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THE ROLE OF EXTENDED SPECTRUM BETA LACTAMASE ENCODING GENES IN CARBAPENEM RESISTANCE IN *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* AT THE AMERICAN UNIVERSITY OF BEIRUT MEDICAL CENTER

by
ARIJE NABIL CHAMSEDDINE

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AMERICAN UNIVERSITY OF BEIRUT

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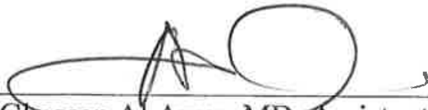

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AN ABSTRACT OF THE THESIS OF

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Title: The role of Extended Spectrum Beta Lactamases encoding genes in Carbapenem resistance in *Escherichia coli* and *Klebsiella pneumoniae* at American University of Beirut Medical Center

Background: Carbapenem resistance is continuously emerging worldwide, in extended spectrum beta lactamase (ESBL) and non-ESBL producing Enterobacteriaceae. Mechanisms of carbapenem resistance have been shown in a previous study done at the Department of Experimental Pathology, Immunology and Microbiology, to be due to the effect of carbapenemases in ESBL and non-ESBL producing *Escherichia coli* and *Klebsiella pneumoniae.* However, carbapenem resistance, has also been observed in this study in ESBL producing *E. coli* and *K. pneumoniae* lacking carbapenemases encoding genes, but having outer membrane porin (OMP) impermeabilities and/or efflux pump (EP) activity. To that purpose, we attempted in this project, to assess by electroporation, 1) the effect of the ESBLs CTXM-15 and TEM-1, encoded by *bla*-CTXM-15 and *bla*- TEM-1genes, and 2) the effect of the carbapenemases OXA-48 and NDM-1, encoded by *bla* -OXA-48 and *bla* -NDM-1 genes, singly and in combination, on carbapenem resistance in electrocompetent cells, lacking OMP impermeabilities and/ or EP activity, in order to determine the sole effect of these enzymes on carbapenem resistance, without the effect of intrinsic mechanisms of resistance in the bacterial cells.
Methods: Seven previously stored ESBL and non ESBL producing and carbapenem resistant *K. pneumoniae* and *E. coli* isolates harboring a selection of ESBL and /or carbapenemases encoding genes, *bla*-CTX-M-15, *bla*-TEM-1, *bla*-NDM-1 and *bla*-OXA-48 were selected in this project. Three control carbapenem resistant isolates lacking ESBL and/or carbapenemases encoding genes, but having OMP impermeabilities and /or EP activity, were also previously stored and used in this project as well. Pulsed Field Gel Electrophoresis (PFGE) analysis was performed. Extracts of total and plasmid DNA were subjected to Polymerase Chain Reaction (PCR) amplification of all encoding genes. Electroporation experiments utilizing *E.coli* GC-10 electrocompetent recipient cells were done. Antimicrobial susceptibility and PCR amplification were performed on cultured recipient electrocompetent cells, to confirm horizontal plasmid mediated transfer of encoded genes for carbapenem resistance.

Results: Plasmid DNA PCR confirmed that CTX-M-15, TEM-1, NDM-1and OXA-48 are plasmid encoded genes. All isolates had diverse genotypic profiles by PFGE analysis. Post electroporation, seven electrocompetent recipient cells showed resistance to ertapenem, with a decreased MIC values as compared to parent cells and harbored transferred genes from parent cells. The control three parent isolates lacking ESBL and/or carbapenemases encoding genes, but showing phenotypically resistance to carbapenems, due only to OMP and/or EP activity, had their electrocompetent recipient cells susceptible to ertapenem.

Conclusion: This study confirmed that carbapenem resistance can be due exclusively to ESBLs without the effect of porin impermeabilities and/or efflux pump activity. It also showed that the simultaneous presence of NDM-1 and OXA-48 in carbapenem resistant isolates confers a higher level or resistance.

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CHAPTER I

INTRODUCTION

Escherichia coli and *Klebsiella pneumoniae* are among the Enterobacteriaceae that are known to be primary or opportunistic human pathogens causing infections. A wide variety of antimicrobial treatment is available for infections caused by Enterobacteriaceae. Of these, β lactams are the first line of treatment especially the third generation cephalosporins. The overuse of these agents along with the transfer of resistance encoding genes have led to bacterial resistance against the β lactams. Resistance to this class of antimicrobials is partially due to the acquisition of extended spectrum beta lactamase enzymes (ESBL) that confer resistance to cephalosporins, leaving the carbapenems, also a type of β lactams, as a last resort of treatment.

Carbapenems have a broad spectrum of activity against many organisms including the Enterobacteriaceae. Nowadays, carbapenem resistance is increasing due to different mechanisms:

1- Production of carbapenemases, β lactamases that hydrolyse carbapenems

2- Decreased permeability of the bacterial outer membrane by porin loss or mutations of encoding outer membrane porin genes

3- Altered affinity of the carbapenem target, the PBP (penicillin binding protein)

4- Efflux pump (EP) expulsion of the carbapenems

The increase in carbapenem resistance is alarming since these agents are the last resort in treating infections caused by multidrug resistant organisms. Therefore an

awareness program should be established as well as further molecular studies conducted in order to better understand these resistance mechanisms. The increase in carbapenem resistance observed in ESBL producing *E.coli* and *K. pneumoniae* at the American University of Beirut Medical Center (AUBMC) since 2008 led to two studies at the Department of Experimental Pathology, Immunology and Microbiology, that determined the prevalence of carbapenem resistance and molecular characteristics of carbapenem resistant isolates and characterized the underlying molecular mechanisms of resistance in these isolates.

This study attempted to assess:

The effect of ESBL encoding genes, *bla*-CTXM-15 and *bla*-TEM-1, and the effect of the carbapenemases, *bla*-OXA-48 and *bla*-NDM-1, singly and in combination on carbapenem resistance. This study was conducted in electrocompetent cells lacking outer membrane porin impermeabilities and/or EP activity, in order to determine the sole effect of these enzymes on carbapenem resistance, without the effect of intrinsic mechanisms of resistance in the bacterial cells.

CHAPTER II

LITERATURE REVIEW

A. Enterobacteriaceae: general characteristics and medical importance

1. General characteristics of the Enterobacteriaceae

Enterobacteriaceae are Gram negative facultative anaerobic rods. They are found worldwide and are part of the normal flora in humans and animals. They can also be primary or opportunistic pathogens causing enteric, urinary tract and respiratory tract infections in humans (1). Enterobacteriaceae are mostly a part of the normal flora of the digestive tract: *Escherichia coli* is the most important Enterobacteriaceae found in the human colon as well as in the colon of some animals (2). This species can also be found in humid areas, constituting a small percentage of the skin normal flora. *E.coli* is one of the most important species of the uro-genital tract normal flora: it colonizes the vagina of pre-pubescent and post-menopausal women, and *E. coli* is occasionally isolated from the upper urethra of humans (3).

The Enterobacteriaceae family contains few species causing diseases. *E. coli* and *Klebsiella pneumoniae* are two of the species known to cause human infections (1).

2. Medical importance of E. coli

E. coli is often encountered as it is the primary Enterobacterial nosocomial pathogen (1). It can cause enteric infections as well as urinary tract infections. Enteric infections

can range from watery diarrhea to inflammatory diarrhea or dysentery; and urinary tract infections include prostatitis and pyelonephritis. This pathogen can also be at the origin of a septicemia (2).

3. Medical importance of K. pneumoniae

Klebsiella species are implicated in nosocomial and community acquired infections like urinary tract infections, burn and wounds complicating infections, septicemia and severe pneumonia.*K. pneumoniae* is implicated in community acquired urinary tract infections and in nosocomial and community acquired pneumonia. Even though this organism rarely causes community acquired pneumonia, when it does, *K. pneumoniae* leaves the lungs severely damaged, resulting in a high mortality rate in untreated patients (1).

B. Treatment of bacterial infections

Bacterial infections are treated by antimicrobial agents that can act in one of two ways: they can either kill (bactericidal) the organism causing the infection or inhibit its growth (bacteriostatic). Choosing the correct agent increases the success rate of the treatment: the agent's mechanism of action and whether or not it has access to its target are two points to be taken into consideration while doing so (4). Three different classifications for antimicrobial agents exist (5). First, they can be classified as natural or synthetic agents. Second, they can be classified as bactericidal or bacteriostatic. Thirdly, they can be classified according to their chemical structure. Fourthly, the most commonly used method of classification, classifies the agents according to their mechanism of action:

1- Agents that interfere with the cell wall synthesis (β-lactams, cephalosporins, carbapenems and glycopeptides).

2- Those that inhibit protein synthesis (aminoglycosides and tetracyclines).

3- Those that interfere with DNA (fluoroquinolones) or RNA synthesis (rifampin).

4- Those that inhibit a metabolic pathway (sulfonamides).

5- Those that disrupt the bacterial cell membrane structure (polymyxins).

1. Treatment of infections caused by Enterobacteriaceae

A wide variety of antimicrobial agents can treat infections caused by Enterobacteriaceae.

Examples of these agents include: The β-lactams (amino-penicillins, cephalosporins, and carbapenems), the fluoroquinolones (ciprofloxacin or levofloxacin), the sulfonamides and the aminoglycosides (6). Although carbapenem resistance is increasing worldwide, these agents are still the treatment of choice for infections caused by a multidrug resistant Gram negative organism. This is because they have a broad spectrum of activity and resistance to them is less common than the resistance to the β-lactams (6, 7, 8, 9). The production of β-lactamases, AmpC enzymes, and/or carbapenemases confers resistance to β-lactams and carbapenems; as a result, infections with organisms resistant to these agents are treated with tigecycline (a tetracycline derivative) (10) and colistin (a polymyxin) or colistin/rifampicin combinations (11).

2. The β-lactams and carbapenems

The β-lactams have a four-membered nitrogen containing ring structure (12). They are bactericidal, resulting in cell death by inhibiting the cell wall synthesis (13, 14). In fact, they irreversibly bind to the penicillin binding proteins (PBPs) (15) that are transpeptidases that catalyze the polymerization of the peptidoglycan layer of bacteria; and in this way, the β-lactams inhibit the transpeptidases' function and disrupt the bacterial cell wall synthesis (13, 14, 15). The β-lactams are a large group of antimicrobial agents that contains the cephalosporins and the carbapenems (12). Impinem, ertapenem, meropenem and doripenem are the clinically used carbapenems (16). Carbapenems are known to have a strong affinity to high molecular weight PBPs (13, 17) and are known to have a broad spectrum of activity against Gram positive organisms, Gram negative organisms and anaerobes (13).

When ESBL organisms and multi-drug resistant organisms are encountered, carbapenems are the treatment of choice. However, with the over use of this class of antimicrobial, resistance to carbapenems is increasing worldwide.

Several different mechanisms of action induce carbapenem resistance:

1- Alteration of the PBP or production of a low affinity PBP

2- Efflux pump expulsion of the agent in Gram negative bacteria

3- Production of extended spectrum β lactamase or carbapenemase that hydrolyze the agent

4- Outer membrane porin loss or a decrease in the outer membrane permeability along with an ESBL or an AmpC β-lactamase.

C. Extended spectrum Beta-lactamases

The β lactamases are enzymes that are capable of hydrolyzing the β lactam agents. They can be classified according to their amino acid sequence homology (Ambler molecular characterization) or to their inhibitor/substrate profiles (Bush-Jacoby-Medeiros functional classification) (18, 19).

The Ambler molecular characterization is the most common used scheme. It classifies the β lactamases into four molecular classes. Classes A, C and D are serine β lactamases. Class B includes the metallo- β lactamases (MBLs) which have an active site requiring zinc as a cofactor (20, 21) as seen in table 1.

Some soil organisms naturally produce β lactams which lead to natural and chromosomal expression of β lactamases in some Gram negative bacteria even before using the β lactams as a treatment (22, 23). The resistance to penicillin and penicillin derivatives due to the production of constitutive and inducible, chromosomally and plasmid mediated β-lactamases from Ambler Classes A (TEM and SHV) and C (AmpC) led to the development of second-, third- and fourth-generation oxyimino-cephalosporins, and to the development of β-lactamase inhibitors such as clavulanic acid and sulbactam as alternative treatment regimens (24).

Nevertheless, extended spectrum oxyimino-cephalosporins were over used to treat infections caused by β lactamases producing Gram negative organisms, leading to cephalosporin resistance with time. This resistance is due to the production of extended spectrum β lactamases (ESBLs), and the first ESBL was isolated in Germany in 1983 from a *K.pneumoniae* (25).

These β lactamases are called extended because they confer resistance not only to penicillin and monobactams such as aztreonam but also to first, second and third

generation cephalosporins. ESBLs don't confer resistance to cephamycins (such as cefoxitin or cefotetan) or carbapenems and are susceptible to β lactamase inhibitors such as clavulanic acid (20, 25).

The ESBLs are mostly classified as part of the Ambler Class A; and they include derivates of plasmid mediated TEM and SHV penicillinase, TEM variants that are β-lactamase inhibitor resistant and plasmid mediated CTX-M β-lactamases. A small part of the ESBLs are classified under the Class D OXA-type β-lactamases (24, 25). This class of ESBLs differs from the Class A because they hydrolyze oxacillin and cloxacillin very efficiently and because they are poorly inhibited by ethylene-diamine-tetra-acetic acid (EDTA), a di-cation chelator used in the treatment of non-competent cells to become competent ones. Class D is also poorly inhibited by β-lactamase inhibitors like clavulanic acid (19, 25).

However, Ambler Class C enzymes (AmpC enzymes) can also confer resistance to cephalosporins especially in Enterobacteriaceae (24). AmpC enzymes can be chromosomal, such as those naturally found in *Pseudomonas aeruginosa*, *E. coli*, and *Enterobacter cloacae*, or acquired, such as CMY, FOX, ACC, LAT, ACT, etc (26). They are inducible upon exposure to β-lactams and, in the case of a mutation in one or more regulatory genes, they may become constitutively over-expressed (26). In addition to conferring resistance to the same antimicrobial agents as the ESBLs, AmpC enzymes confer resistance to cephamycins. But unlike ESBL producers, AmpC producers are susceptible to fourth generation cephalosporins and are inhibited by aztreonam (26) and in general are not inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam, or by EDTA (26,27, 28).

The ESBL enzymes present along with porin protein alterations or outer membrane impermeabilities play a role in carbapenem resistance (19).

D. Carbapenemases

Carbapenems are the antimicrobial agents used to treat infections caused by ESBL producing organisms; and carbapenem hydrolyzing enzymes (carbapenemases) are increasing, thus making carbapenem resistance alarming and clinically important (29). According to the Ambler classification, carbapenemases are part of the class A, B and D; and they belong to the functional groups 2 and 3 according to the Bush-Jacoby-Medeiros functional classification (21). Carbapenemases are widely active and some of them hydrolyze all β lactams and are uninhibited by β lactamase inhibitors (21).

1. Class A Carbapenemases

Some of the class Acarbapenemases existed before imipenem was clinically used. This can be explained by the selective pressure from soil organisms that produce thienamycin from which imipinem is derived (30, 31). Class A carbapenemases share with the β lactamases the ability to hydrolyze penicillins, cephalosporins and aztreonam and they are inhibited by clavulanic acid. This class of carbapenemase can hydrolyze imipenem resulting in elevated yet susceptible imipenem minimal inhibitory concentrations (MICs) to completely resistant MICs.

2. Class B Metallo-β-lactamases

The class B Metallo-β-lactamases are so called in the Ambler classification and, and their corresponding functional group is 3 in the Bush-Jacoby-Medeiros classification.

They only hydrolyze β lactams in the presence of Zn^{2+} (18, 19, 32).These enzymes can be chromosomally or plasmid encoded, depending on the organisms (32, 33).

a. Chromosomal Metallo-β-lactamases

Around 40 years ago, the chromosomal MBLs were first discovered in some environmental and opportunistic organisms such as *Bacillus cereus*, *Aeromonas* species, and *S. maltophilia* (21, 34, 35, 36, 37). Most of the time, these chromosomal MBLs were found with serine β lactamase. They are both induced once β lactams are added. The MBLs were found to need Zn^{2+} at their active site in order to hydrolyze the β lactams, and to be inhibited by EDTA, clavulanic acid and tazobactam (21).

b. Acquired or transferrable Metallo-β-lactamases

In 1998, the first plasmid mediated MBL was identified from a *P. aeruginosa* in Japan. This enzyme is now called IMP-1. It hydrolyzes penicillins, extended spectrum cephalosporins, cephamycins and imipenem, but not aztreonam and piperacillin. It is inhibited by EDTA, but not by clavulanic acid and sulbactam (32, 38).

Chromosomal MBLs are not easily transferrable, however these enzymes are spread between bacteria and species worldwide. In reality, this is explained by the fact that these tranferrable enzymes are encoded by what is called *bla* genes on class 1 integrons that when associated with plasmids or transposons make the MBLs easily transferrable (21, 32).

In 2009, a Swedish patient of Indian origin was hospitalized in New Delhi, India. It was the first time that the novel metallo-β-lactamase, *bla*-NDM-1 was isolated from this patient who had acquired a urinary tract infection caused by a *K. pneumoniae*

strain (39). *E. coli* isolates from this patient's intestinal flora showed as well the presence of *bla*-NDM-1 gene but on a different plasmid size; this means that the *bla*-NDM-1 gene is transferred in vivo (39).

Since 2009, NDM-1 has been found worldwide in India, Pakistan and the USA; and in 2011, NDM-2, a variant of NDM-1, has been found in an *A. baumannii* isolate coming from a patient previously hospitalized in Egypt (40, 41).

3. Class D OXA-type carbapenemases

OXA type carbapenemases are found worldwide. More than 102 OXA enzymes have been identified nowadays from which 37 possess carbapenem hydrolytic activity. The OXA enzymes can be divided to 9 subgroups according to their amino acids homology. The members of the same subgroup share at least 50% similarities in their amino acids composition (21, 42, 43, 44). While the majority of the OXA carbapenemase are found to be chromosomally encoded, some plasmid mediated enzymes have been identified and they are associated with insertion sequences (42, 45, 46).

These OXA enzymes provide a very high level of resistance to penicillins like ampicillin, amoxicillin and ticarcillin. They hydrolyze narrow and extended spectrum cephalosporins and very poorly hydrolyze monobactams. These enzymes poorly and slowly hydrolyze carbapenems but still at a higher level than they hydrolyze cephalosporins. The OXA carbapenemases are unaffected or barely affected by β lactamase inhibitors such as clavulanic acid and tazobactam (21, 42, 47, 48). For a higher resistance to carbapenems due to the OXA carbapenemases, these enzymes should be accompanied by outer membrane permeability alterations or alteration in the affinity to PBP's or alteration in their production (21, 42).

E. The role of the outer membrane: porin channels and efflux pumps

Gram negative bacteria have an outer membrane that constitutes a permeability barrier. In other words, antimicrobial agents, like the β lactams for example, have to cross the barrier through porin channels before they can reach their target (49). The decrease in the outer membrane porin channels and the over expression of efflux pumps play a role in the bacterial membrane impermeabilities; and thus play a primordial role in conferring resistance against certain antimicrobial agents (26, 49, 50, 51).

1. Porin channels

E. coli produces three major types of porins, the OmpF, OmpC and PhoE (53, 54, 55); while *K.pneumoniae* produces two major porins, the OmpF called OmpK35 and the OmpC called OmpK36 (53). Porins are not really specific but they prefer certain substrate types.

For example, both OmpF and OmpC prefer cations whereas PhoE prefers inorganic phosphate and anions. OmpF is expressed under low osmotic pressure and has a large porin channel that allows larger substrates to pass through compared to OmpC, which is preferably expressed under high osmotic pressure (53, 54, 56).

Most antimicrobial agents have to cross the bacterial outer membrane by passing through the OmpF and OmpC porin subfamilies. This is also the case of the β lactams in Enterobacteriaceae (53, 55).

Exchange, mutation, decreased level of expression, and alteration in the porin channel all contribute to unfunctional porins and in this way renders the outer membrane impermeable (53). The outer membrane impermeability affects the susceptibility of Enterobacteriaceae to cephalosporins and carbapenems. In fact, the loss

of both the OmpF and OmpC porin proteins has been associated with very high level resistance to carbapenems, with ertapenem MICs ≥16 μg/ml, while changes in OmpF or OmpC alone have been associated with lower level resistance to ertapenem (57).

a. Alterations in porin expression

When comparing a quiescent *K. pneumoniae* isolate possessing the OmpK37 porin (and no OmpK35 and OmpK36) with another strain expressing the porins OmpK35 and OmpK36, the susceptibility to the β lactams appears to be lower in the quiescent isolate where the major porins have been exchanged (58, 59).

In another study, a multi-drug resistant *K. pneumoniae* isolate was studied and its porin expression was determined. This isolate showed a decrease in the expression of the larger channel porin OmpK35 and overexpression of the smaller channel porin OmpK36. Along with these alterations, a decrease in the intracellular antimicrobial concentration, including that of the cephalosporins, was observed (60).

An *Enterobacter aerogenes* isolate resistant to imipinem and meropenem was studied and put under different conditions. In the absence of imipenem, the isolate was found to express a porin similar to the OmpC. In the presence of imipinem, the isolate did not express any porins: neither OmpC nor OmpF. The carbapenem resistance was shown to be more probably correlated to the absence of porins (61).

b. Extended spectrum β-lactamases and porin alterations

The presence of one or more ESBL, especially a class A ESBL or an AmpC cephalosporinase, along with porin alterations where shown to play a role in carbapenm resistance in *E. coli* and *K. pneumoniae* isolates (62, 63, 64).

In the United Kingdom for example, more than 200 carbapenem resistant *K. pneumoniae* were found to present porin alterations and CTXM-15, sometimes with TEM-1 and OXA-1 (24, 65).

In Enterobacteriaceae such as *Enterobacter*species and *K. pneumonia*, resistance to ertapenem was observed to be due to active drug efflux combined with the loss/alteration in porins and the presence of a class A ESBL or AmpC enzyme (66, 67, 68).

However, the combination of the alteration, or loss in porins, and the active drug efflux confer the organism a resistance to a wide variety of antimicrobial agents belonging to different classes (68).

2. *Efflux pumps and the efflux pump inhibitor Phenylalanine-Arganine β-Naphthylamide (PAβN)*

Having antimicrobial agents as substrates, efflux pumps in Enterobacteriaceae confer resistance to many of these agents including the β lactams (60, 69).The overexpression of one kind of efflux pumps makes the organism resistant to different classes of antimicrobial agents (70, 71, 72, 73).

Ertapenem resistant *E. cloacae* isolates were studied and observed to have porin alterations. The ertapenem MICs decreased significantly when an efflux pump inhibitor was used, suggesting that efflux pumps may be playing a role in carbapenem resistance (66).

F. Alteration of the carbapenem target: the PBPs

Alteration in the PBPs is one of the carbapenem resistance mechanisms in Gram negative bacteria. It has rarely been reported because it is surpassed by the other resistance mechanisms stated above (74, 75). More cases have been reported for non-Enterobacteriaceae, such as *A. baumannii* and *P. aeruginosa* than for Enterobacteriaceae (76, 77, 78). The first and only case reported in Enterobacteriaceae was in 1995 (79). Two strains of *Proteus mirabilis* were found to produce less of a PBP inhibited by imipenem, the PBP 1A. Only one of those strains produced the PBP 2 that is an essential PBP for imipenem binding, however, it showed less affinity for imipenem (79, 80).

G. Fundamentals of bacterial genetics

Bacteria are prokaryotic microorganisms and generally have one single and circular chromosome. Plasmids are independent molecules of DNA that replicate autonomously in the cytoplasm. Plasmids carry different genes than those present on the chromosomes, and some of them contain resistance genes conferring antimicrobial resistance.

The process of introducing a piece of DNA into a host living cell is called transformation (81,82).

1. Transformation

Bacterial transformation may occur naturally and spontaneously but it can also be controlled under laboratory conditions. It begins when the donor bacterial cell gives up a piece of DNA. This DNA is released in the surroundings, and is fragile and prone to

degradation. If the piece of DNA escapes any alterations and arrives to the recipient cell, then it is taken up by this second bacterial cell and recombines with the homologous portion of the recipient chromosome (82).

2. Transduction

Transduction is the process by which DNA is transferred from one bacterial cell to another one by means of a bacteriophage (82).

3. Conjugation

Conjugation is a unidirectional DNA transfer from a donor cell to a recipient one by a direct cell to cell contact or via the pilus . It starts at a certain point on the DNA fragment and progresses in a linear way. A part of the plasmid or the whole plasmid can be transferred via conjugation, and sometimes the plasmid is accompanied by a part of the chromosome as well (81).

4. Electroporation

Electroporation is an electrical process to transform a cell, mostly Gram negative bacteria. A very high voltage (2500 V) is delivered by a capacitor into a medium containing the recipient cells and the genetic material to transfer. Once the electric current is generated, it alters and damages the cells and not all survive the current. Among the survivors, some develop pores in the cell membrane and take up the plasmids that are present at high concentrations (82).

5. Plasmids

Plasmids are autonomous pieces of DNA that self-replicate. Bacteria carry numerous plasmids of which some are conjugative (F plasmid) and some are not (81).

In the 1950s, in some Japanese isolates of *Shigella* strains, simultaneous resistance to different antimicrobial agents was observed. Researchers noted that the resistance to the combination of the antimicrobial agents was reproducible, meaning that it was not due to random mutations to each agent individually. The DNA coding for this resistance appeared to be plasmid DNA. Both ESBLs and carbapenemases are examples of enzymes which are expressed on plasmids and confer these types of resistance combinations in *E. coli* and *K. pneumoniae* (81).

Many studies were conducted to determine the plasmid size carrying the ESBL enzymes and the carbapenemases.

In a study conducted by Beyrouthy *et al*. (2014) in the north of Lebanon, the *bla*-OXA-48 gene was found to be on a 62 Kb conjugative plasmid.

In Taiwan, *bla*-NDM-1gene was found in two isolates, one *E.coli* and one *K.pneumoniae*.

In the first one, the *bla*-NDM-1 was carried on a circular plasmid of almost 54 bp. And in the second isolate the *bla*-NDM-1 was carried on a circular plasmid of almost 56 bp (84).

In India, *E.coli* harboring both the latter genes was found to have higher MICs against carbapenems than the isolate harboring the *bla*-NDM-1 gene alone (85). The dissemination of ESBLs and carabapenemase is due to plasmid dissemination between species and makes antimicrobial resistance harder to overcome.

CHAPTER III
MATERIALS AND METHODS

A. Bacterial isolates

Ten *E.coli* and *K.pneumoniae* isolates previously collected at the Clinical Microbiology Laboratory (Department of Pathology and Laboratory Medicine) at the AUBMC were used in this study (IMP 02, IMP 21, IMP30, IMP 31, IMP 33, IMP 42, IMP 44, IMP 53, IMP 54, IMP 56).

Seven isolates harbored ESBL and/ or carbapenem resistance encoding genes (IMP 2, IMP 21, IMP 33, IMP 53, IMP 54, IMP 56). Three isolates lacked resistance genes (IMP 30, IMP 31, IMP 42).

Isolates were chosen according to genetic profile and their resistance to carbapenems. Ertapenem was used to detect resistance to carbapenem by the disk diffusion agar method and all isolates showing a diameter ≤ 19mm and an MIC (minimal inhibitory concentration) of ≥ 1µl/ml by E-test were considered resistant according to the Clinical and Laboratory Standards Isolates (CLSI) guidelines 2011.

B. Sample handling and bacterial identification

The ten isolates were previously stored in Brucella Broth (Becton, Dickinson and Company, Sparks, Maryland) containing 15% Glycerol and kept at -20˚C. They were thawed, gram stained, inoculated onto a MacConkey Agar (BBL, Becton, Dickinson and Company, Sparks, Maryland) plate and incubated at 37˚C, under aerobic conditions, for 24 hours.

The following day, isolates were identified to the species level using API20E kits (Biomérieux, SA69280 Marcy-l'Etoile, France) and were stored in Brucella Broth (Becton, Dickinson and Company, Sparks, Maryland) containing 15% Glycerol to be kept at -20˚C.

C. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed following CLSI guidelines 2011 using the Kirby-Bauer Disk Diffusion agar method on Mueller Hinton II Agar (BBL, Becton, Dickinson and Company, Sparks, Maryland) plates. Antimicrobial susceptibility test disks (BBL, Becton, Dickinson and Company, Sparks, Maryland; AB Biodisk, Solna, Sweden) chosen for susceptibility testing included: imipenem 10µg, meropenem 10µg, ertapenem 10µg, cefotaxime 30µg, cefuroxime 30µg, ceftazidime 30µg, cefepime 30µg, ciprofloxacin 5µg, cefpodoxime 10µg, aztreonam 30µg, sulfamethoxazole/ trimethoprim 23.75µg/1.25µg, and tobramycin 10µg. Ceftazidime 30µg disks containing 10µg clavulanic acid and cefotaxime 30µg disks containing 10µg clavulanic acid (BBL, Becton, Dickinson and Company, Sparks, Maryland) were used in order to identify carbapenem resistant and ESBL producing isolates.

To further confirm carbapenem resistance, the MICs of three carbapenems—ertapenem, imipenem and meropenem—were determined using E-test strips. The ATCC *E. coli* strain #25922 was used as a control for the tests.

Following the CLSI guidelines, the bacterial isolates were cultured and incubated aerobically at 37˚C. The next day, the , fresh overnight cultures were inoculated in Mueller Hinton II Broth (BBL, Becton, Dickinson and Company, Sparks, Maryland) tubes and were adjusted to an optical density of 0.5 MacFarland.

The isolates were then sterilely swabbed onto Mueller Hinton II Agar (BBL, Becton, Dickinson and Company Sparks, Maryland) plates in order to produce confluent growth. Plates air dried for five minutes under sterile conditions and then, the antimicrobial disks and the E-tests were dispensed onto the plates containing the bacteria. After an aerobically overnight incubation at 37˚C, the zones of inhibition around the antimicrobial disks were measured and the MICs were recorded.

Interpretation of susceptibility for the antimicrobial disks and E-tests was according to the CLSI 2011 breakpoints.

D. Genomic DNA extraction

The DNA of the isolates was extracted using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK Limited Little Chalfont Buckinghamshire) as follows:

1. Harvesting of Bacterial Culture

- An overnight bacterial culture (1.5 ml) in LB broth (Becton, Dickinson and Company, Sparks, Maryland) was transferred to a 1.5 ml microcentrifuge tube.
- The culture was the centrifuged for 1 minute at 12,000xg.
- The supernatant was discarded without disturbing the pellet.

2. Lysis

Bacterial cells are lysed by salts and detergents,

- Lysis buffer type 2 (40µl) were added and immediately mixed by vortexing till no visible pellet is present.

- After re-suspension, 10µl of Proteinase K (20mg/ml) were added and the sample was vortexed for 10 seconds.
- Lysis buffer type 3 (10µl) were added to the sample and mixed by vortexing for 10 seconds.
- The sample was incubated for 15 minutes at 55ºC with 10 seconds of vortexing after the first 7 minutes.

3. Purification

Chaotropic salts present in Buffer type 4 help the binding of genomic DNA to the silica membrane.

- Lysis buffer type 4 (500µl) was added to the sample and mixed by vortexing for 10 seconds.
- The sample was incubated for 10 minutes at room temperature, with a short vortexing pause after the first 5 minutes.
- The sample was then transferred to mini column placed inside one collection tube and then spinned for 1 minute at 11,000xg.
- The flowthrough was discarded and the column was placed back into the collection tube.

4. Wash and Dry

Lysis Buffer type 4 removes proteins and other contaminants from the membrane bound DNA.

The Wash Buffer type 6 removes salts and other contaminants and dries the silica membrane at the same time.

- 500µl of Lysis buffer type 4 was added to the column inside the collection tube
- The sample was centrifuged for 1minute at 11000xg.
- The flowthrough was discarded by emptying the collection tube and the column was then placed back in it.
- Wash buffer type 6 (500µl) was added to the column.
- The sample was then spinned for 3 minutes at 12,000xg.
- The collection tube was discarded and the column transferred to a fresh DNase free 1, 5 ml microcentrifuge tube.

5. Elution

- Low ionic strength Elution buffer type 5 was pre-heated to 70˚C and 160µl of it was then added directly to the column.
- The sample was incubated for 1 minute at room temperature and then spinned for 1 minute at 11,000g in order to recover the purified DNA as flowthrough.
- The eluted DNA was finally aliquoted and stored at -20˚C till it was needed.

E. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was carried out on all 10 isolates to determine their genomic relatedness using the standard operating procedure for Pulsenet.

1. Materials needed

- 10× TBE (Tris-Boric Acid-EDTA), volume of 500 ml:
    - Tris Base (60.55 g) (Amresco, USA)
    - Boric Acid (30.99 g) (Amresco, USA)

- Disodium EDTA (1.85 g) (Amresco, USA)
- Distilled water (500 ml) was added and the mixture was autoclaved

- 0.5 × TBE: 10 × TBE was diluted using autoclaved distilled water
- Cell Lysis Buffer (CLB) (all components were placed in an autoclaved flask), volume of 500 ml:
  - Sarcosyl (5 g) (N-Lauroylsarcosine sodium salt) (Sigma Chemical Co., St. Louis, MO).
  - Tris (25 ml, 1M, pH 8) (Amresco, USA)
  - EDTA (50 ml, 0.5 M, pH 8) (Amresco, USA)
  - Completed with 425 ml double distilled water
- Cell Suspension Buffer (CSB) (all components were placed in an autoclaved flask), volume of 100 ml:
  - Tris (10 ml, 1M, pH 8) (Amresco, USA)
  - EDTA (20 ml, 0.5 M, pH 8) (Amresco, USA)
  - Completed with 70 ml autoclaved distilled water
- TE buffer (for washing and plugs preparation), volume of 500 ml:
  - Tris (5 ml, 1M, pH 8) (Amresco, USA)
  - EDTA (1 ml, 0.5 M, pH 8) (Amresco, USA)
  - Autoclaved distilled water (494 ml)
- Seakem Gold (SKG) Agarose for PFGE (Lonza, USA)
- Ladder: BAA 664 (Braendrup) Salmonella species
- Autoclaved distilled water
- Nuclease free water
- Proteinase K (20 mg/ml; Thermo Scientific Inc., USA)

- 10 × Tango buffer (Fermentas, USA)
- Incubation buffer mix (1 × Tango buffer), a volume of 2 ml (enough for 10 samples): prepared by mixing 200 µl with 1800 µl nuclease free water and mixed by vortexing.
- Restriction mix (for 10 samples), volume of 2 ml:
    - Nuclease free water (1755 µl)
    - Restriction enzyme buffer (200 µl ,10 × Tango buffer)
    - Bovine Serum Albumin (BSA) (20 µl) (Amresco, USA)
    - XbaI enzyme (25 µl) (Fermentas, USA)
    - Mixed by pipetting the mixture up and down followed by swirling
- Ethidium bromide (Amresco, USA)

2. Protocol:

a. <u>Preparation of Seakem Gold Agar Gel for plugs</u>

- The gel (1%) was prepared by mixing 0.5 g of Seakem Gold Agarose in 50 ml TE buffer and the preparation was mixed gently to disperse the agarose.
- The mixture was heated in the microwave (with regular stirring) to dissolve the agarose.
- The gel was incubated at 50 ºC in a water bath for 20 minutes or until ready to use.

b. <u>Preparation of Cell suspension</u>

- In different test tubes labeled with name of the isolates including the ladder, 2 ml CSB was added

- A few colonies from a fresh culture of each isolate including the ladder grown on MacConkey agar plate were inoculated in their corresponding tubes containing the CSB. The suspension was vortexed to evenly disperse the bacterial cells.
- The concentration of the cell suspension for the samples was adjusted to 2 McFarland, while that of the ladder was adjusted to 2.5 McFarland.

c. Plug casting

- Adjusted cell suspensions (400 µl) was added to labeled autoclaved 1.5 ml microcentrifuge tubes
- Proteinase K (20 µl of 20 mg/ml) was added to each microcentrifuge tube and the mixture was pipetted up and down.
- The agarose gel was removed from the water bath and 400 µl of the gel was transferred to each microcentrifuge tube and the mixture was pipetted up and down a few times.
- The mixture (400 µl) in each microcentrifuge tube was transferred into separate wells in the plug mold.
- The mold was incubated at 4 ºC for 10 minutes until the plugs solidified.

d. Cell lysis of agarose plugs

- CLB (5 ml) was transferred to labeled falcon tubes. Subsequently, 25 µl of Proteinase K (20 mg/ml) was added to each falcon tube.
- After solidification, the plugs were removed from the plug mold and dropped into their corresponding falcon tube, making sure they were completely immersed in the buffer.

- The falcon tubes were incubated in a shaker incubator with constant vigorous agitation (150-175 rpm) for 2 hours at 54 ºC.

e. Washing of plugs after cell lysis

- After incubation, the CLB was poured off carefully from each falcon tube and 5 ml of pre-heated (54-55 ºC) autoclaved water was added to each falcon tube. Subsequently, the tubes were incubated in a shaker incubator for 15 minutes at 54ºC, after which the water was discarded. The washing with autoclaved water was pursued twice.
- After washing the plugs with water, 5 ml preheated TE (54-55 ºC) was added to each falcon tube. The tubes were incubated in a shaker incubator for 15 minutes at 54 ºC, after which the TE was discarded. The washing step with TE was performed 4 times.
- After the last incubation step with TE, the TE was discarded and 5 ml of TE was added to each falcon tube. The falcon tubes were incubated overnight at 4 ºC.

f. Plug cutting

- On the second day, 200 µl of 1 × Tango buffer (incubation buffer mix) was added to labeled microcentrifuge tubes.
- The falcon tubes containing the plugs were removed from the refrigerator and the plugs were cut into 2 mm thick plug using a blade.
- Each cut plugs was placed in the tango buffer found in the corresponding microcentrifuge tube using a spatula.

- The microcentrifuge tubes were incubated in a walk-in incubator at 37ºC for 15 minutes.

g. Digestion of DNA in agarose plugs

- After incubation, the incubation buffer was discarded from each tube without disturbing the plug, 200 µl of restriction buffer mix was added into each tube, and the tubes were incubated at 37 ºC for 3 hours (XbaI enzyme works at 37 ºC).

h. Preparation of 1% Seakem Gold Agarose

- An hour before the incubation period ended, agarose for gel preparation and for covering the wells were prepared.

i. Agarose for gel preparation

- Seakem Gold Agarose (0.5 g) was mixed in 100 ml of 0.5 × TBE and the preparation was swirled gently to disperse the agarose.
- The mixture was heated in the microwave (with frequent stirring) until the agarose dissolved.
- The gel was incubated at 56 ºC in a water bath for 20 minutes before pouring.

j. Agarose preparation for covering the wells in the gel

- Seakem Gold Agarose (0.1 g) was mixed in 50 ml 0.5 × TBE and the preparation was mixed gently to disperse the agarose.
- The mixture was heated in the microwave (with regular stirring) to dissolve the agarose.

- The gel was incubated at 56 ºC in a water bath until use.

k. Agarose gel casting

- After cooling, the gel was poured into a gel cast with the corresponding comb.
- The gel was covered with an aluminum foil and left to dry at room temperature for 30-45 minutes.
- After the 3 hours incubation period, the restriction mix was discarded from each microcentrifuge tube without disrupting the plug and 200 µl of 0.5 × TBE was added to each tube.
- The microcentrifuge tubes containing the plugs were incubated at room temperature for 10 minutes.

l. Plug loading into wells

- Each plug was removed from its microcentrifuge tube using a sterile spatula and placed into a well in the gel.
- The wells containing the plugs were completely covered by filling the wells of the gel with 50 µl agarose prepared for well covering. The agarose was left for 3-5 minutes to harden.

m. Running of the gel

- 0.5 × TBE (2200 ml) was poured into the electrophoretic cell (Bio-rad, USA) and the buffer was allowed to cool to 14 ºC (by turning on the cooling module (Bio-rad, USA)). The gel was then removed from the casting mold and placed in the electrophoretic cell.

- The program for Non O157 Shiga toxin producing Escherichia coli (STEC) was entered on the CHEF MAPPERTM (Bio-rad, USA) by selecting the following conditions:
    - Auto Algorthim

        50 kb- low MW

        400 kb- high MW
    - Default values were selected except where noted by pressing "Enter"
    - Run Time of 18 hours was entered

        (Default values: Initial switch time= 6.76 s; Final switch time= 35.38s).
- The program was started and the run was carried out overnight.

n. Staining of the PFGE agarose gel

- On the second day, after the run was over, the machine was turned off and the gel was placed in a jar filled with 400 ml distilled water. 8 drops of ethidium bromide were then added.
- The jar was placed on a rocker machine and incubated for 20 minutes.
- After incubation, the ethidium bromide was discarded.

o. 13. Destaining of the PFGE agarose gel

- Distilled water (500 ml) was added to the jar containing the gel. The gel was placed on a rocker machine and incubated for 20 minutes. This was repeated 2 more times.

After discarding the water from the jar for the last time, a picture of the gel was captured using the Gel Doc XR + system Machine (Bio-rad, USA) and the bands were visualized and analyzed with "Quality one" and "Bionumerics" software respectively.

F. Plasmid DNA extraction

The plasmid DNA of the samples was extracted using the illustraplasmidPrep Mini Spin Kit (GE Healthcare, UK Limited Little Chalfont Buckinghamshire) , it's a three steps process involving lysis, purification and de-salination.

1. Preparation of bacterial culture:

- The samples were freshly cultured on MacConkey Agar (BBL, Becton, Dickinson and Company, Sparks, Maryland) plates.
- For each sample, a single colony was picked and inoculated into 2-5 ml of Luria-Bertani (LB) Broth (Difco, Becton, Dickinson and Company, Sparks, Maryland) flasks.
- The samples were then incubated for 8 hours at 37˚C with constant shaking at 225rpm.
- After the incubation, each starter culture was diluted 1 in 1000 into new LB broth flasks.
- The cultures were incubated again at 37˚C for 18 hours with shaking at 225rpm.

2. Harvesting of bacterial culture

- The following day, cultures were transferred into polypropylene tubes.
- The samples were centrifuged for 15min at 5,000xg and 4˚C.
- The supernatant was discarded and the pellet left as dry as possible.

3. *Re-suspension of bacterial culture*

- The pellet was re-suspended in 6 ml of Re-suspension buffer type 1 containing RNase1.
- Complete re-suspension of the bacterial pellet was done by pipetting up and down.

4. *Cell lysis*

Lysis occurs in alkaline conditions with SDS detergent: genomic DNA and proteins are denatured and RNA is hydrolysed.

- Lysis buffer type 2 (6 ml) were added.
- Samples were mixed thoroughly by inverting sharply inverting the tubes several times.
- Samples were incubated for exactly 5 minutes at room temperature.

5. *Neutralization of cell lysis reaction*

Potassium acetate solution neutralizes alkaline conditions and genomic DNA, proteins, cell debris and potassium dodecyl sulfate (KDS) precipitate.

- Pre-chilled Neutralization buffer 3 (6 ml) was added.
- The tubes were sharply inverted till they become less viscous.
- Samples were incubated for 15 minutes on ice.

6. *Clarification of lysate and preparation of fast flow plasmid 250 column*

The lysis reaction is clarified by high speed centrifugation in order to remove genomic DNA, proteins and KDS precipitate.

The purification process is facilitated by the pre-equilibrated fast flow plasmid 250 purification column.

- The lysate was centrifuged for 30 minutes at 20,000xg and 4˚C and the supernatant was clear.
- During the above step, the Fast flow plasmid 250 column were allowed to empty completely of excess packing buffer by gravity flow by carefully cutting the tip of each column.

7. Purification- Load

The resin is an anion exchange matrix that binds plasmids and prevents the binding of most contaminants.

- 10ml of the clarified lysate were pipetted into the Fast flow plasmid 250 column and were allowed to enter the resin by gravity flow.
- The rest of the clarified lysate was then also pipetted into the column to enter the resin by gravity flow.

8. Purification- Wash

- Wash buffer (10ml) was added to the columns.
- Columns were allowed to empty completely of buffer by flow gravity.
- The wash was repeated with additional 10ml of the buffer.

9. Purification- Elute

- 4ml of the high salt elution buffer were added to the columns.
- The eluate was collected by flow gravity into polypropylene tubes.

10. Concentration- Isopropanol precipitation

- The eluate was transferred into centrifuge tubes.
- 4ml of room temperature isopropanol were added.
- The samples were mixed by inversion.
- The tubes were put in the centrifuge for 30 minutes at 15,000xg and at 4˚C.
- Supernatant was immediately carefully pipetted.

11. Desalination- 70% ethanol wash

- At room temperature, 5ml of 70% ethanol were added to wash the DNA pellet.
- The tubes were centrifuged for 10 minutes at 15,000xg and at 4˚C.
- After centrifugation, the supernatant was aspirated with a pipette.

12. Drying and re-suspension

- The pellet was air dried for 5 to 10 minutes.
- The plasmid DNA was dissolved in 1ml low ionic strength TE (Tris-EDTA) buffer.
- The samples were then stored at -20˚C for downstream applications.

G. PCR detection of ESBL, carbapenemase and porins encoding genes

β lactamases, carbapenemases, porins encoding genes and the novel metallo-β-lactamase NDM-1; were detected by PCR on the genomic and plasmid DNA of the isolates as well as on the electrocompetent cells. Sequences for the primers used are found in the table below.

1. Reaction mixtures

Polymerase chain reactions for all genes except, OmpC, OmpF and *bla*-NDM-1 were performed in 50 microliter reaction mixtures using the following reagents:

- DNA template (2.5µl), with a final concentration of 10µg/l
- Forward primer (5 µl) and reverse primer (5 µl), with a final concentration of 1 µM in solution
- Starting from a concentration of 5U/µl ,0.25 µlTaq DNA polymerase stored in buffer containing 0.1mM EDTA, 50mM tris HCL, 5mM dithiothereitol stabilizers and 50% glycerol(Fermentas Life Sciences, Burlington, Ontario, Canada) with a final concentration of 1.25U in 50 µl
- 10X Taq Polymerase buffer (5 µl) (MBI Fermentas Life Sciences, Burlington, Ontario, Canada)
- 4 µl MgCl2 (MBI Fermentas Life Sciences, Burlington, Ontario, Canada) with a final concentration of 2 mM in solution
- Deoxynucleoside triphosphate dNTP mixture (2 µl), (MBI Fermentas Life Sciences, Burlington, Ontario, Canada) with a final concentration of 0.2 mM of each dNTP in solution
- Water, sterile, nuclease free (26.25 µl) (Amresco, Solon, OH 44139, USA)

To increase the sensitivity of the reaction, volumes and final concentrations in solution of the reagents were optimized for OmpF, OmpC and *bla*-NDM-1 as follows:

2. PCR cycling conditions

Amplification was achieved using the PCR Sprint Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions were previously optimized in house for all genes amplified and were as follows:

H. Gel electrophoresis

To determine the amplification of a gene in question, the PCR products were run into a 1.5% agarose gel.

The agarose gel was prepared under the following steps:

- SeaKem® LE Agarose (1.5 grams) (FMC BioProduct, Rockland, ME, USA) were weighted
- The LE Agarose was added to 100 ml 1× TBE buffer (PH 8.3.Tris Boric acid 0.089 M, Tris Base 0.089M, EDTA 0.002 M)
- To completely dissolve the agarose, the gel mixture was microwaved for approximately 2 minutes
- Ethidium bromide, 10µl of 0.625 mg/ml, (Amresco, Solon, OH 44139, USA) were then added to the dissolved agarose for staining
- The gel was poured into the electrophoretic chamber
- The gel solidified and then submerged into 1x TBE buffer

Once the gel was ready, the samples were run in parallel horizontally according to the steps below:

- Ten microliters of the sample were mixed with two microliter of the loading dye (Fermentas Life Sciences, Burlington, Ontario, Canada)
- They were pipetted up and down before being loaded into respective wells

- A 100 base pair ladder (Fermentas Life Sciences, Burlington, Ontario, Canada) was run in parallel to the samples and served as a size marker for amplicons
- Amplicons were then observed using UV light, and were photographed using a digital camera (Olympus)
- A permanent record was saved using the Digi-Doc It program

I. Electroporation

Electroporation is the procedure of introducing the already extracted plasmid DNA into the Thunderbolt GC-10 electrocompetent cells (Sigma-Aldrich Co, St Louis, Missouri) by an electric pulse.

The extracted plasmid of the ten isolates was introduced into the electrocompetent cells following the steps below:

- The SOC medium stored at 4˚C (Sigma-Aldrich Co, St Louis, Missouri) was warmed to room temperature (20-25 ˚C).
- The plasmid DNA was removed from the -80 ˚C freezer and the tubes were immediately placed on wet ice to thaw for around 10 minutes.
- Ten standard cuvettes of 1mm and autoclaved microcentrifuge tubes were placed were placed on ice, one for each reaction.
- SOC medium (960µl) was placed in each microcentrifuge tube and left to room temperature.
- The Thunderbolt cells were resuspended by tapping on the vials and thawed.
- The electrocompetent cells (40µl) were transferred into the chilled microcentrifuge tube containing the plasmid DNA.

- The DNA/cells mixture was pipetted into the chilled 1mm cuvette and placed into the electroporator (BioRad Gene Pulser).
- The electroporator was set as follows: 2.5 kV, 25 mF, 100 W, 1 mm cuvette.
- The set were then removed from the cuvette and added to the microcentrifuge tubes containing the 960µl SOC medium.
- The tubes were then placed into the microcentrifuge and the cells were incubated and shaked for one hour at 37 ˚C and 220-225 rpm.
- The electrocompetent cells (100µl) were pipetted and evenly spread onto LB agar plate containing Ceftazidime.
- Note that the LB agar plates were pre-warmed at 37˚C for optimal colony growth.
- Growth on the LB agar containing the antibiotic shows that the Thunderbolt cells have taken up the plasmid.

CHAPTER IV

RESULTS

A. Bacterial isolates

Genus and species of all ten ESBL producing and non-ESBL producing carbapenem resistant isolates were confirmed phenotypically.

B. Antimicrobial susceptibility

Antimicrobial susceptibility testing confirmed that:

- The three non ESBL producing isolates were resistant to all three tested carbapenems
- Two of the ESBL producing isolates were resistant to all three tested carbapenems
- Three of the ESBL producing isolates were resistant to Meropenem and Ertapenem but sensitive to Imipenem.

Disk diffusion susceptibility profiles for all the isolates are seen in Table 4.

However, the isolates showed different MIC profiles to the three tested carbapenems as shown in table 7:

- All ten isolates were resistant to Ertapenem with an MIC $\geq$ 1 µg/ml.
- IMP 54 and IMP 56 were resistant to Meropenem with an MIC $\geq$ 4 µg/ml, and IMP 53 was highly resistant with an MIC $\geq$ 32 µg/ml.
- IMP 42 was resistant to Imipenem with an MIC $\geq$ 4 µg/ml, and IMP 53, IMP 54, IMP 56 were highly resistant to Imipenem with MIC $\geq$ 32 µg/ml.
- IMP 2 and IMP 30 were intermediate to Meropenem.

- IMP 31 and IMP 44 were intermediate to Imipenem.

C. Pulsed field gel electrophoresis

All isolates showed diversity in their genomic DNA patterns, denoting that they all belonged to different strains (Figure 1).

D. Electroporation experiments

All ten isolates were electroporated. The susceptibility to Ertapenem in each receiver cell isolate was reproducible even though the MIC's of all isolates were decreased, but they all however remained resistant to it. Table 3 shows the MIC comparison between the donor cells and the receiver ones.

The presence of *bla*-CTX-M-15, *bla*-TEM-1, *bla*-NDM-1 and *bla*-OXA-48 was also identical between the donor and the recipient cells as shown in table 8.

E. Detection of resistance encoding genes and porin encoding genes by PCR

The three control isolates (IMP 30, IMP 31, IMP 42) only harbored the porin encoding genes and none of the ESBLs or carbapenemases encoding genes. IMP 30 and IMP 42 possess both porin proteins OMP-C and OMP-F. IMP 31 harbors only the OMP-C porin protein and has lost the OMP-F (Figures 3,4, 5).

Both porin encoding genes *bla*-OMP-C and *bla*-OMP-F were found in three isolate: IMP 21, IMP44 and IMP 53. The *bla*-OMP-C gene was present alone in IMP 2, IMP 33 and IMP 54. The isolate IMP 56 has lost both of its porin encoding genes: *bla*-OMP-C and *bla*-OMP-F (Figures 3,5).

Different ESBLs and carbapenemase combinations were present in these isolates that showed different antimicrobial susceptibility profiles. The ESBL CTXM-15 was present in three of the isolates: IMP 2, IMP 33 and IMP 54. Only IMP 2 and IMP 21 harbored the TEM-1 ESBL. Concerning the carbapenemases, the OXA-48 was found in the IMP 44, IMP 53 and IMP 54 whereas the NDM-1 was harbored by the IMP 54 and IMP 56 (Figures 4, 5).

To sum it up, the isolates chosen showed different carbapenem resistance levels along with different genetic profile that was maintained the same in after electroporation in electrocompetent recipient cells as shown in table 7 and table 8.

Table 1: Summary of ESBL and carbapenemases

Gene	Enzyme Type, Ambler Class	Cofactor needed	Plasmid mediated/Chromosomal mediated
***bla*-CTX-M**	ESBL; A	Serine	Plasmid
***bla*-TEM-1**	ESBL; A	Serine	Plasmid
***bla*-NDM-1**	MBL; B	Zinc	Plasmid
bla*-*OXA48	Carbapenemase; D	Serine	Plasmid

Table 2: Outer membrane porin genes, carbapenemases and ESBL gene primers

Primer	Gene, Enzyme Type, Ambler Class	Sequence (5'-3')	Size (bp)
CTX-M-F **CTX-M-R**	*bla* - CTX-M*;* ESBL; A	5'-GGTTAAAAAATCACTGCGTC-3' 5'-TTACAAACCGTCGGTGACGA-3'	874
OT-3 **OT-4**	*bla* -TEM-1*;* ESBL; A	5'-ATGAGTATTCAACATTTCCG-3' 5'-CCAATGCTTAATCAGTGAGG-3'	1143
NDM-1-F **NDM-1-R**	*bla*-NDM-1*;* MBL; B	5'-GGAAACTGGCGACCAACG-3' 5'-ATGCGGGCCGTATGAGTGA-3'	678
OXA-48-A **OXA-48-B**	*bla*-OXA48*;*carbapenemase; D	5'-TTGGTGGCATCGATTATCGG-3' 5'-GAGCACTTCTTTTGTGATGGC-3'	744
OmpF-A **OmpF-B**	OmpF	5'-CAGGTACTGCAAACGCTGC-3' 5'-GTCAACATAGGTGGACATG-3'	953
OmpC-F **OmpC-R**	OmpC	5'-GAACTGGTAAACCAGACCCAG-3' 5'-GTTAAAGTACTGTCCCTCCTG-3'	1086

Table 3: Reaction mixtures of *bla* -OmpF, *bla* -OmpC and *bla*NDM-1

Gene	DNA template (µl)	Primers	Taq DNA Polymerase (µl)	10x Taq Polymerase buffer (µl)	MgCl2 (µl)	dNTP (µl)	Nuclease free H2O (µl)
***bla* -OmpF**	2.5,10 µg/l	2.5 each, 0.5 µM final concentration	0.25	5	4	2	31.25
***bla* -OmpC**	5, 60-120 µg/ml	2.5 each, 0.5 µM final concentration	0.25	5	4	2	28.75
***bla*-NDM-1**	5, 10 µg/l	2.5 each, 0.5 µM final concentration	0.25	5	4	2	28.75

Table 4: PCR cycling conditions

Gene(s)	Initial denaturation 1 cycle	Denaturation (1)	Annealing (2)	Polymerization (3)	# of cycles for 1,2,3	Final extension, 1 cycle
***bla*-CTX-M-15, *bla*-TEM-1**	94°C for 10 minutes	94°C for 30 seconds	50°C for 40 seconds	72°C for one minute	35	72°C for 10 minutes
***bla*-OXA-48**	94°C for 7 minutes	94°C for 45 seconds	56°C for 45 seconds	72°C for 1 minutes	35	72°C for 10 minutes
OmpF	95°C for 7 minutes	95°C for 45 seconds	56°C for 45 seconds	72°C for 1 minutes	35	72°C for 7 minutes
OmpC	95°C for 7 minutes	95°C for 45 seconds	50°C for 45 seconds	72°C for 1 minutes	35	72°C for 10 minutes
***bla*-NDM-1**	95°C for 7 minutes	95°C for 45 seconds	60°C for 45 seconds	72°C for 45 seconds	35	72°C for 7 minutes

Table 5: Antimicrobial susceptibility profiles of all ten isolates as determined by Disk Diffusion
R=resistant, S=sensitive, I=intermediate, >5=ESBL producer, <5=non ESBL producer

	IMP 2	IMP 21	IMP 30	IMP 31	IMP 33	IMP 42	IMP 44	IMP 53	IMP 54	IMP 56
Profile	*E. coli*	*E. coli*	*E. coli*	*K. pneumonia*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
Disk diffusion										
Meropenem	R	R	R	R	R	R	R	R	R	R
Ceftazidime	R	R	R	R	R	R	S	R	R	R
Ertapenem	R	R	R	R	R	R	R	R	R	R
Imipenem	S	R	S	S	S	S	R	R	R	R
Cefipime	R	R	R	R	R	S	I	R	R	R
Ciprofloxacin	R	R	R	R	R	R	S	R	R	R
Bactrim	S	R	R	R	R	S	R	R	R	R
Tobramycin	R	R	R	R	R	R	S	R	R	R
Aztreonam	R	R	R	R	R	S	I	R	R	R
Cefpodoxime	R	R	R	R	R	R	R	R	R	R
Cefotaxime	R	R	R	R	R	R	R	R	R	R
Cefuroxime	R	R	R	R	R	R	R	R	R	R
CTX-CLA	>5	>5	>5	>5	>5	>5	>5	<5	<5	<5
CAZ-CLA	>5	>5	>5	>5	>5	>5	<5	<5	<5	<5

Table 6: Genetic profile of all ten isolates.

	IMP 2	IMP 21	IMP 30	IMP 31	IMP 33	IMP 42	IMP 44	IMP 53	IMP 54	IMP 56
Profile	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
Genes										
OmpC	positive	positive	positive	positive	positive	positive	positive	positive	positive	negative
OmpF	negative	positive	positive	negative	negative	positive	positive	positive	negative	negative
Oxa-48	negative	negative	negative	negative	negative	negative	positive	positive	positive	negative
TEM-1	positive	positive	negative	negative	negative	negative	negative	negative	negative	negative
CTXM-15	positive	negative	negative	negative	positive	negative	negative	negative	positive	negative
NDM-1	negative	negative	negative	Negative	negative	negative	negative	negative	positive	positive

Table 7: Comparison of the MICs of the parent cells and the electrocompetent recipient cells.

	IMP 2	IMP 21	IMP 30	IMP 31	IMP 33	IMP 42	IMP 44	IMP 53	IMP 54	IMP 56
Profile	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
MIC µg/ml Parent Cells										
Meropenem	2	0.75	2	0.5	0.38	1	0.5	32	16	16
Imipenem	0.5	0.5	0.5	1.5	0.38	4	1.5	>32	32	32
Ertapenem	16	8	>32	16	3	24	2	>32	>32	32
MIC µg/ml *E.coli* Electrocompetent recipient Cells										
Ertapenem	2	0.5	0.38	0.38	0.5	0.38	2	2	16	8

Table 8: Comparison of the genetic profile of the parent cells and the electrocompetent recipient cells.

	IMP 2	IMP 21	IMP 30	IMP 31	IMP 33	IMP 42	IMP 44	IMP 53	IMP 54	IMP 56
Profile	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
Genes of Parent Cells										
CTXM-15	positive	negative	negative	negative	positive	negative	negative	negative	positive	negative
TEM-1	positive	positive	negative	negative	negative	negative	negative	negative	negative	negative
Oxa-48	negative	negative	negative	negative	negative	negative	positive	positive	positive	negative
NDM-1	negative	negative	negative	negative	negative	negative	negative	negative	positive	positive
Genes of *E.coli* Electrocompetent recipient Cells										
CTXM-15	positive	negative	negative	negative	positive	negative	negative	negative	positive	negative
TEM-1	positive	positive	negative	negative	negative	negative	negative	negative	negative	negative
Oxa-48	negative	negative	negative	negative	negative	negative	positive	positive	positive	negative
NDM-1	negative	negative	negative	negative	negative	negative	negative	negative	positive	positive

Table 9: Efflux pumps in parent cells

	IMP 2	IMP 21	IMP 30	IMP 31	IMP 33	IMP 42	IMP 44	IMP 53	IMP 54	IMP 56
Profile	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
EP in Parent Cells	positive	positive	positive	negative	negative	positive	negative	positive	negative	negative

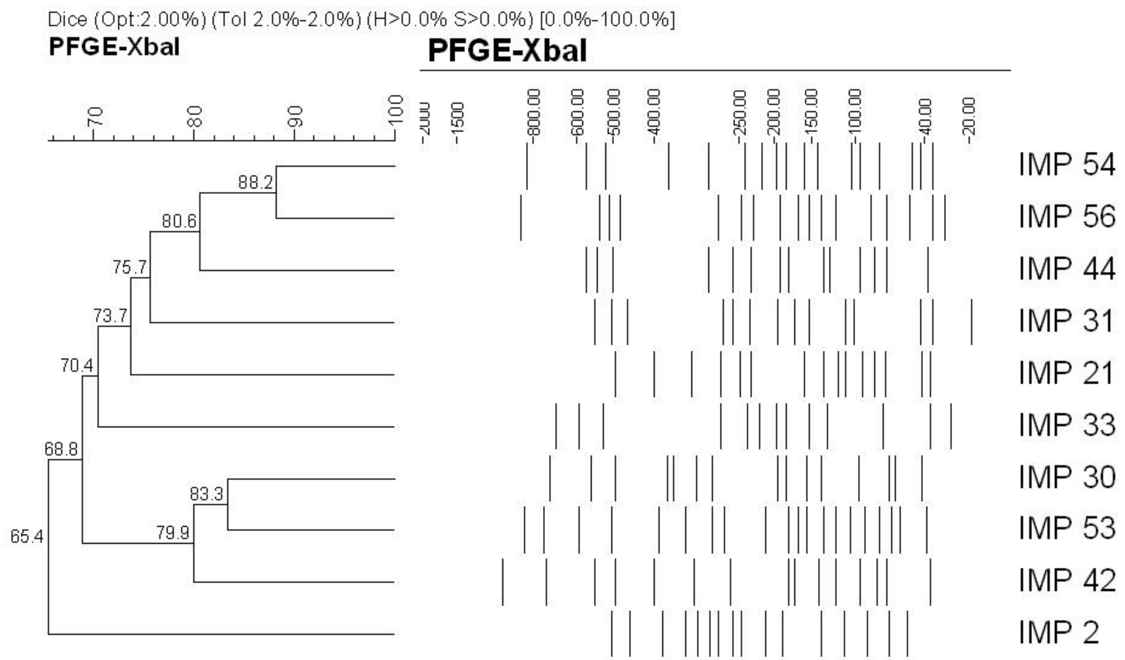


Figure 1: PFGE analysis of the ten carbapenem resistant isolates.

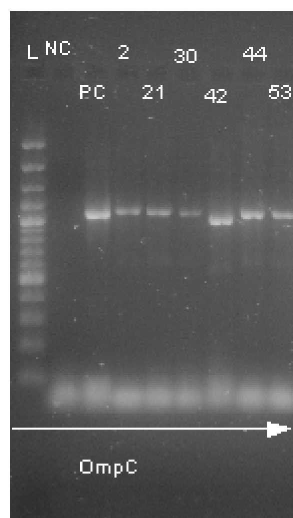
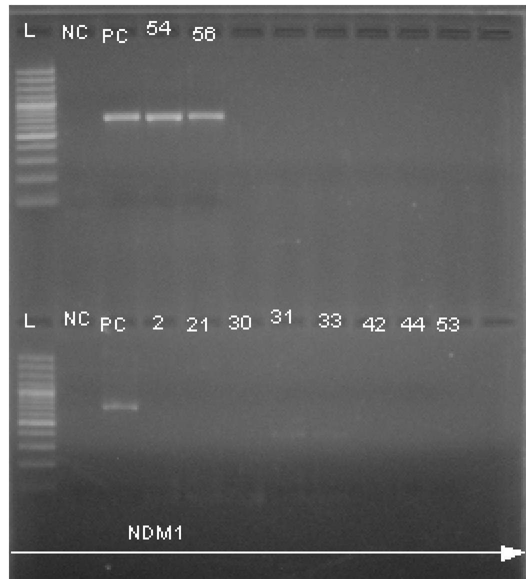
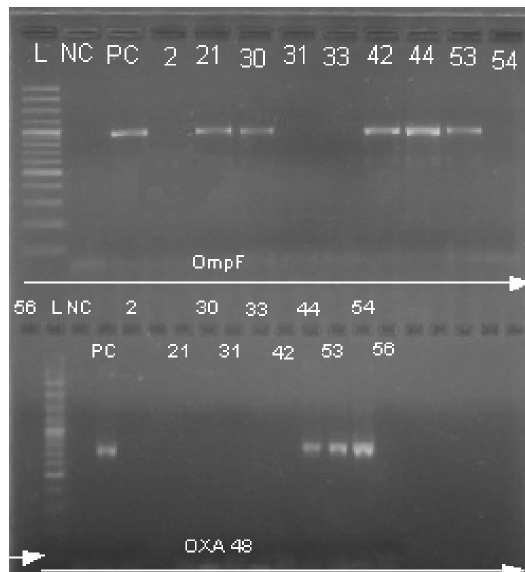


Figure 2: Gel electrophoresis of *bla*-OmpC for parent cells.
L=ladder, NC= negative control, PC=positive control.



<u>Figure 3:</u> Gel electrophoresis of *bla*-NDM-1gene for parent cells. L=ladder, NC=negative control, PC= positive control.



<u>Figure 4:</u> Gel electrophoresis of OmpF and *bla*-OXA-48 genes for parent cells. L=ladder, NC=negative control, PC= positive control.

CHAPTER V

DISCUSSION

The ten selected ESBL and non-ESBL producing, carbapenem resistant isolates, collected from a previous study by Baroud *et al*. (2011), belonging to diverse genotypes as determined by PFGE analysis, indicated different levels of resistance to carbapenems. Resistance in seven of these isolates is due to either carbapenemases encoded by *bla*-OXA-48 or *bla*-NDM-1or to ESBLs encoded by *bla*-CTXM-15 or *bla*-TEM-1, along with outer membrane proteins (Omp-F and Omp-C) impermeability or loss, while resistance of the three control isolates lacking the resistance genes, it is due to OMP impermeabilities and / or EP activity (Baroud *et al*. 2011).

For all ten isolates, the genetic profile of electrocompetent cells remained similar to parent cells after electroporation. The *bla*-CTX-M-15, *bla*-OXA-48, *bla*-NDM-1and *bla*-TEM-1 genes were all transferred to recipient electrocompetent cells and exhibited resistance to carbapenems as detected by antimicrobial susceptibility testing and PCR amplification of transferred genes. This confirms further that the genes are plasmid encoded.

The antimicrobial susceptibility profile of electrocompetent recipient cells showed resistance to ertapenem with a reduction of MICs in comparison to parent cells, due exclusively to the effect of the ESBL and/ or carbapenemases, since outer membrane proteins are available in electrocompetent cells facilitating the entry of the antimicrobial agent that is not expulsed by efflux pumps.

Post electroporation, only five (IMP 2, IMP 44, IMP 53, IMP 54, IMP 56) of the ten isolates remained resistant to ertapenem with an MIC $\geq$ 1µg/ml. For all five of

these isolates, the MIC values observed in parent cells were decreased in electrocompetent recipient cells.

In the case of the IMP 2 elctrocompetent recipient cell, the MIC decreased from 16µg/ml to 2µg/ml.

IMP 2 parent cell, an ESBL producing isolate, harbors only the porin protein Omp-C encoding gene and lost the Omp-F porin protein. This observation explains that the high MIC of 16µg/ml in parent cells is due to the Omp-F loss along with the presence of ESBL TEM-1 and CTXM-15. The decrease in the ertapenem MIC to 2µg/ml after electroporation in IMP 2 electrocompetent recipient cell, suggests that the loss of Omp-F in the parent isolate was playing a role in its resistance, because the GC10 electrocompetent cells contain both porins and both ESBLs have been taken up.

Both IMP 54 and IMP 56 isolates ~~both~~ remained resistant to ertapenem after electroporation in electrocompetent cells, although there was a decrease in their MICs. IMP 54 parent cell possesses the Omp-C porin protein and the *bla*-OXA-48 gene along with the *bla*-NDM-1gene and the *bla*-CTX-M-15. On the other hand, the IMP 56 lacks the two porin proteins in parent cell and carries the *bla*-NDM-1gene exclusively. The ertapenem MIC of the IMP 54 decreased from ≥ 32µg/ml to 16µg/ml in electrocompetent recipient cell, whereas the ertapenem MIC of the IMP 56 decreased from 32µg/ml to 8µg/ml.

At a first sight, this seems contradictory because the IMP 56 has lost both of its main porins when compared to the IMP 54 and yet has a lower ertapenem MIC in electrocompetent recipient cell. A study by Khajuria*et al* (2014) on *E. coli* isolates containing either both *bla*-OXA-48 along with the *bla*-NDM-1, or *bla*-NDM-1 alone found that the presence of the *bla*-OXA-48 along with the *bla*-NDM-1confers higher

carbapenem resistance as compared to the exclusive presence of *bla*-NDM-1. Moreover, IMP 54 has in addition the ESBL CTXM-15 which confers additional increase in carbapenem resistance, denoting the effect of ESBL on increase of carbapenem resistance. This could explain why IMP 54 had a higher MIC than IMP56 both in parent and electrocompetent recipient cells regardless of the porin presence.

As to IMP 44 and IMP 53, both isolates have both porins proteins and harbor exclusively *bla*-OXA-48. However, their respective ertapenem MIC differs significantly. The IMP 44 has an MIC of 2µg/ml that remained unchanged in both parent and electrocompetent recipient cells, whereas the ertapenem of the IMP 53 has dropped from ≥ 32µg/ml parent cell to 2µg/ml in electrocompetent cell. This difference in the ertapenem MIC can be explained by the fact that the IMP 53 has a higher MIC in parent cell due to a possible over expression of the *bla*-OXA-48 compared to the IMP 44. This is possibly due to an insertion sequence resulting in the up regulation of the *bla*-OXA-48 which is present only in the IMP 53 isolate. This insertion sequence could be determined by further studies and is different than the IS*1999* detected in both isolates in a previous study done by Baroud*et al*. (2011).

With regard to IMP 44, it is resistant to cefotaxime and susceptible to ceftazidime by the disc diffusion method. In a study done by Kariuki*et al.* (2001),*K.pneumoniae*were isolated from an outbreak that happened in six newborns in Kenya. The isolates were screened to be ESBL resistant to cefotaxime and susceptible to ceftazidime and they harbored the *bla*-CTXM-12 gene that was detected for the first time then. In Bae*at al.* (2006) showed that, three ESBL *E.coli* were isolated from Korea and presented the same antimicrobial susceptibility and genetic profile as above.

Therefore, a possible presence of the *bla*-CTXM-12 gene could be an explanation of the antimicrobial profile in the IMP 44 isolate. This observation requires further studies.

In conclusion, this study demonstrated that ESBLs such as CTXM-15 and TEM-1 are plasmid encoded and confer resistance to carbapenems singly and in combination without the effect of outer membrane proteins and efflux pumps. In addition, OXA-48 and NDM-1, are plasmid encoded carbapenemases, and demonstrated to have a synergistic effect on carbapenm resistance, when harbored in the same isolates leading to a higher levels of carbapenem resistance.

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