2010	
80083	ONT	34	F	CTX-M-15, OXA-1	2008	ST648
92885	BC/AB	46	F	Unknown	2010	
79652	QC/Marit.	86	M	CMY-2	2008	
79692	QC/Marit.	75	M	-28 (G to A), +81 (G to A)	2008	
80057	ONT	81	F	CMY-2	2008	ST10
76897	QC/Marit.	77	M	CMY-2	2007	
79078	QC/Marit.	70	F	CTX-M-15, TEM-1	2008	ST38
83204	SK/MB	67	M	CMY-2	2008	
81687	QC/Marit.	78	M	CTX-M-15, OXA-1	2008	ST617
84814	BC/AB	85	F	CMY-2	2009	
95090	QC/Marit.	46	M	Unknown	2010	ST131
80386	BC/AB	72	M	+22 (C to T), +26 (T to G), +27 (A to T), +32 (G to A), +70 (C to T)	2008	

FIGURE 10: Genetic relationships among CRS-*K. pneumoniae*. Isolate number, region, age, sex, genotype, and year of isolation are also included. The line represents 80% similarity.

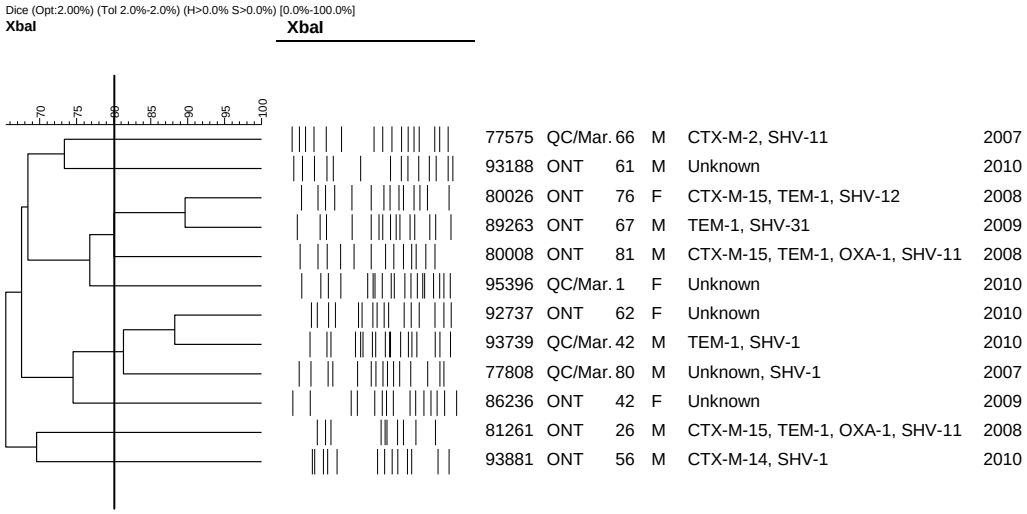
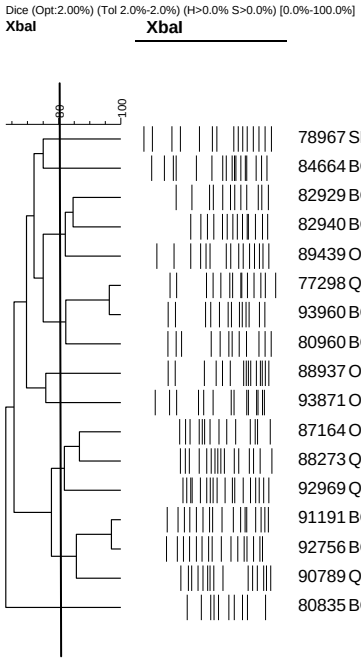
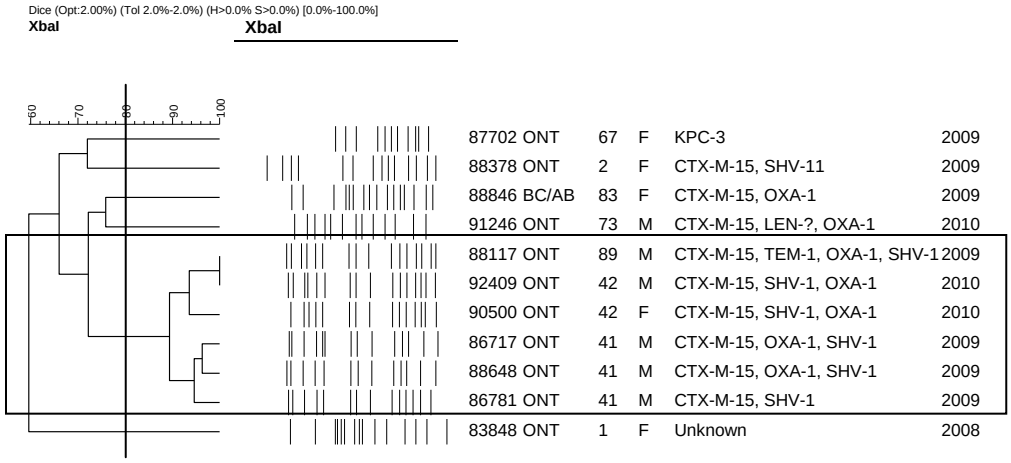


FIGURE 11: Genetic relationships among CIR-*E. coli*. Isolate number, region, age, sex, genotype, and year of isolation are also included. The line represents 80% similarity.



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	
84664	BC/AB	36	M	CTX-M-15, OXA-1	2009	ST405
82929	BC/AB	77	M	CTX-M-15, TEM-1, OXA-1	2008	ST405
82940	BC/AB	44	F	+70 (C to T)	2008	
89439	ONT	80	F	CTX-M-15, OXA-1	2009	ST405
77298	QC/Marit.	58	M	CMY-2	2007	
93960	BC/AB	61	F	CMY-2	2010	
80960	BC/AB	76	M	CTX-M-15, TEM-1	2008	ST38
88937	ONT	2	F	CMY-2	2009	
93871	ONT	33	M	Unknown	2010	
87164	ONT	1	F	CTX-M-15, TEM-1	2009	ST131
88273	QC/Marit.	25	F	CTX-M-15, TEM-1, OXA-1	2009	ST131
92969	QC/Marit.	68	M	CTX-M-15, OXA-1	2010	ST131
91191	BC/AB	66	M	CTX-M-14	2010	
92756	BC/AB	66	M	CTX-M-14, TEM-1	2010	
90789	QC/Marit.	77	M	TEM-1, KPC-3	2010	ST131
80835	BC/AB	3	F	CMY-2	2008	ST38

FIGURE 12: Genetic relationships among CIR-*K. pneumoniae*. Isolate number, region, age, sex, genotype, and year of isolation are also included. The line represents 80% similarity.



9. Discussion

The main hypothesis in this thesis was that carbapenem resistance in Canada is emerging as a result of carbapenemase production or ESBL and/or AmpC β-lactamase production coupled with porin loss or alteration. This thesis focuses on *E. coli* and *K. pneumoniae* isolates that have reduced susceptibility (CRS) and resistance (CIR) to carbapenems to address the prevalence, epidemiology, resistance mechanisms including the role the major outer membrane porins of these organisms play in carbapenem resistance.

9.1. Carbapenem Resistance and Reduced Susceptibility in *E. coli* and *K. pneumoniae* from Canadian Hospitals

For more than 2 decades, carbapenems have been considered the last line of therapy for multidrug resistant infections caused by Enterobacteriaceae. However, over the past decade, resistance to carbapenems have emerged and appears to be increasing among these pathogens, particularly in *K. pneumoniae* (94). The main mechanism of resistance to carbapenems in Enterobacteriaceae is through production of a carbapenemase (particularly KPCs and the newly emerging NDM) or through the production of an ESBL and/or AmpC β-lactamase with porin alteration. In Canada, there has only been a handful of reports of KPC producing organisms (36, 60, 94). The more common mechanism of resistance to carbapenems among the Enterobacteriaceae is through porin alteration combined with an ESBL and/or AmpC β-lactamase. The prevalence of KPC-producing organisms in Canada is currently unknown because laboratories may not be using the most sensitive methods and previous studies have demonstrates the difficulties in identifying KPC-producing strains (3, 10, 12, 36). Strains may not demonstrate frank resistance to the carbapenems

and may have only reduced susceptibility to the carbapenems. Laboratories should screen and confirm using the old CLSI guidelines since confirmation using the MHT is not required in the most current CLSI guidelines (24, 25). Carbapenemase production (more commonly KPC-production) should be suspected if *in vitro* testing shows resistance or reduced susceptibility to any of the carbapenems (94). Isolates (particularly *K. pneumoniae* and *E. coli*) resistant to any of the extend-spectrum cephalosporins but test susceptible to the carbapenems should also be evaluated for possible KPC production (79). Screening with ertapenem is preferred since, compared to the other carbapenems, it is more sensitive to KPC hydrolysis.

In this study we used ertapenem to screen for resistance and reduced susceptibility to the carbapenems. Isolates with ertapenem MICs of 0.12 and 0.25 μg/ml were considered carbapenem reduced susceptible (CRS) and MICs of ≥0.5 μg/ml were considered intermediate or resistant (CIR) according to current CLSI guidelines (25). In this study from 2007 to 2010, we found 17/4807 (0.35%) *E. coli* resistant to ertapenem with MICs ranging from 0.5 to 4 μg/ml and 11/1419 (0.78%) *K. pneumoniae* with MICs ranging from 0.5 to 16 μg/ml. Only 1 *E. coli* and 1 *K. pneumoniae* were confirmed to be KPC producers. Resistance in the other organisms was mainly due to ESBL and/or AmpC production coupled with porin alteration. We also found 40/4807 (0.83%) CRS-EC and 12/1419 (0.85%) CRS-KP over the study period from 2007 to 2010. No other studies in Canada have monitored isolates we defined as CRS. It is important that these organisms be monitored for infection control purposes as many of these reduced susceptible organisms may be missed by routine susceptibility testing (36, 94).

9.2. The Prevalence of CRS and CIR Isolates in Canadian Hospitals

As mentioned in the previous section, the prevalence of carbapenem-resistant and reduced susceptible organisms in Canada is currently unknown due to lack of monitoring of these types of organisms especially those with carbapenem resistance and reduced susceptibility as a result of ESBL and/or AmpC production with porin loss. Over the study years, no trend was observed when the cohorts were separated into CRS-EC, CRS-KP, CIR-EC, and CIR-KP. However, when analyzing all isolates with ertapenem MICs ≥0.12 μg/ml (ie. grouped CRS and CIR *E. coli* and CRS and CIR *K. pneumoniae*), there was an increase seen in the prevalence observed for CRS/CIR *K. pneumoniae* (2007: 1.1%, 2008: 1.3%, 2009: 2.5%, 2010: 2.6%). The same was not observed within the *E. coli* CRS/CIR group (2007: 1.3%, 2008: 2%, 2009: 1.4%, 2010: 1.2%). This is as expected as the development of carbapenem resistance, both through KPC-production and porin alteration, is most common amongst *K. pneumoniae* isolates compared to *E. coli* based on our porin analysis data which will be discussed in detail in later sections. Isolates sent to Centers for Disease Control and Prevention (CDC) for reference testing also showed that KPC enzymes seem to occur most commonly in *K. pneumoniae* (73). Data regarding nosocomial infections reported to the CDC showed that the overall prevalence of carbapenem resistance among *K. pneumoniae* isolates rose from <1% in 2000 to 8% in 2007 (4). The highest prevalence of KPC producing organisms to date was in regions such as Greece, Israel, and north-eastern United States (68). Compared to these regions just mentioned, the overall prevalence of carbapenem resistance in Canada is low as shown by our study.

9.3. Changing Carbapenem Breakpoints and Detection of Carbapenem Producing Organisms

Recent changes in CLSI guidelines have been the cause of debate. Based on pharmacokinetic/pharmacodynamic properties, limited clinical data, and MIC distributions for recently described carbapenemase producing isolates, CLSI recently revised the interpretive criteria for carbapenems which were first published in June 2010 (25). The exact changes were described in section 4 of this thesis. With these changes made, even though there is an increase in sensitivity, the specificity goes down. A higher percentage of isolates will be reported as resistant and this may decrease the potential benefit that patients may get from treatment with a carbapenem. On the other hand, a proportion of isolates will be reported as susceptible to carbapenems despite the presence of a carbapenemase and thus a number of patients may be treated with carbapenems, a therapeutic approach not considered appropriate by some experts (28, 79). This highlights the importance of confirmatory tests to detect these important resistance mechanisms in terms of spread and patient safety. For the purpose of this study, we screened any *E. coli* or *K. pneumoniae* with an ertapenem MIC of ≥0.12 μg/ml (reduced susceptibility to carbapenems [CRS]) as a potential carbapenemase producer and performed the additional confirmatory tests to confirm the presence of carbapenemase production.

9.4. Antimicrobial Susceptibilities Among CRS and CIR Isolates

Resistance rates among CRS and CIR isolates were very high, leaving only a very few therapeutic options. Consistent with our hypothesis, frequently these organisms were MDR (defined as concomitant resistance to ≥3 different classes of antimicrobial agents).

Compared to the 100 wild type *E. coli* and *K. pneumoniae* (50 of each *E. coli* and *K. pneumoniae*) we tested as controls, which were 100% susceptible to cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, we found 21/40 (52.5%), 5/12 (41.7%), 11/17 (64.7%), and 9/11 (81.8%) of CRS-EC, CRS-KP, CIR-EC, and CIR-KP to be MDR, respectively. The higher resistance rates in *K. pneumoniae* were consistent with our findings that porin alteration is more commonly seen in this organism compared to *E. coli* and may contribute to the MDR phenotype allowing for resistance to a greater range of drug classes. Along with porin alteration a high percentage of the CRS and CIR isolates were also positive for ESBL- or AmpC- production. These genes are carried on plasmids which also carry resistance determinates to other classes of antimicrobials including fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole so the MDR phenotype is not surprising putting all these mechanisms together (75). When looking specifically at ertapenem MICs, we found CRS and CIR strains to have substantially higher ertapenem MICs at 16-32x and 64-2048x higher, respectively.

Although MDR rates are high in CRS and CIR *E. coli* and *K. pneumoniae*, some therapeutic options still remain. Amikacin, colistin (polymyxin E), tigecycline, and the carbapenems remain viable options. Over the course of the study, a high percentage of organisms remained susceptible to these agents.

9.5. ESBL and AmpC Genes Amongst CRS and CIR Isolates

Since enzyme mediated carbapenem resistance in Canada is rare (ie. KPC production), the main mechanism of resistance to carbapenems is through porin alteration plus β-lactamase production (36, 79). This is consistent with our study in that only 1 KPC-producing *E. coli* (1/4807, 0.02%) and 1 KPC-producing *K. pneumoniae* (1/1419, 0.07%)

was found over the 4 year study period. Rather than carbapenemase production, CRS and CIR *E. coli* and *K. pneumoniae* were found to be highly associated with plasmid mediated ESBL and AmpC β-lactamases or associated with promoter and/or attenuator mutations of the *ampC* gene. When looking at all (CRS and CIR) *E. coli* and *K. pneumoniae* isolates, 22/57 (38.6%) and 12/23 (52.2%) were producing CTX-M-15, respectively. Another 22/57 (38.6%) *E. coli* were found to have a plasmid mediated CMY-2 or mutations of the promoter and/or attenuator of the *ampC* gene. A very few isolates were found to produce CTX-M-14, CTX-M-2, and CTX-M-3. Others had unknown mechanisms of resistance. This along with the porin data supports the main mechanism of reduced susceptibility to carbapenems in Canada which will be discussed further in the next section.

9.6. Contribution of Porin Alteration Combined with ESBL and AmpC β-lactamase Production in Carbapenem Resistance and Reduced Susceptibility

The role that ESBL and/or AmpC production coupled with porin alteration in the development of carbapenem resistance is well documented (11, 18, 26, 34, 35, 50, 57, 59, 70, 90, 104, 105). Porin alteration alone cannot significantly enhance resistance to antibiotics and the combination of porin loss and β-lactamase production does not always result in carbapenem resistance (20, 104). A study by Mammeri *et al* showed that imipenem resistance may occur mostly among plasmid mediated AmpC producers of CMY-2, ACT-1, and DHA-1 types, particularly giving importance to the carbapenemase properties of CMY-2 since it is widely distributed throughout the world among humans and animals (57). We found 13/40 (22.8%) *E. coli* isolates with reduced susceptibility to carbapenems that were producing CMY-2, only 1 (7.69%) of which had a porin alteration within the *ompF* gene. However, 11/13 (84.6%) CMY-2 producing *E. coli* were CRS and

had ertapenem MICs of 0.12 and 0.25 μg/ml, a result indicating that the reduced susceptibility may have to do with the level of porin expression which we did not look at.

A study by Girlich *et al* showed that ertapenem may select for mutant strains with decreased susceptibility by modification of porin expression. This group showed that *E. coli* and *K. pneumoniae* with decreased susceptibilities to ertapenem had similarly decreased expression of *ompF* and *ompC* and *ompK35* and *ompK36* genes, respectively, no matter which CTX-M was expressed (CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-15) (35). This group also indicated that CTX-M β-lactamases may also contribute to decreased ertapenem susceptibility by binding to ertapenem with high affinity, even if poorly hydrolyzed by these enzymes (ie. ertapenem has a strong inhibitory effect on CTX-Ms). In this study, we found a stronger association of carbapenem reduced susceptibility coupled with CTX-M expression compared to AmpC expression. When looking at all *E. coli* isolates, 26/57 (45.6%) were found to be CTX-M-producers, 9/26 (34.6%) of which also had an associated porin alteration in one of the nonspecific porin, OmpF or OmpC. When considering all *K. pneumoniae* isolates, 14/23 (73.9%) were found to be CTX-M-producers, 10/14 (71.4%) of which were associated with porin alteration in either OmpK35 and/or OmpK36. These results suggest the association of carbapenem resistance and reduced susceptibility with ESBL production plus porin alteration.

9.7. Dissemination of CRS and CIR Isolates

Pulsed-field gel electrophoresis was done to determine the genetic relationships among isolates. Whether looking at CRS- EC or KP or CIR- EC or KP, generally the isolates were unrelated meaning they had less than 80% similarity, although small clusters of related isolates were observed. This means that the spread of these isolates in Canada is

mainly due to polyclonal spread rather than due to a single clone as shown in Figures 9, 10 and 11 although some clonal spread seems to have occurred in 2009 and 2010 within Ontario among the CIR-*K. pneumoniae* as shown in Figure 12. Since a high percentage of these isolates are ESBL or AmpC producers, polyclonal spread of these isolates has also been shown in previous studies by work done in our lab (88). This may explain why the prevalence of CRS and CIR isolates in Canada is so low.

9.8. New Emerging Issue: New Delhi Metallo-β-Lactamase

Perhaps the newest emerging issue worldwide is the New Delhi Metallo-β-lactamase (NDM-1) which is a new class B enzyme that was recently characterized from a *K. pneumoniae* isolate from Sweden, imported from India (63, 72). Particularly NDM-1 is endemic to India but due to international travel, it is emerging as an important clinical threat worldwide (72). It's been found in, but not limited to, U.K., Austria, Belgium, Denmark, the Nordic countries, Germany, Italy, The Netherlands, Slovenia, Spain, Australia, U.S.A., Canada, Japan, Taiwan, Singapore, China, Kenya, Oman, Israel, and Turkey. NDM-1 has been recovered from *K. pneumoniae, K. oxytoca, E. coli, C. freundii, Morganella morganii, Providencia* spp, *Proteus* spp., *E. cloacae* and *A. baumannii.* Although we have not found an NDM producing organisms in this study, it has been found by others in Canada which is a cause for concern. It has been suggested that NDM-1 is the new CTX-M-15 and will soon become global (97). It has even been recently shown to be associated with the highly successful, virulent clone, ST131, a well established clone worldwide including Canada (76). It may even soon surpass the success of CTX-M-15 as plasmids carrying NDM also carry significantly more resistance determinants (106).

10. Conclusions

In conclusion, this thesis presents data showing the prevalence of *E. coli* and *K. pneumoniae* isolated from Canadian hospitals from 2007-2010 with reduced susceptibility and resistance to carbapenems and the molecular mechanisms leading to this resistant phenotype. Even though the prevalence is low for CRS-EC (0.83%), CRS-KP (0.85%), CIR-EC (0.35%), and CIR-KP (0.78%), there has been an increase observed over the 4 year study period when looking at all (CRS and CIR combined) *K. pneumoniae* isolates (1.1% in 2007, 1.3% in 2008, 2.5% in 2009, and 2.6% in 2010). CRS isolates were mainly found in people ≥18 years of age whereas CIR isolates were mainly found in the ≤17 years age group. CRS and CIR *E. coli* were mainly isolated from BC/AB in contrast to CRS and CIR *K. pneumoniae* which were isolates mainly from Ontario. Wound and respiratory specimens from ICUs seems to be the main source for these types of organisms.

Therapeutic options for these types of infections are limited as they are frequently MDR. Carbapenems remains the best drug of choice for ESBL and AmpC producing *E. coli* and *K. pneumoniae* although emerging resistance may limit the use of this drug when treating these types of infections. However, a few options do remain including tigecycline, colistin, amikacin, and as we've seen from the work in this thesis is that carbapenems still remain a good option for treating MDR *E. coli* and *K. pneumoniae* in Canada.

Most CRS and CIR were found to be associated with ESBL and AmpC β-lactamases. The most predominate among these were CMY-2 type AmpC β-lactamases and the CTX-M-15 β-lactamase. Carbapenem reduced susceptibility and resistance was found to be more strongly associated with presence of CTX-M combined with porin alteration compared to CMY-2 expression combined with porin alteration.

The dissemination of CRS and CIR *E. coli* and *K. pneumoniae* was mainly due to polyclonal spread rather than from a single clone.

Although the prevalence of these types of organisms is low, new emerging issues like the changes in CLSI carbapenem breakpoints may facilitate the spread of these types of organisms. The elimination of the confirmatory tests may allow for organisms producing carbapenemase to slip right past us allowing for silent spread. Also it has been suggested that NDM-1 is the new CTX-M-15 and will soon become global is another cause for concern. This demonstrates the need for increased surveillance and understanding of these emerging pathogens. The continued surveillance of CRS and CIR isolates of *E. coli* and *K. pneumoniae* and understanding their molecular mechanisms of resistance and spread will help guide proper infection control procedures and identify optimal treatment for these clinically important pathogens in Canadian hospitals.

11. Future Research

Although this research provides insight in the prevalence and molecular mechanisms of reduced susceptibility to carbapenems and resistance in *E. coli* and *K. pneumoniae* to the carbapenems, there still are many unanswered questions. Other work that can be done is as follows:

a. Continued surveillance of carbapenem resistance in Canadian hospitals

Carbapenem resistance in Canada is an emerging issue and continued surveillance is a necessary component to help limit the spread of these types of organisms. In particular the implementation of the new CLSI breakpoints and elimination of confirmatory tests to identify carbapenemase producers in clinical microbiology laboratories may have a huge impact in terms of infection control. Understanding the molecular epidemiology and mechanisms of resistance play a pivotal role in controlling spread and guiding antimicrobial therapy.

b. Outer membrane protein expression studies

One of the limitations of this study was the link between porin expression levels and carbapenem reduced susceptibility. When looking at expression, SDS-PAGE alone cannot tell you much. Many isolates in the study, especially the *E. coli* isolates had both porins intact and didn't show any signs of mutations within the porin genes. This indicates that carbapenem reduced susceptibility may have something to do with the level of porin expression. These types of studies would help tie things together.

c. Sequencing of the promoter and attenuator regions of the porin genes

Along with the expression studies, we could also look at mutations within the promoter and attenuator regions of the *ompF*, *ompC*, *ompK35*, and *ompK36* genes could be studied to determine the exact changes resulting in changes in expression levels.

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