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Whole-Genome Sequence Analysis of Carbapenem-Resistant *Enterobacteriaceae* Recovered from Hospitalized Patients in Lebanon

By

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I dedicate this work to my beloved parents.

Whole-Genome Sequence Analysis of Carbapenem-Resistant *Enterobacteriaceae* Recovered from Hospitalized Patients in Lebanon

Maria K. El Khoury

ABSTRACT

Antimicrobial resistance is one of the greatest threats to public health. Carbapenems are among the few useful antibiotics against multidrug resistant *Enterobacteriaceae*. This study aimed at characterizing the plasmid content and resistome of clinical carbapenem-resistant *Enterobacteriaceae* recovered in the period between 2016-2019 from hospitalized patients in Lebanon. The studied population included: 27 *Escherichia coli*, 24 *Klebsiella pneumoniae*, one *K. quasipneumoniae*, three *Morganella morganii*, three *Citrobacter freundii*, five *Enterobacter hormaechei*, and two *Serratia marcescens*. Antimicrobial susceptibility testing using the disk diffusion assay, carbapenemase confirmatory testing, and in silico whole-genome based analysis revealed that the majority of the isolates (87.7%; n=57) were carbapenem resistant harboring a broad range of carbapenemases. *bla*$_{\text{OXA-48}}$ (33.8%; n=22) and *bla*$_{\text{OXA-48-like}}$ genes were among the detected resistance determinants, with two isolates additionally co-harboring the *bla*$_{\text{NDM-5}}$. All OXA-48 producing isolates were ESBL and/or AmpC producers and were additionally resistant to non-β-lactam antibiotics. Carbapenem resistance determinants related to *bla*$_{\text{NDM}}$ were also detected including *bla*$_{\text{NDM-1}}$ (16.9%; n=11), *bla*$_{\text{NDM-5}}$ (9.2%; n=6), *bla*$_{\text{NDM-7}}$ (9.2%; n=6), and *bla*$_{\text{NDM-19}}$ (4.6%; n=3). A wide variety of ESBLs (OXA, SHV, TEM, CTX-M) and AmpC β-lactamases (CMY, DHA, ACT) were among the detected resistance determinants and of which *bla*$_{\text{CTX-M-15}}$ (58.5%;

n=38) and *bla*$_{\text{OXA-1}}$ (53.8%; n=35) were the most common. Our study revealed a wide variety of incompatibility groups to be associated with carbapenem resistance with the IncFIB(K) (43.1%; n=28) being the most prevalent, followed by IncFIA (40.0%), IncL (35.4%), IncX3 (32.3%), IncI1 (32.3%), IncFIIK (29.2%). It was also the largest genome-based study in the region, revealing the tremendous heterogeneity, plasmid content, and various resistance determinants circulating in the country. High-risk carbapenem-resistant Enterobacteriaceae clones pose a significant threat to patients and healthcare systems in Lebanon. The observed plethora of plasmid-encoded resistance genes shed light on implementing corrective measures to mitigate the spread of carbapenem resistance.

Keywords: carbapenem resistance, NDM, ESBL, AmpC β-lactamases, OXA-48, whole-genome sequencing, Lebanon.

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LIST OF ABBREVIATIONS

AmpC: Class C β-lactamase

AMR: Antimicrobial resistance

bla: Beta lactamase

BLAST: Basic Local Alignment Search Tool

CDC: Centers for Disease Control and Prevention

CGE: Center for Genomic Epidemiology

CLSI: Clinical & Laboratory Standard Institute

CRE: Carbapenem Resistant *Enterobacteriaceae*

CPE: Carbapenemase Producing *Enterobacteriaceae*

CTX-M: Cefotaxime resistance

DTA: deep tracheal aspirate

EDTA: Ethylenediaminetetraacetic acid

ESBL: Extended Spectrum β-lactamase

IMP: Imipenemase

Inc: Incompatibility

IS: Insertion Sequence

Kbp: Kilo-Base Pairs

KPC: *Klebsiella pneumoniae* Carbapenemase

K-type: Capsular type

LPS: Lipopolysaccharide

MBL: Metallo-β-Lactamase

MDR: Multi-drug resistant

MLST: Multi-locus sequence typing

NCBI: National Center of Biotechnology Information

NDM: New-Delhi Metallo-β-Lactamase

OmpK: Outer membrane porin protein

OXA: Oxacillin-hydrolyzing β-lactamases

PBP: Penicillin-binding proteins

PBRT: PCR-based replicon typing

PCR: Polymerase Chain reaction

PFGE: Pulsed-field gel electrophoresis

PT: Pulsotype

RAST: Rapid Annotation using Subsystem Technology

SHV: Sulfhydryl variable

TEM: Temoneria β-lactamase

Tn: Transposon

TSA: Tryptic soy agar

UTI: Urinary tract infections

WGS: Whole genome sequencing

WHO: World Health Organization

Chapter One

Introduction

1.1 Antibiotic Resistance Overview

Instead of observing a remarkable drop in bacterial diseases after the "golden era" of antibiotics, the emergence of a threatening and universal health concern has taken place throughout the years in the form of antibiotic resistance (Aslam, et al., 2018). Antibiotic resistance occurs when bacteria evolve in response to the use of antibacterial agents which become ineffective (Christaki, Marcou, & Tofarides, 2019). The first antibiotic, called penicillin, was discovered in 1928 by Alexander Fleming and recovered from *Penicillium notatum* (Fleming, 1929). Afterwards, several antibiotics were developed and classified according to their structure and degree of affinity to the target sites. These antibiotics, chemically or naturally synthesized, act through different mechanisms and interfere with the cell wall and nucleic acid synthesis, disrupt protein synthesis and metabolic pathway, and disarrange the cell membrane structure (Sultan, et al., 2018). For a growing number of infections caused by multi-drug resistant (MDR) pathogens, treatment becomes more difficult, if not impossible, with a notable increase in morbidity and mortality (Christaki, Marcou, & Tofarides, 2019). As estimated by the Centers for Disease Control and Prevention (CDC), around two million individuals are infected with antibiotic resistant microorganisms yearly in the U.S., with at least 23,000 deaths cases each year arising from these infections (CDC, 2013). It is also estimated that antibiotic resistance is accountable for 700,000 deaths per year worldwide which

could escalate by 2050 to 10 million (O'Neill, 2014). The World Health Organization (WHO) predicted that infections caused by resistant bacteria will become the foremost cause of fatality globally even ahead of cancer or cardiovascular diseases if it continues to grow at this rate (O'Neill, 2014).

1.1.1 Causes of Antimicrobial Resistance

Antibiotic resistance is the resultant of the intensive use or misuse of antimicrobial drugs in human medicine, veterinary, agriculture, and industry (Harbarth, et al., 2015). This can basically be due to unrestricted use, improper consumption, and inaccurate prescription of antibiotics (Reygaert, 2018). Various studies showed that treatment length and instructions as well as drug options were unsuitably prescribed in 30% to 50% of the cases (Lushniak, 2014). Numerous other factors also contribute to antimicrobial resistance (AMR) including negligent hygiene, overcrowding, expanded global travel and migration, selection pressure, and poor waste dumping systems (Aslam, et al., 2018).

1.1.2 Intrinsic and Acquired Resistance

Bacterial resistance to antibiotics may be due to intrinsic or acquired resistance mechanisms exhibited by bacteria (Lee, 2019). Intrinsic resistance, generally defined as a feature commonly exhibited in bacterial species, is unassociated with horizontal gene transfer but related to natural inherent properties. Multi-drug efflux pumps and decreased outer membrane permeability, mainly lipopolysaccharide (LPS), are prevalent mechanisms of induced resistance (Cox & Wright, 2013). Acquired resistance is another trait involved in the dissemination of the resistance genes mainly through horizontal

gene transfer by three main mechanisms: transformation, transduction, and conjugation. Additionally, bacteria can acquire resistance through chromosomal mutations (Holmes, et al., 2016; Munita & Arias, 2016). Mobile genetic elements such as plasmids, transposons and integrons are responsible for the spread of resistance genes (Partridge, Kwong, Firth, & Jensen, 2018). Plasmids are circular double-stranded DNA that are distinct from chromosomal DNA and replicate independently in a bacterial cell (Keyes, Lee, & Maurer, 2003). Based on the expression of replication factors, plasmids are classified into incompatibility (Inc) or plasmid replicon groups. Incompatibility refers to the inability of plasmids with the same replicon to steadily propagate in the same cell (Johnson, et al., 2007). Some of the Inc groups that are regularly detected in *Enterobacteriaceae* are: IncF, IncA/C, IncL, IncM, IncN, IncH, and IncI (Carattoli, 2009). Plasmids are mainly associated with transposons, integrons, and insertion sequences that are capable of mobilizing antibiotic resistance genes from the chromosome to the plasmid (vice versa) or within the plasmids by different mechanisms (Partridge, Kwong, Firth, & Jensen, 2018). Transposons, within the plasmids, are transposable DNA fragments that contain integrons associated with insertion sequences and encodes for multidrug resistance by expressing and excising resistance genes (Sultan, et al., 2018). Insertion sequences (IS), bounded by inverted repeats (IR), excise and paste resistance genes between the plasmids. It is made up of four classes that are associated with resistance where class 1 integron harbors the *intI1* integrase gene and gene cassettes encoding β-lactams, trimethoprim, and streptomycin resistance (Deng, et al., 2015; Sultan, et al., 2018).

1.2 Mechanisms of Resistance in *Enterobacteriaceae*

Resistance mechanisms acquired by some bacterial strains include group transfers where enzymes cause chemical substitution thus inactivating the drug, target modification where antibiotic cannot bind properly to the target site upon its modification, and via inactivation of antibiotics by redox processes or hydrolysis (enzymes), which is known as the most common mechanism (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015).

1.2.1 Extended Spectrum β-lactamase

The primary antibiotic to be delineated was the β-lactam. β-lactam resistance can be exhibited through various mechanisms linked to the mode of action of these agents (King, Sobhanifar, & Strynadka, 2017). β-lactams bind to penicillin-binding proteins (PBPs), that are responsible for the bacterial cell wall synthesis, in order to halt cell division and growth (Tipper & Strominger, 1965).The foremost effective method for microorganisms to counteract these antibiotics has been by producing β-lactamases (Jacoby & Munoz-Price, 2005). These enzymes inactivate the drugs by targeting the four-membered azetidinone ring, breaking its amide bond, and adding a water molecule to the ring-opened molecule (Bush & Bradford, 2020). β-lactamases are generally classified into two broad schemes: Ambler molecular classification scheme and Bush-Jacoby-Medieros functional classification system. The Ambler molecular classification scheme does not rely on phenotypic characterization but on amino acid similarity meaning protein homology, and it divides β-lactamases into four significant classes from A to D. β-lactamases of classes A, C, and D are serine β-lactamases, while the enzymes classified as class B are metallo- β-lactamases (Ambler, et al., 1991). The Bush-Jacoby-Medieros functional classification scheme sorts the β-lactamases based on the profile of

the substrate and the inhibitor meaning functional similarities, which is of more significant relevance to the microbiologist or physician (Bush, Jacoby, & Medeiros, 1995; Rasmussen & Bush, 1997).

In Early 1980s, the emergence of the third-generation cephalosporins antibiotics was due to the heightened prevalence of β-lactamases in some organisms such as β-lactamases causing ampicillin hydrolysis in *Escherichia coli* and *Klebsiella pneumoniae*. However, plasmid-encoded β-lactamases capable of hydrolyzing third-generation cephalosporins, especially oxyimino cephalosporins, were later discovered and then cited as Extended Spectrum β-lactamases (ESBLs) (Paterson & Bonomo, 2005). ESBLs are a group of plasmid-encoded enzymes that are capable of breaking down the β-ring of the β-lactam antibiotics belonging to the penicillin, first-, second-, third-, few fourth-generation cephalosporins, and the monobactam aztreonam, rendering them to become ineffective (Rupp & Fey, 2003). ESBLs, designated as group 2be, are usually inhibited by β-lactamase inhibitors, such as clavulanic acid, tazobactam, and sulbactam as well as newer FDA approved inhibitors like relebactam, avibactam, and vaborbactam but does not confer resistance to carbapenems or cephamycins (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015; Bush & Bradford, 2020). ESBLs carried on plasmids also harbor genes for resistance to other types of antibiotics such as aminoglycosides, trimethoprim, quinolones, tetracyclines, and sulfonamides (Paterson, 2000). The multi-drug resistant ESBL producing *Enterobacteriaceae* were linked to longer hospital stays and increased mortality (Paterson & Bonomo, 2005). ESBL producing *E. coli* and *K. pneumoniae* cause a broad spectrum of diseases ranging from colonization to life-threatening type of

infections such as hospital-acquired pneumoniae, bacteremia, and most importantly urinary tract infections (UTIs) (Chong, 2012).

1.2.1.1 Types of ESBLs

1.2.1.1.1. SHV

The SHV-type ESBLs are more often present in clinical isolates compared to other ESBL types (Jacoby, 1997). SHV refers to sulfhydryl variable and is detected in a broad range of *Enterobacteriaceae,* mostly *K. pneumonia*e. SHV-1 β-lactamase gene was originally carried on the chromosome of *K. pneumoniae* strains and was later incorporated into a plasmid and subsequently dispersed to other *Enterobacteriaceae* (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). It is accountable for around 20% of the plasmid-mediated ampicillin resistance in *K. pneumonia*e species (Tzouvelekis & Bonomo, 1999). The first SHV-type ESBL (SHV-2) was discovered in 1983 and was found in a *K. ozaenae* isolate from Germany (Knothe, Shah, Krcmery, Antal, & Mitsuhashi, 1983). SHV-2 differed from SHV-1 by replacement of glycine by serine at position 238 (G238S) (Bradford, 2001) and was capable of efficiently hydrolyzing cefotaxime and to a lesser degree ceftazidime (Knothe, Shah, Krcmery, Antal, & Mitsuhashi, 1983). *bla*$_{SHV}$ and the *bla*$_{TEM}$ subgroups were the outcome of point mutations in these genes thus causing the appearance of different subtypes (Bradford, 2001).

1.2.1.1.2. TEM

TEM-type ESBL was derived from TEM-1, the first common β-lactamase, and TEM-2. In 1965, TEM-1 was discovered in an *E. coli* isolate from a patient named

Temoneira (accordingly abbreviated as TEM) in Greece (Datta & Kontomichalou, 1965). TEM-1 has an insignificant effect against extended-spectrum cephalosporins, but it hydrolyzes ampicillin at a higher rate than oxacillin, carbenicillin, or cephalothin. TEM-13 share analogous hydrolytic profile to TEM-1 and TEM-2. These three mentioned enzymes are not considered to be in the ESBL's category (Paterson & Bonomo, 2005). However, the first TEM-type ESBL was extracted from a *K. oxytoca* that was harboring a plasmid with a gene that mediated ceftazidime resistance (Bois, Marriott, & Amyes, 1995). TEM-3, originally called CTX-1, was reported from a *K. pneumoniae* strain in 1987 and differed from TEM-2 by substitution of two amino acids (Sougakoff et al., 1988). More than 100 types of TEM with majority being ESBLs were discovered (Paterson & Bonomo, 2005).

1.2.1.1.3. CTX-M

CTX-M-type enzymes, standing for its ability to hydrolyze cefotaxime, are the most frequent ESBLs expanding worldwide and are commonly present in *E. coli and K. pneumoniae* as well as other *Enterobacteriaceae* species (Peirano & Pitout, 2019). Some CTX-M producing organisms also encoded resistance to the cephalosporin ceftazidime (MICs as high as 256 μg/ml) (Sturenburg, 2004) and cefepime with an MIC higher than that seen in any other ESBL producing bacteria (Yu, Pfaller, Winokur, & Jones, 2002). Ceftazidime is hydrolyzed at a higher rate by CTX-M-15 rather than CTX-M-3, which might be a contributing element for the dissemination of the most predominant CTX-M-15 enzyme (Poirel, 2002). CTX-M-type ESBLs are inhibited by the commercially present β-lactamase inhibitors along with the newly added inhibitors such as avibactam and vaborbactam (Drawz, Papp-Wallace, & Bonomo, 2013). They are divided into five

distinct groups according to their amino acid sequences including: CTX-M group 1, 2, 8, 9, 25 (Bonnet, 2004). CTX-M β-lactamases are close to the β-lactamases of *Kluyvera* spp. by which CTX-M-8 shares a 99% similarity in amino acid identity to extended-spectrum β-lactamase KLUG-1, the chromosomally encoded β-lactamase gene of *Kluyvera georgiana* (Poirel, Kämpfer, & Nordmann, 2002). They are acquired by horizontal gene transfer where plasmids, insertion sequences (IS*Ecp1*-like, IS*CR1*) and class 1 integron harbor the $bla_{\text{CTX-M}}$ gene and promote its mobilization (Poirel, Bonnin, & Nordmann, 2012).

1.2.1.1.4. OXA

The OXA-type β-lactamases of class D and group 2d, were commonly found in *Pseudomonas aeruginosa,* but were also identified in different Gram-negative bacteria (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). The most frequent OXA-type β-lactamase is OXA-1 that showed a high hydrolytic activity against cefepime (Castanheira, Farrell, Deshpande, Mendes, & Jones, 2013). It is prevalent among *Enterobacteriaceae* carrying $bla_{\text{CTX-M-15}}$ (Castanheira, Farrell, Deshpande, Mendes, & Jones, 2013) and has been identified in one to ten percent of *E. coli* isolates (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). OXA β-lactamases have the ability to hydrolyze oxacillin as well as cloxacillin, but most of these β-lactamases do not efficiently hydrolyze extended-spectrum cephalosporins and therefore are not ESBLs. However, OXA-10 and other OXA ESBLs, such as OXA-11 and OXA-14 to OXA-20, have the ability to confer resistance to cefotaxime and aztreonam (Paterson & Bonomo, 2005). The activities of these enzymes can be inhibited *in vitro* by sodium chloride, which is an effective *in vitro* identification method, even though they are not inhibited by the β-

lactamase inhibitors such as tazobactam, sulbactam, and clavulanic acid (Drawz & Bonomo, 2010). Furthermore, some of the β-lactamases belonging to class D were shown to confer resistance to carbapenems. OXA-23 was the first group of carbapenem-resistant OXA-type β-lactamases to be detected. Several class D β-lactamases with carbapenemase activity have been then identified including OXA-48, OXA-232, and OXA-181 (Evans & Amyes, 2014).

1.2.2 AmpC β-lactamases

AmpC β-lactamases, belonging to Ambler molecular class C, were originally located on the chromosome of several *Enterobacteriaceae* species including *E. coli*, *C. freundii* and *Enterobacter* spp., and then transferred to plasmids, thus enhancing their spread (Rupp & Fey, 2003). Plasmid-mediated AmpC β-lactamases have been globally found, in nosocomial and non-nosocomial isolates, and includes FOX-, ACC-, LAT-, MIR-, ACT-, MOX-, DHA-, and CMY- with CMY-2 having the widest geographic distribution. Both chromosomal and plasmid-mediated AmpC β-lactamases are capable of hydrolyzing penicillin, cephamycin, aztreonam and oxymino-β-cephalosporins. Unlike ESBLs, AmpC β-lactamases are resistant to the inhibition by clavulanic acid and other inhibitors but are mainly inhibited by cloxacillin and boronic acid (Jacoby, 2009). *bla*$_{\text{AmpC}}$ gene mobilization is linked to its association with insertion sequences such as IS*Ecp1* with many CMY alleles (CMY-2, CMY-14, CMY-31) as well as IS*CR1* adjacent to different CMY varieties, DHA-1, and MOX-1 (Jacoby, 2009;Toleman, Bennett, & Walsh, 2006). However, CMY-13 is surrounded by directly repeated IS*26* elements mainly composed of *tnpA* with flanking inverted terminal repeat segments (Miriagou, et al., 2004).

1.2.3 Carbapenem Resistance

The extensive prescription of carbapenems globally is on account of its potency in treating lethal infections caused by ESBL-producing organisms (Kanj & Kanafani, 2011). Carbapenems are broad-spectrum and last-line antibiotics that are classified into three groups depending on the stability and inhibitory characteristics of these β-lactam antibiotics (Papp-Wallace, Endimiani, Taracila & Bonomo, 2011; El-Gamal, et al., 2017). The main difference between carbapenems and other β-lactams is the carbon present at the fourth position of the lactam ring in place of sulfone (Kattan, Villegas & Quinn, 2008). The most clinically used carbapenems include ertapenem, imipenem, meropenem, and doripenem (Medeiros, 1997). However, there has been a worldwide increase in carbapenem-resistant *Enterobacteriaceae* (CRE) as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* causing high mortality cases ranging from 6.6% to 20% (Zhang et al., 2016; Livorsi et al., 2018). Thereafter, they have been ranked by WHO as priority 1 crtitical pathogen (Tacconelli et al., 2018). Some *Enterobacteriaceae* resist the carbapenem activity by primarily producing carbapenem-hydrolysing β-lactamases that belong to three Ambler classes and functions by discrete mechanisms where classes A and D have serine at their active cites, while class B have zinc ions at their active sites (Queenan & Bush, 2007; Öztürk, Ozkirimli & Özgür, 2015). Resistance to carbapenem may also be attributed to AmpC type enzymes, ESBLs, permeability defects, and efflux pumps (Girlich, Poirel & Nordmann, 2008). Carbapenemase genes are highly transmissible between the same or different bacterial species via mobile genetic elements (Partridge, Kwong, Firth & Jensen, 2018). CRE can also confer resistance to multiple non-β-lactam antibiotics including fluoroquinolones,

aminoglycosides, and colistin hence narrowing therapeutic options (Bratu et al., 2005; Ah, Kim & Lee, 2014). Even though CRE have initially been isolated from hospital-acquired infections, *Enterobacteriaceae* causes as well community-acquired infections, enhancing the dissemination of CRE into the community (Gupta, Limbago, Patel & Kallen, 2011).

1.2.3.1 Types of Carbapenemases

The first Class A serine carbapenemase was detected in 1982 (Yang, Wu & Livermore, 1990). A diversity of enzymes have been described. Some were chromosomally encoded and detected in clinical and environmental Gram-negative bacteria (Nordmann, Naas & Poirel, 2011), and others were plasmid-encoded and reported in clinical *Enterobacteriaceae, P. aeruginosa,* and *A. baumannii* (Naas, Dortet & I. Iorga, 2016). Class A carbapenemases consist of the following enzymes: not a metalloenzyme carbapenemase class A (NMC-A), *Serratia marsescens* enzyme (SME), and imipenem hydrolyzing (IMI), *Klebsiella pneumoniae* carbapenemase (KPC), and Guiana extended-spectrum (GES). These enzymes effectively hydrolyze all β-lactams including carbapenems and monobactams plus fluoroquinolones and aminoglycosides, but they are inhibited by clavulanic acid and tazobactam (El-Herte, Kanj, Matar & Araj, 2012). The most clinically common and globally distributed enzyme in this family is the KPC. The first acquired KPC enzyme (KPC-2) was initially discovered in 1996 in North Carolina from an isolate of *K. pneumoniae* (Yigit, et al., 2001). To date, more than 20 KPC variants were reported with KPC-2 and KPC-3 being the most commonly spread variants (Tijet, et al., 2014). Interestingly, the successful spread of the bla_{KPC} genes has been mainly due to the dissemination of *K. pneumoniae* isolates associated with ST258

that has caused several hospital outbreaks in the United States (Munoz-Price, et al., 2013). ST11 and ST101 were also reported as predominant types in causing pandemic spread of KPCs (Roe, Vazquez, Esposito, Zarrilli, & Sahl, 2019).

Class B carbapenemases, or metallo-β-lactamases (MBLs), depend on the active site zinc ions (Zn^{2+}) for breaking the β-lactam ring and hydrolyzing all β-lactams except aztreonam (Meini, Llarrull, & Vila, 2015; Walsh, Toleman, Poirel, & Nordmann, 2005). They are not hydrolyzed by the commercially available β-lactamase inhibitors, but rather inhibited by metal ion chelators such as dipicolinic acid (DPA) and ethylenediaminetetraacetic acid (EDTA) (Bush & Jacoby, 2009). The most commonly reported MBLs include Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP), and New Delhi metallo-β-lactamase (NDM) (Bush & Jacoby, 2009). IMP-1 was the first acquired MBL that was identified in 1991 in Japan from an isolate of *Serratia marcescens* and was found on a class 1 integron (Ito, et al., 1995). Subsequently, IMP and VIM showed to be endemic to Greece, Japan, and Taiwan and resulted in outbreaks and single reports in numerous other countries (Queenan & Bush, 2007; Walsh, Toleman, Poirel, & Nordmann, 2005). In 2009, an NDM-1 positive *K. pneumoniae* isolate was discovered in Sweden from an Indian patient (Yong, et al., 2009). Thereafter, the gene was endemically disseminated to all areas of India and Pakistan followed by northern Europe, Balkan states, Arabian Peninsula, Africa, and additional countries (Nordmann & Poirel, 2014). bla_{NDM} is carried on plasmids that often harbor several resistance genes including other carbapenemases genes, ESBL genes, and sulfamethoxazole resistance genes (Nordmann, Poirel, Toleman, & Walsh, 2011). Additionally, there has been a robust association with the existence of NDM-1

and aminoglycoside resistance genes (ArmA, RmtB, or RmtC 16S rRNA methylases) (Serio, Keepers, Andrews, & Krause, 2018). Twenty-four NDM variants have been discovered and spread worldwide since the first report of NDM-1 (Wu, et al., 2019). In comparison with NDM-1, variants like NDM-4, NDM-5, and NDM-7 have an elevated activity towards carbapenems (Rahman, et al., 2014).

Class D β-lactamases, also termed as oxacillinases (OXAs), constitute at least 200 enzymes with a minor selection having a carbapenemase functional activity (Nordmann, et al., 2012). Among these, OXA-48 is the most prevalent variant that was initially identified from a clinical *K. pneumoniae* isolate in Turkey in 2003 (Poirel, Héritier, Tolün, & Nordmann, 2004). It has been reported afterwards in other countries in Europe, Africa, and the Mediterranean area, but it is detected less often in the American continent (Mairi, Pantel, Sotto, Lavigne, & Touati, 2017). OXA-48 significantly hydrolyzes penicillins and imipenem, but less effectively resist meropenems and broad-spectrum cephalosporins (Poirel, Héritier, Tolün, & Nordmann, 2004). Even though OXA-48 enzymes weakly hydrolyze carbapenems, their coexistence with other mechanisms such as permeability defects, perhaps confer high-level resistance (Poirel, Bonnin, & Nordmann, 2012). OXA-181, the second most common OXA-48 derivative, also shared equivalent carbapenemase activity and was detected in *K. pneumoniae, C. freundii,* and other *Enterobacteriaceae* species. It was first recovered from India and then disseminated to France, New Zeeland, Netherland, and Oman (Sekar, Shanthi, Arunagiri , & Bramhne, 2013). Other most common OXA-48-like carbapenemases include OXA-162, OXA-204, OXA-232, and OXA-244 (Pitout, Peirano, Kock, Strydom, & Matsumura, 2019).

1.2.3.2 Other Mechanisms of Carbapenem Resistance

Apart from carbapenemase production, Gram-negative bacteria have alternative mechanisms that enable them to possess the carbapenem resistance phenotype (Krishnappa, Marie, & Al Sheikh, 2015). Primarily, a deletion or reduction in porin channels, such as OmpK35 and OmpK36, due to the loss of expression of the genes encoding porins, and their association with the upregulated production of ESBLs or AmpC β-lactamases, may lead to carbapenem resistance (Nordmann, Doret, & Poirel, 2012). Mutation is a form of porin regulation that might result either in total depletion of a porin due to premature truncation or an inefficient one (Krishnappa, Marie, & Al Sheikh, 2015). A less frequent non-enzymatic carbapenem resistance mechanism might involve defects in efflux pumps causing their overexpression. In turn, the upregulation of efflux pump, such as the AcrAB-ToIC pump found in *Enterobacteriaceae*, induce the export of carbapenems from the bacterial cell (Baroud et al., 2012). They are encoded by the following genes: *OqxAB, AcrAB*, and *MdtABC* (Nordmann, Doret, & Poirel, 2012; Li & Nikaido, 2009). Both overexpression of efflux pumps and porin loss coupled with carbapenem resistance may also confer resistance to several antibiotic groups including other β-lactams (Moyá, et al., 2012). Another rare carbapenem resistance mechanism observed in *E. coli* isolates involve modifications or mutations that disrupt the binding affinity of PBPs (Yamachika, Sugihara, Kamai, & Yamashita, 2013).

1.2.3.3 Plasmids Associated with Carbapenemases

IncF plasmids are mostly found to contain MDR regions including $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, and aac(6′)-Ib-cr, in addition to aminoglycoside and tetracycline resistance genes (Coque, Baquero, & Cantón, 2008). Currently, several studies have

been concentrating on these plasmids in taking part in the dissemination of carbapenemases mainly NDM and KPC variants in *K. pneumoniae* and *E. coli* isolates (Huang, et al., 2013; Pitart et al., 2014; Yoon et al., 2018). A 73.6 kb plasmid (pBK30661) of IncFIA replicon group carried $bla_{\text{KPC-3}}$ in *K. pneumoniae* with eight other antibiotic resistance genes such as $bla_{\text{TEM-1}}$, $bla_{\text{OXA-9}}$, *aacA4*, *aadA1*, *strA*, *strB*, *sul2*, and *dfrA14* (Chen et al., 2014). Moreover, IncFIB and IncFII plasmids carrying NDM variants in *K. pneumoniae* and *E. coli* respectively have been described since 2012 (Huang, et al., 2013; Pitart et al., 2014; Sugawara et al., 2017). However, IncX3 type plasmids are predominantly reported to carry $bla_{\text{OXA-181}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{NDM-5}}$ carbapenemase genes in *Enterobacteriaceae* (Kopotsa, Osei Sekyere & Mbelle, 2019). In China, an IncX plasmid co-harbored $bla_{\text{NDM-5}}$ and *mcr-1* an *E. coli* isolate (Zhang et al., 2017). Also, other NDM variants including $bla_{\text{NDM-4}}$ and $bla_{\text{NDM-7}}$ were found on IncX3 plasmids according to a study done in Myanmar (Sugawara et al., 2017). The dissemination of $bla_{\text{OXA-48}}$ was mainly associated with the spread of IncL/M within Tn*1999* and did not carry any other resistance genes on the same plasmid (Bonnin, Nordmann, Carattoli, & Poirel, 2012; Beyrouthy, et al., 2014). The IncA/C plasmid type are generally correlated with the spread of bla_{CMY} cephalosporinases and bla_{NDM} (Carattoli, Villa, Poirel, Bonnin & Nordmann, 2011). Other carbapenemase genes such as bla_{IMP} and bla_{VIM} are found on the broad host range IncN plasmids (Kopotsa, Osei Sekyere & Mbelle, 2019).

1.3 CRE: a rising health problem

Carbapenem resistance mostly appears in hospital settings among the *Enterobacteriaceae* family which is a large family of Gram-negative, non-spore forming

rod-shaped, and facultative anaerobic bacteria (Nordmann, Doret, & Poirel, 2012). It consists of microorganisms of different genera such as *Enterobacter, Escherichia, Klebsiella, Proteus, Salmonella, Shigella, Citrobacter, Yersinia,* and *Serratia* (Farmer, Farmer, & Holmes, 2010). CDC have declared *Enterobacteriaceae* as a high-risk factor contributing in more than 21.3% of hospital-associated infections (Hidron, et al., 2008). Listed as critical priority group, CRE cause a wide range of severe infections like complex urinary tract and intra-abdominal infections, ventilator associated pneumonia, as well as bloodstream infections (Suay-García & Pérez-Gracia, 2019). Fatality rates correlated to CRE infections have reached around 44% which revealed the significance of these bacteria (Correa & Fortaleza, 2019). They are able to grow and survive even in the presence of clinically relevant concentrations of carbapenems (Durante-Mangoni, Andini, & Zampino, 2019). Among *Enterobacteriaceae*, the predominant CRE is *K. pneumoniae*, which counts for around 60%, followed by *E. coli* and *E. cloacae* (Zhang, et al., 2018; Grundmann, et al., 2017). According to the estimations performed by the CDC, they showed that carbapenem-resistant *Klebsiella* and *Escherichia* species are responsible for as much as 9000 healthcare-acquired infections annually in the U.S. (CDC, 2013). Based on data published between 2009 and 2010 by the National Healthcare Safety Network (NHSN), it revealed that central line-associated bloodstream infections (CLABSIs) or catheter-associated urinary tract infections (CAUTIs), reported in 20% of hospitals were linked to carbapenem-resistant *Klebsiella* spp. (Sievert, et al., 2013). Patel et al. showed that death rates reached 40% to 50% in patients with bloodstream carbapenem-resistant *K. pneumoniae* infection (Patel, Huprikar, Factor, Jenkins, & Calfee, 2008).

1.4 ESBLs and Carbapenem Resistance in Lebanon

Resistant strains of *Enterobacteriaceae* are continuously being recorded worldwide. Borg et al. have revealed that the emergence of resistant pathogens in the Mediterranean area is highly correlated to the extensive consumption of antimicrobial agents (Borg, Zarb, Ferech, & Goossens, 2008). Various studies have reported the distressing rates of exploitation and mishandling of antibiotics, especially in Lebanon (Kanafani, Mehiosibai, Araj, Kanaan, & Kanj, 2005; Daoud, Moubareck, Hakime, & Doucet-Populaire, 2006). There has been a drastic expansion in the frequency of infections caused by ESBL producing organisms in Lebanon (Moubareck, et al., 2005). In 1994, a study performed at the American University of Beirut Medical Center (AUBMC) revealed that as high as 65% *E. coli* isolates were ampicillin resistant (Araj, Uwaydah, & Alami, 1994), which increased to become 72% after ten years (Araj & Ibrahim, 2008). A four year study done at the same hospital showed a rise in the rate of ESBL producing *E. coli* from 3% to 5% as well as an increase in *K. pneumoniae* from 6.4% to 13% (Samaha-Kfoury & Araj, 2003). Additionally, ESBL positive strains, particularly *E. coli* (40%) and *K. pneumoniae* (33%), were detected in patients with ventilator-associated pneumonia also at AUBMC (Kanafani, Kara, Hayek, & Kanj, 2003). Furthermore, in 2006, a study aimed to examine the intestinal carriage of ESBL producing strains of patients in the intensive care units (ICU) of five Lebanese healthcare facilities. They revealed that 80.5% of *E. coli*, 13.6% of *K. pneumonia*e, and 5.6% *E. cloacae* were recognized as ESBL producers (Daoud, Moubareck, Hakime, & Doucet-Populaire, 2006). However, this alarming rate increase led to an instant attention because of the danger they inflicted on hospitalized patients (Salem, Dahdouh, & Daoud,

2013). Moreover, a study conducted in Saint George Hospital in Beirut over a period of nine years (2000-2009) showed an increase in ESBL positive organisms isolated from Urinary Tract Infections (UTI) from 2.3% to 16.8% (Daoud & Afif, 2011). Nevertheless, and according to molecular analysis, several studies suggested that the most prevalent β-lactamase gene in hospital settings in Lebanon as well as in poultry and cattle is the CTX-M-15 (Salem, Dahdouh, & Daoud, 2013; Dandachi, Fayad, El-Bazzal, Daoud, & Rolain, 2019). Matar et al. also showed that 2.5% of *E. coli* and 7.84% of *K. pneumoniae* were carbapenem resistant and ESBL producers (Matar, et al., 2010). More recently, ESBL production rate in *E. coli* was reported to be as high as 32.3% and 29.25% for *Klebsiella* spp. (Chamoun, et al., 2016), whereas carbapenem susceptibility reached 70% and 12% in isolates recovered from Lebanon during 2015-2016 (Moghnieh, et al., 2019). The appearance of carbapenem-resistant *Enterobacteriaceae* in Lebanon and adjacent countries is a threat to public health (El-Herte, Kanj, Matar & Araj, 2012). The first carbapenemase in Lebanon was detected in 2007 in a *K. pneumoniae* isolate that carried $bla_{\text{IMP-1}}$ and $bla_{\text{CTX-M}}$ genes (Daoud, Hobeika, Choucair, & Rohban, 2008). Later on, Lebanon's first OXA-48 (class D) report was in 2008 detected in *K. pneumoniae* (Matar, et al., 2008) followed by another report in 2010 revealing the presence of $bla_{\text{OXA-48}}$ in *E. coli* and *K. pneumoniae* (Matar, et al., 2010). On the other hand, a study, done at Nini Hospital in North Lebanon between 2008 and 2012, aimed to investigate the carbapenem resistance profile of *Enterobacteriaceae* isolates recovered from hospitalized patients. An increase in ertapenem resistance from 0.4% (2008-2010) to 1.6% (2012) was detected (Beyrouthy, et al., 2014). In addition, an elevation in carbapenem resistance has been observed in *E. coli* and *Klebsiella* spp. from

0.8 and 2% (2011–2013) to 3 and 4% (2015–2016) respectively, based on data retrieved from 13 hospital laboratories in Lebanon (Chamoun, et al., 2016). Arabaghian et al. did a whole-genome-based characterization of 34 carbapenem resistant *Klebsiella* spp. recovered from clinical samples in a Lebanese Hospital between 2012 and 2015. All of the isolates were resistant to ertapenem with 61.8% and 17.6 % harboring the OXA-48 and NDM carbapenemases, respectively (Arabaghian, et al., 2019). The notable increase in carbapenem resistant *Enterobacteriaceae* in Lebanon and other countries impose a serious threat that requires an instant action (El-Herte, Kanj, Matar & Araj, 2012).

1.5 Treatment

Different strategies are followed to treat infections caused by CRE. Limited preexisting antibiotics such as fosfomycin, tigecycline, and colistin are still useful against CRE (Kanj & Kanafani, 2011). Fosfomycin is still effectual against 80% of CRE with urinary tract infections (UTIs) (Vardakas, Legakis, Triarides, & Falagas, 2016). High doses of tigecycline are used to treat ventilator-associated pneumonia (Pascale, et al., 2014). Colistin remains the most common drug used in treating CRE, especially *K. pneumoniae* harboring carbapenemases, but is also considered as the last resort drug due to its side effects including respiratory conditions, neurotoxicity, and nephrotoxicity. However, monotherapy revealed to have a reduced efficiency because of resistance development (Sader, Castanheira, Duncan, & Flamm, 2018). Therefore, dual therapy with another carbapenem (meropenem) is applied and which exhibited a decrease in mortality rates (Daikos, et al., 2014). Also, new combinations of β-lactam/β-lactamase inhibitor, for example ceftazidime/avibactam and meropenem/vaborbactam, have been developed to treat CRE infections (Suay-García & Pérez-Gracia, 2019).

Objectives of the Study

The aim of this study was to characterize 65 multi-drug resistant (MDR) *Enterobacteriaceae* isolates, where 58 of the isolates were carbapenem resistant, and recovered in the period between 2016-2019 from hospitalized patients at a large tertiary hospital in Lebanon. Whole-genome sequencing (WGS) was used to identify and investigate the resistant determinants present in the collected isolates. It was also used to study in details the plasmid contents and their incompatibility groups in addition to determining the genetic profiles of the carbapenemase genes and the flanking mobile genetic elements (MGEs) (transposons and insertion sequences). Thus, the interplay between the resistance genes, plasmids, and strains have been determined. Typing and clonal relatedness were also investigated as well as the spread of the CRE in Lebanon.

Chapter Two

Materials and Methods

2.1. Bacterial Isolates

A total of 27 *E. coli*, 24 *K. pneumoniae*, one *K. quasipneumoniae*, three *M. morganii*, three *C. freundii*, five *E. hormaechei*, and two *S. marcescens* were recovered from hospitalized patients at the MGH (Makassed General Hospital) between 2016 and 2019. *E. coli* isolates were designated as EC1-EC27, *K. pneumoniae* as KP1-KP24, *K. quasipneumoniae* as KQ1, *M. morganii* as MM1-MM3, *C. freundii* as CF1-CF3, *E. hormaechei* as EH1-EH5, and *S. marcescens* as SM1-SM2. The isolates were gathered from multiple body sites including sputum, urine, blood, wound, fluid, and deep tracheal aspirate (DTA). The mean age of patients was 62 years old, ranging from 1 month to 94 years with 40.3% of the patients being males (Table 1). Isolates were first identified to the species level using API20E kits (bioMeÂrieux, Marcy l'EÂtoile, France) and following the manufacturer's instructions.

Table 1: Bacterial isolates, source of isolation, age, and gender. Mean pateints' age and age range, gender, collection sites of the isolates, and department. Ur, urine; Wd, wound; Fl, fluid; Bl, blood; Sp, sputum; DTA, deep tracheal aspirate; ISO; isolation room; ICU, intensive care unit; OUT, outpatient treatment unit; SI, surgery; SVI, internal medicine and surgery; NI, internal medicine and isolation; NII, internal medicine (North 2); NV, hematology and oncology; NVI, internal medicine and surgery. Others include pediatric Hemato-Oncology unit, pediatric intensive care unit, obstetrics and gynecology.

Parameter	Category	Value
Age		
	Mean	62
	Range	1 month-94 years
Gender [no. (%)]		
	Male	25 (40.3)
	Female	37 (59.7)
Collection site [no. (%)]		
	Ur	27 (43.5)
	Wd	16 (25.8)
	Fl	4 (6.5)
	Bl	5 (8.0)
	Sp	4 (6.5)
	DTA	6 (9.7)
Department [no. (%)]		
	ISO	7 (11.3)
	ICU	2 (3.2)
	OUT	6 (9.7)
	SI	4 (6.4)
	SVI	5 (8.1)
	NI	11 (17.7)
	NII	5 (8.1)
	NV	6 (9.7)
	NVI	7 (11.3)
	Others	9 (14.5)

2.2. Antimicrobial Susceptibility Testing

All 65 isolates classified as being members of the family *Enterobacteriaceae* were tested for resistance to antibiotics through the disk agar diffusion assay using Mueller–Hinton agar plates against 11 different antibiotic disks belonging to six drug categories: carbapenems (ertapenem, imipenem, meropenem), monobactam (aztreonam), quinolones (norfloxacin, ciprofloxacin), aminoglycosides (tobramycin, amikacin, gentamicin), tetracycline, and sulfonamides (trimethoprim/sulfamethoxazole). The

inhibition zone diameters for each antimicrobial agent were measured and interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017).

2.3. Confirmation of Carbapenemase Activity

Colonies were grown overnight on TSA agar plates. Inhibitor-based phenotypic test was performed on Mueller–Hinton agar plates with EDTA (Lee, Lim, Yong, Yum, & Chong, 2003), phenylboronic acid (Doi et al., 2008), and temocillin disc assays (Glupczynski et al., 2012) were used to test for the production of different carbapenemases (MBLs, OXA-48, and KPC).

2.4. Bacterial DNA extraction

Bacterial DNA purification and extraction was then performed using NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) in accordance to the manufacturer's instructions. The extracted DNA was utilized for PCR assays and whole-genome sequencing.

2.5. PCR assays and sequencing-ESBL and Carbapenemase Gene Detection

ESBL and carbapenemase encoding genes namely bla_{OXA} and bla_{NDM} were screened, amplified and sequenced as previously described (Lim, Yasin, Yeo, Puthucheary, & Thong, 2009; Poirel, Walsh, Cuvillier, & Nordmann, 2011). Primers used are listed in Table 2.

Table 2: Resistance genes amplification, their corresponding primer sequences and amplicon sizes.

Target	Primer	Sequence 5'-3'	Amplicon size (bp)
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621
	NDM-R	CGGAATGGCTCATCACGATC	
bla$_{OXA}$	OXA-F	ACACAATACATATCAACTTCGC	814
	OXA-R	AGTGTGTTTAGAATGGTGATC	

2.6. PFGE fingerprinting

Pulse Field Gel Electrophoresis (PFGE) typing and determination of genetic relatedness was performed for *E.coli* and *K. pneumoniae*; the two most recovered isolates in this study.The restriction enzyme *Xba*I (ThermoScientific, Waltham, MA, USA) was used. 1% SeaKem agarose gel and *Salmonella enterica* susbp. enterica serovar Braenderup (ATCC BAA664TM) was used as the universal laboratory standard and isolates were processed according to the standard PulseNet protocol (http:/www.pulsenetinternational.org). Electrophoresis was performed using the Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA). The gels were stained using ethidium bromide and de-stained before visualization. The PFGE profiles were later analyzed using the BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and pulsotypes were clustered through dice correlation coefficients with an optimization of 1.5% and tolerance of 1.5%. Fingerprints with three or more different bands were assigned a different pulsotype (Tenover et al., 1995).

2.7. Plasmid replicon typing

Plasmid identification was performed for all 65 isolates using the DIATHEVA PCR-Based Replicon Typing (PBRT) kit (Diatheva, Fano, Italy). The PBRT method uses eight multiplex PCRs along with positive controls for all reactions targeting the 28 plasmid replicon groups found in the family *Enterobacteriaceae*: FIA, FIB, FIC, FII, FIIS, FIIK, HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, A/C, T, K, HIB-M, FIB-M, W, Y, P, B/O, U, R. All PCR reactions were performed according to the manufacturer's instructions and visualized on a 2.5% agarose gel stained with ethidium bromide.

2.8. Whole-genome sequencing

Genomic DNA (gDNA) was used as input for library preparation using the Illumina Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). The kit was used to simultaneously fragment and tag the library, as per the manufacturer's instruction. The library was normalized by bead-based affinity and then sequenced using the MiSeq version 3 600-cycle kit (Illumina) to perform 300 bp paired-end sequencing on the MiSeq instrument (Illumina), according to the manufacturer's instructions. Genome assembly was performed *de novo* using Spades Genome Assembler Version 3.6.0 (Bankevich et al., 2012). Quality control checks on the obtained raw sequence data was performed using FastQC version 1.0.0 (Andrews, 2010).

2.9. Genome assembly and annotation

RAST server (http://rast.nmpdr.org) was used for genome annotation. The SEED Servers offer open access platforms to regularly updated data and annotate prokaryotic genomes (Aziz et al., 2012). Goseqit (https://www.goseqit.com) and ResFinder 3.0 (Zankari et al., 2012) available on the Center for Genomic Epidemiology website

(www.genomicepidemiology.org) were used to detect resistance genes. *in silico* Multi-locus sequence typing (MLST) was performed on all whole-genome sequenced isolates using MLST2.0 on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) (Larsen et al., 2012). The identification of *E. coli* serotypes was done using SerotypeFinder2.0. (Joensen, Tetzschner, Iguchi, Aarestrup, & Scheutz, 2015). PhylotypeFinder accessible on Goseqit (https://www.goseqit.com) was also used to detect *E. coli* phylogenetic groups. Potential K-types of *K. pneumoniae* were inferred with the Kaptive online tool which analyses the entire *cps* locus and assigns capsular locus types (https://github.com/katholt/Kaptive) (Wyres et al., 2016).

2.10. Plasmid Finders

The presence of plasmids in the genomic sequences was determined using PlasmidFinder v.2.1. (Carattoli et al., 2014). PlasmidSPAdes version 3.12.0 was utilized to capture the plasmids and assemble them into separate contigs (Antipov et al., 2016). The output is then visualized through Bandage (Wick, Schultz, Zobel, & Holt, 2015). Plasmid sequences were extracted and aligned with corresponding reference strains using multiple genome alignment tools namely Mauve v2.4.0 (Darling, 2004) and BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Annotation of the plasmid sequence was manually curated, and the complete circularized sequence was visualized using the GenomeVx tool (Conant & Wolfe, 2008). EasyFig, a genome comparison visualizer tool, was also applied in order to generate linear comparison figures (Sullivan, Petty, & Beatson, 2011). IS-finder was used to identify insertion sequences (ISs) and IS-families (Siguier, Perochon, Lestrade, Mahillon & Chandler, 2006).

2.11. Pan Genome Analysis

E. coli and *K. pneumoniae* genomes were annotated using Prokka version 1.13. with a similarity cutoff e-value 10^{-6} and minimum contig size of 200 bp (Seemann, 2014). Annotated GFF3 files were piped into the rapid large-scale prokaryote pan genome analysis (Roary) pipeline version 3.12.0. by choosing a minimum blastp identity of 95 and core gene prevalence in all (>99%) of the isolates (Page et al., 2015). The following command flags were used in pan-genome analysis: *roary -f outdir -e --mafft* 27 *-v *.gff*. A maximum-likelihood phylogenetic tree based on the core genome alignment was visualized using the web-based tool Phandango (Hadfield et al., 2017).

Chapter Three

Results

3.1. Antimicrobial susceptibility testing

Disk diffusion assay was used to screen for carbapenem resistance. Results obtained showed that 76.9% (n= 50) and 10.8% (n= 7) of the isolates were either resistant or intermediate resistant to ertapenem respectively. Less resistance was detected against meropenem and imipenem where 46.2% (n=30) and 23.1% (n=15) of the isolates showed resistance and intermediate resistance to meropenem respectively, while 44.6% (n=29) and 23.1% (n=15) of the isolates were resistant or intermediate resistant to imipenem respectively. It is noteworthy that the studied isolates also showed high level of resistance to aztreonam (n=39, 60%), norfloxacin (n=51, 78.5%), tobramycin (n=28, 43.1 %;), trimethoprim/sulfamethoxazole (n=43, 66.2%), tetracycline (n=44, 67.7%), ciprofloxacin (n=50, 76.9%), gentamicin (n=27, 41.5%), and amikacin (n=12, 18.5%) (Table 3).

Table 3: Antimicrobial susceptibility testing results based on a sample of 65 sequenced isolates using 11 different antibiotics.

		Disk Agar Diffusion Test										
Isolate number #	Species	ERTAPENEM	MEROPENEM	IMIPENEM	AZTREONAM	NORFLOXACIN	TOBRAMYCIN	Sulfamethoxazol+Trimethroprim	AMIKACIN	GENTAMICIN	TETRACYCLINE	CIPROFLOXACIN
KP1	*K. pneumoniae*	R	I	R	S	I	I	R	S	R	R	I
KP2	*K. pneumoniae*	R	R	R	R	R	R	R	S	R	R	R
EC1	*E.coli*	R	I	I	R	R	S	S	S	S	S	R
CF1	*C. freundii*	R	I	R	R	R	R	S	R	R	S	R
EC2	*E.coli*	S	S	S	R	R	R	R	S	R	R	R
EC3	*E.coli*	R	S	S	R	R	S	S	S	S	S	R
EC4	*E.coli*	I	S	S	R	R	I	R	S	R	R	R
EC5	*E.coli*	R	S	I	R	R	S	R	S	S	R	R
EC6	*E. coli*	R	S	S	R	R	S	S	S	S	S	R
EC7	*E.coli*	I	S	S	R	R	S	R	S	S	R	R
EH1	*E. hormaechei*	R	R	R	I	R	R	R	S	R	R	I
KP3	*K. pneumoniae*	R	R	R	R	R	R	R	R	R	R	I
KP4	*K. pneumoniae*	R	I	S	R	R	R	I	S	R	S	R
KP5	*K. pneumoniae*	R	R	R	R	R	R	R	R	R	S	R
SM1	*S. marcescens*	R	R	R	S	S	S	S	S	S	I	S
EC8	*E.coli*	R	I	I	R	R	S	R	S	S	R	R
KP6	*K. pneumoniae*	R	R	R	R	R	I	S	S	S	R	R
EC9	*E.coli*	R	S	S	R	R	R	R	S	R	R	R
EC10	*E.coli*	R	S	S	R	R	R	R	S	R	R	R
KP7	*K. pneumoniae*	R	R	I	S	R	I	R	S	S	S	R
KP8	*K. pneumoniae*	R	R	I	R	R	R	R	S	S	R	R
EC11	*E.coli*	R	S	S	R	R	R	R	S	S	R	R
KP9	*K. pneumoniae*	R	R	R	S	S	S	S	S	R	R	S
EC12	*E.coli*	R	S	S	R	R	S	S	S	S	S	R
EC13	*E.coli*	R	R	R	R	R	S	S	S	S	R	R
EC14	*E.coli*	R	R	R	S	R	S	S	S	S	R	R
EC15	*E.coli*	I	S	S	R	R	S	S	S	R	R	R
EC16	*E.coli*	R	R	R	R	R	S	R	S	S	R	R
KP10	*K. pneumoniae*	R	R	R	I	R	R	R	S	S	R	R
KP11	*K. pneumoniae*	R	R	R	R	R	R	S	R	S	S	R
KQ1	*K. quasipneumoniae*	R	I	R	R	I	R	R	S	R	R	R
EC17	*E.coli*	S	S	S	R	R	R	R	S	S	S	R
KP12	*K. pneumoniae*	S	S	S	S	S	S	R	S	S	S	S
EC18	*E.coli*	R	R	R	S	R	R	R	R	R	R	R
KP13	*K. pneumoniae*	R	R	R	I	R	I	R	S	S	R	R
EC19	*E.coli*	R	R	R	S	R	I	R	S	R	R	R
EC20	*E.coli*	R	R	R	R	R	S	S	S	S	R	R
KP14	*K. pneumoniae*	R	R	R	I	R	I	R	S	S	R	R
KP15	*K. pneumoniae*	R	R	R	R	R	R	R	S	S	R	R
KP16	*K. pneumoniae*	R	R	R	R	R	I	R	S	S	R	R
KP17	*K. pneumoniae*	R	R	R	R	R	S	S	R	R	S	R
EC21	*E.coli*	R	R	R	R	R	S	R	S	S	S	R
EC22	*E.coli*	R	R	R	S	R	S	S	S	S	S	R
EC23	*E.coli*	R	I	I	R	R	S	S	S	S	S	R
KP18	*K. pneumoniae*	S	S	S	S	R	S	R	S	S	S	R
MM1	*M. morganii*	S	S	S	S	R	S	R	S	S	R	R
KP19	*K. pneumoniae*	I	S	S	I	I	R	R	S	R	R	I
SM2	*S. marcescens*	I	S	S	S	S	S	S	S	S	R	S
EC24	*E.coli*	R	I	I	R	R	R	R	S	S	S	R
EH2	*E. hormaechei*	R	I	I	I	R	R	R	R	R	R	R
KP20	*K. pneumoniae*	S	I	S	R	R	R	R	R	R	R	R
KP21	*K. pneumoniae*	I	S	I	R	S	S	R	S	R	R	S
EH3	*E. hormaechei*	R	I	I	I	S	R	R	S	R	R	S
CF2	*C. freundii*	R	R	R	S	S	R	S	R	R	S	S
CF3	*C. freundii*	R	R	I	S	I	I	R	S	S	R	S
EC25	*E.coli*	R	R	R	R	R	R	R	R	R	R	R
EH4	*E. hormaechei*	I	I	I	S	S	S	S	S	S	S	S
KP22	*K. pneumoniae*	R	R	I	R	R	S	R	R	S	R	R
MM2	*M. morganii*	S	S	S	S	S	S	S	S	S	R	S
EC26	*E.coli*	R	I	S	R	R	R	R	S	S	R	R
EC27	*E.coli*	R	R	R	R	R	R	R	R	R	R	R
KP23	*K. pneumoniae*	R	R	R	R	R	S	S	S	S	S	R
MM3	*M. morganii*	S	S	S	S	S	S	R	S	R	R	S
KP24	*K. pneumoniae*	R	I	I	I	R	R	R	S	S	R	R
EH5	*E. hormaechei*	R	I	I	S	R	R	R	S	R	R	R

Resistant
Intermediate
Sensitive

Dark blue: resistant; light blue: intermediate; white: sensitive.

3.2. Resistance Genes

in silico resistome analysis using ResFinder and Goseqit as well as PCR assays targeting the resistance genes (*bla*$_{NDM}$ and *bla*$_{OXA}$) revealed that the majority of the isolates (87.7%; n= 57) harbored at least one carbapenemase gene. The most common carbapenemase was *bla*$_{OXA-48}$ detected in 22/65 (33.8%) of the isolates. Other isolates carried *bla*$_{OXA-48-like}$ genes such as *bla*$_{OXA-181}$ (*K. pneumoniae* n=1 and *E. coli* n= 7) and *bla*$_{OXA-244}$ (*E. coli* n=3). Interestingly, one *K. pneumoniae* isolate co-carried *bla*$_{NDM-5}$ and *bla*$_{OXA-48}$, while an *E. coli* isolate was positive for *bla*$_{NDM-5}$ and *bla*$_{OXA-181}$. Other carbapenem resistance determinants related to *bla*$_{NDM}$ were also detected such as: *bla*$_{NDM-1}$ (16.9%; n=11), *bla*$_{NDM-5}$ (9.2%; n=6), *bla*$_{NDM-7}$ (9.2%; n=6), and *bla*$_{NDM-19}$ (4.6%; n=3). A variety of β-lactamases were also present including *bla*$_{CTX-M}$ (72.3%), *bla*$_{OXA}$ (61.5%), *bla*$_{TEM}$ (50.8%), *bla*$_{SHV}$ (36.9%), *bla*$_{CMY}$ (26.2%), *bla*$_{ACT}$ (7.7%), *bla*$_{DHA}$ (10.8%), *bla*$_{LEN}$ (3.1%), *bla*$_{SRT}$ (1.5%), *bla*$_{SST}$ (1.5%), and *bla*$_{OKP-B1}$ (1.5%) (Table 4 and 5). *bla*$_{CTX-M-15}$ was the most frequently identified ESBL variant (n=38/65; 58.5%) followed by *bla*$_{OXA-1}$ (n=35/65; 53.8%). Six variants of the *bla*$_{CMY}$ (*bla*$_{CMY-2, 6, 42, 48, 75, 150}$) were detected with *bla*$_{CMY-2}$ and *bla*$_{CMY-42}$ being the most common AmpC β-lactamases.

in silico resistome analysis also revealed the presence of 64 distinct genes with each conferring resistance to one of the following ten antimicrobial agents: aminoglycosides, quinolones, Fosfomycin, macrolides, lincosamides, chloramphenicol, sulphonamides, tetracyclines, trimethoprim, and rifampicin (Tables 6 and 7). Among the 21 identified aminoglycoside resistance genes, *aac(6')-Ib-cr* (50.8%; n=33/65) was the

most common, followed by *aph(3'')-Ib* (27.7%; n=18/65), *aph(3')-Ia* (27.7%; n=18/65), *aph(6)-Id* (26.2%; n=17/65), and *aac(3)-Iia* (26.2%; n=17/65). The majority of the isolates (67.7%; n=44/65) harbored more than one aminoglycoside resistance gene.

Four different Fosfomycin resistance genes were detected with *fosA* being the most common and was found in 44.6% (n=29/65) of the isolates. It is noteworthy that EC1, EC19, and KP19 harbored *fosA3* (n=1/65), *fosA4* (n=1/65), and *fosA5* (n=1/65) respectively.

The core chromosomal genes, *oqxA* and *oqxB*, conferring low-level resistance to quinolones, were present in approximately 46.2% (n=30/65) of the isolates, with 96.7% (n=29/30) carrying at least one additional quinolone resistance determinant mainly *aac(6')-Ib-cr*, *qnrS1*, and/or *qnrB1*.

Six genes were identified that are related to macrolide resistance. *mdf(A)* was the most common and was found in sixty of the studied isolates (92.3%). The remaining macrolide resistance genes include *mph(A)*, *mph(E)*, *msr(E)*, *erm(B)*, *ere(A)*. Interestingly, EC19 was the only isolate that harbored the *lnu(F)* gene conferring resistance to lincosamides.

catB3 was the most frequent chloramphenicol resistance encoding gene identified (52.3%; 34/65) among the other four (*catA1*, *catA2*, *cmlA1*, and *floR*). For trimethoprim, six *dfr* variants (*drfA1, dfrA12, dfrA14, dfrA15, dfrA17 and dfrA27*) were detected with *dfrA14* and *dfrA17* being the most common variant (24.6%; n=16/65). However, *tet(A)*, present in 38.5% (n=25/65) of the isolates, was the most common tetracycline resistance encoding gene (*tet(B), tet(A), tet(D)* and *tet(41)*).

Sulphonamides and rifampicin on the other hand, showed little variability with only three variants for sulphonamides (*sul1*, *sul2*, and *sul3*) and two variants for rifampicin (*arr-2* and *arr-3*) being detected. *sul1* (64.6%; n=42/65) and *arr-3* (10.8%; n=7/65) were the most common for each antimicrobial agent, respectively.

One *K. pneumoniae* isolate KP4 of MLST sequence type ST147 showing resistance to ertapenem and intermediate resistance to meropenem based on disk diffusion assay, did not carry detectable carbapenemase-encoding genes. Major porins, OmpK35 and OmpK36, were then analyzed using reference genomes (accession # MG197742.1 and KY086537.1) downloaded from NCBI. We didn't detect any non-synonymous mutation and both genes, *ompK35* and *ompK36*, looked intact in KP4. Further investigation revealed that the isolate carried a number of ESBLs ($bla_{\text{CTXM-15}}$, $bla_{\text{SHV-67}}$, $bla_{\text{OXA-1}}$) and multi-drug efflux pumps (EmrAB-OMF, AcrAB-TolC, MdtABC-TolC, AcrEF-TolC, and MexAB), which could be involved in the effective export of carbapenems from the bacterial cell.

Table 4: MLST of the *E. coli*, *E. hormaechei*, and *C.freundii* isolates and their resistance profiles including carbapenemase encoding and β-lactam resistance genes.

	ST	Isolate number #	Carbapenemase genes	β-lactam resistance genes
***E. coli* (n=27)**	ST648	EC6	*bla*$_{OXA-244}$	*bla*$_{CMY-42}$
		EC15	*bla*$_{OXA-244}$	*bla*$_{CTX-M-15}$; *bla*$_{TEM-1B}$
		EC23	*bla*$_{OXA-244}$	*bla*$_{CMY-42}$
		EC27	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-10}$; *bla*$_{OXA-1}$; *bla*$_{CMY-42}$
	ST405	EC2	-	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
		EC17	-	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$
		EC25	*bla*$_{NDM-5}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
	ST410	EC5	*bla*$_{OXA-181}$	*bla*$_{CMY-2}$
		EC9	*bla*$_{OXA-181}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{CMY-2}$; *bla*$_{TEM-1B}$
		EC10	*bla*$_{OXA-181}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{CMY-2}$; *bla*$_{TEM-1B}$
		EC11	*bla*$_{OXA-181}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{CMY-2}$; *bla*$_{TEM-1B}$
		EC12	*bla*$_{OXA-181}$	*bla*$_{CMY-42}$
	ST167	EC16	*bla*$_{NDM-5}$	*bla*$_{CTX-M-3}$
		EC19	*bla*$_{NDM-5}$	*bla*$_{CMY-2}$; *bla*$_{TEM-176}$
		EC20	*bla*$_{NDM-19}$	*bla*$_{CTX-M-15}$; *bla*$_{TEM-1B}$
		EC21	*bla*$_{NDM-19}$	*bla*$_{CTX-M-15}$; *bla*$_{TEM-1B}$; *bla*$_{SHV-12}$
		EC22	*bla*$_{NDM-19}$	-
	ST354	EC3	*bla*$_{OXA-48}$	*bla*$_{CTX-M-24}$; *bla*$_{CMY-2}$
		EC7	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{CTX-M-24}$; *bla*$_{TEM-1B}$
		EC8	*bla*$_{OXA-48}$	*bla*$_{CTX-M-24}$; *bla*$_{TEM-1B}$
	ST38	EC26	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$; *bla*$_{CMY-42}$
	ST940	EC14	*bla*$_{NDM-5}$	*bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
	ST205	EC4	*bla*$_{OXA-181}$	*bla*$_{CTX-M-15}$;*bla*$_{CMY-42}$; *bla*$_{TEM-1B}$
	ST457	EC1	*bla*$_{OXA-48}$	*bla*$_{CTX-M-27}$
	ST46	EC24	*bla*$_{NDM-5}$; *bla*$_{OXA-181}$	*bla*$_{CTX-M-15}$
	ST8717	EC13	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$;*bla*$_{CMY-2}$
	ST224	EC18	*bla*$_{NDM-1}$	*bla*$_{OXA-10}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$; *bla*$_{LEN12}$
***E.hormaechei* (n=5)**	ST114	EH1	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{ACT-16}$
		EH5	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{ACT-16}$
	ST171	EH2	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{OXA-10}$; *bla*$_{ACT-7}$
	ST134	EH3	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{ACT-7}$; *bla*$_{TEM-1B}$
	Novel ST	EH4	*bla*$_{OXA-48}$	*bla*$_{ACT-7}$
***C. freundii* (n=3)**	ST415	CF1	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{CMY-48}$
	ST91	CF2	*bla*$_{NDM-1}$	*bla*$_{CMY-150}$
	ST112	CF3	*bla*$_{NDM-1}$	*bla*$_{CMY-75}$; *bla*$_{DHA-1}$; *bla*$_{TEM-1C}$

Table 5: MLST of the *Klebsiella spp.*, *S. marcescens*, and *M. morganii* isolates and their resistance profiles including carbapenemase encoding and β-lactam resistance genes.

	ST	Isolate number #	Carbapenemase genes	β-lactam resistance genes
Klebsiella spp. **(n=25)**	ST45	KP7	*bla*$_{OXA-181}$	*bla*$_{SHV-27}$; *bla*$_{OXA-1}$
		KP12	-	*bla*$_{DHA-1}$; *bla*$_{LEN12}$
	ST16	KP6	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
		KP8	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{DHA-1}$; *bla*$_{TEM-1B}$
		KP13	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
		KP14	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{OXA-405}$; *bla*$_{TEM-1B}$
		KP15	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
		KP16	*bla*$_{NDM-7}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
	ST17	KP20	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{CTX-M-14b}$; *bla*$_{SHV-94}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
		KP22	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{CTX-M-14b}$; *bla*$_{SHV-94}$
	ST147	KP4	-	*bla*$_{CTX-M-15}$; *bla*$_{SHV-67}$; *bla*$_{OXA-1}$
		KP5	*bla*$_{NDM-5}$;*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-67}$; *bla*$_{OXA-9}$; *bla*$_{TEM-1B}$
		KP23	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-67}$; *bla*$_{OXA-1}$
	ST15	KP11	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-28}$; *bla*$_{OXA-1}$; *bla*$_{CMY-6}$; *bla*$_{TEM-1B}$
		KP24	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-28}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
	ST1065	KP19	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-26}$; *bla*$_{OXA-1}$; *bla*$_{TEM-205}$
	ST307	KP10	*bla*$_{OXA-48}$	*bla*$_{CTX-M-14b}$; *bla*$_{SHV-28}$; *bla*$_{OXA-1}$
	ST394	KP3	*bla*$_{NDM-1}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-81}$; *bla*$_{OXA-1}$; *bla*$_{DHA-1}$; *bla*$_{TEM-1B}$
	ST870	KP9	*bla*$_{OXA-48}$	*bla*$_{SHV-1}$; *bla*$_{TEM-1B}$
	ST101	KP17	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{CTX-M-14b}$; *bla*$_{SHV-28}$; *bla*$_{TEM-1A}$
	ST392	KP2	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-67}$; *bla*$_{OXA-1}$; *bla*$_{TEM-1B}$
	ST1844	KP18	-	*bla*$_{SHV-28}$; *bla*$_{OXA-1}$
	ST35	KP21	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{SHV-33}$; *bla*$_{TEM-1B}$
	ST2923	KP1	*bla*$_{OXA-48}$	*bla*$_{SHV-40}$; *bla*$_{OXA-1}$
	ST1907	KQ1	*bla*$_{OXA-48}$	*bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$; *bla*$_{OKP-B-1}$; *bla*$_{TEM-1B}$
S. marcescens **(n=2)**	-	SM1	*bla*$_{OXA-48}$	*bla*$_{SRT-1}$
		SM2	*bla*$_{OXA-48}$	*bla*$_{SST-1}$
M. morganii **(n=3)**	-	MM1	-	*bla*$_{DHA-13}$; *bla*$_{OXA-1}$
		MM2	-	*bla*$_{DHA-20}$
		MM3	-	*bla*$_{DHA-9}$

Table 6: Distribution of antibiotic resistance genes based on *in silico* analysis. Antimicrobial categories are marked as: A, when it is an aminoglycoside resistance gene; Q, quinolone resistance genes.

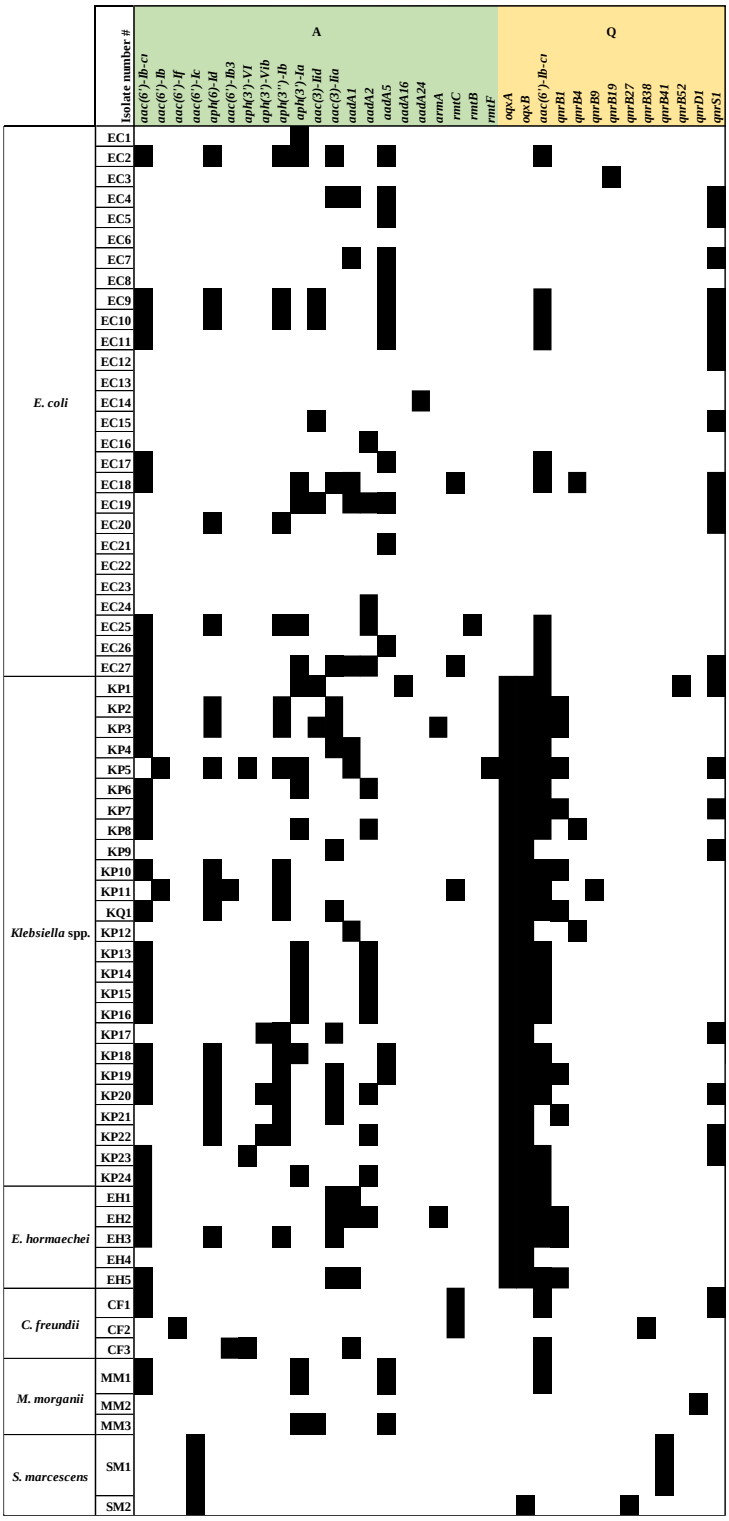
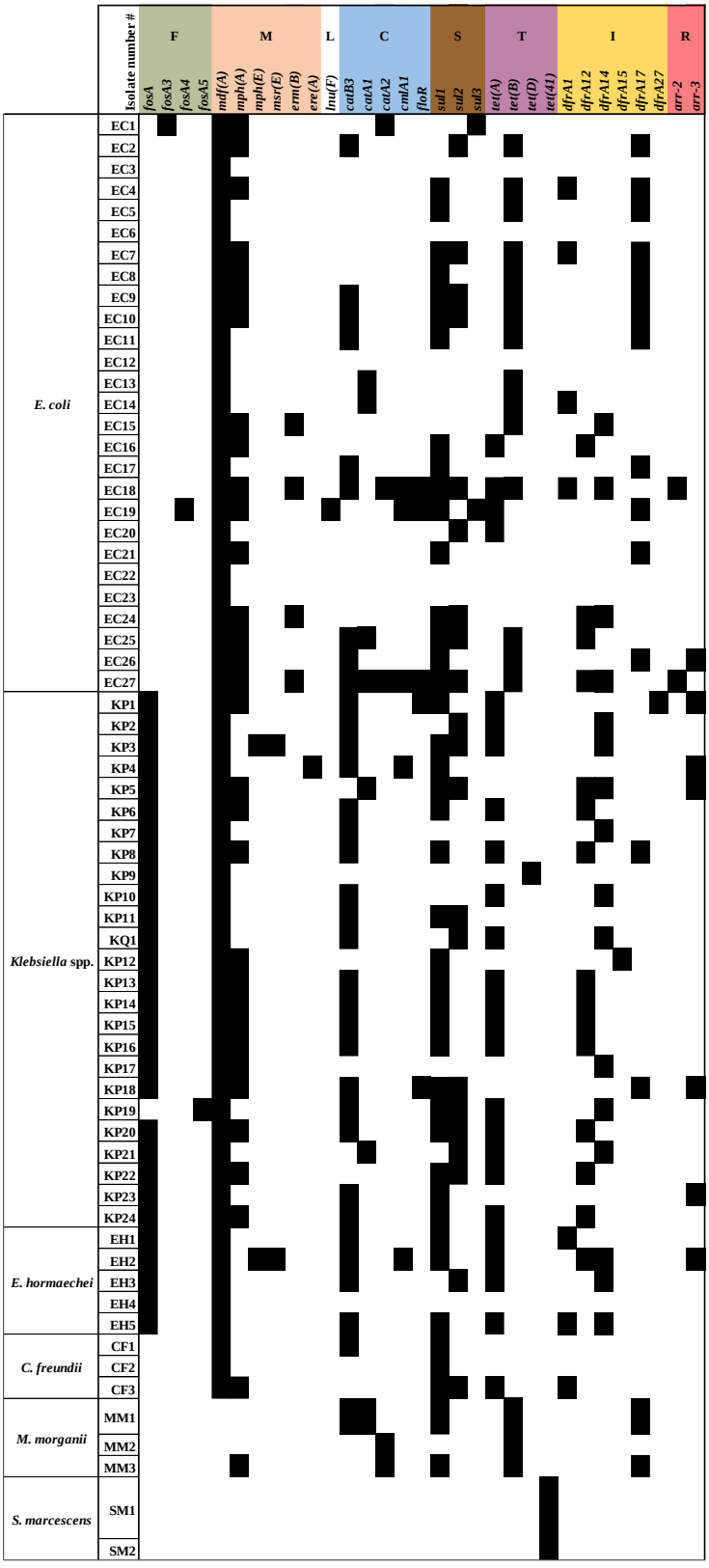

A
Q
Isolate number #
aac(6')-Ib-cr
aac(6')-Ib
aac(6')-If
aac(6')-Ic
aph(6)-Id
aac(6')-Ib3
aph(3')-VI
aph(3')-VIb
aph(3'')-Ib
aph(3')-Ia
aac(3)-IId
aac(3)-IIa
aadA1
aadA2
aadA5
aadA16
aadA24
armA
rmtC
rmtB
rmtF
oqxA
oqxB
aac(6')-Ib-cr
qnrB1
qnrB4
qnrB9
qnrB19
qnrB27
qnrB38
qnrB41
qnrB52
qnrD1
qnrS1
E. coli
EC1
EC2
EC3
EC4
EC5
EC6
EC7
EC8
EC9
EC10
EC11
EC12
EC13
EC14
EC15
EC16
EC17
EC18
EC19
EC20
EC21
EC22
EC23
EC24
EC25
EC26
EC27
Klebsiella spp.
KP1
KP2
KP3
KP4
KP5
KP6
KP7
KP8
KP9
KP10
KP11
KQ1
KP12
KP13
KP14
KP15
KP16
KP17
KP18
KP19
KP20
KP21
KP22
KP23
KP24
E. hormaechei
EH1
EH2
EH3
EH4
EH5
C. freundii
CF1
CF2
CF3
M. morganii
MM1
MM2
MM3
S. marcescens
SM1
SM2

Table 7: Distribution of antibiotic resistance genes based on *in silico* analysis. Antimicrobial categories are marked as: F, when it is a Fosfomycin resistance genes; M, macrolide resistance genes; L, lincosamide resistance genes; C, chloramphenicol resistance genes; S, sulphonamide resistance genes; T, tetracycline resistance genes; I, trimethoprim resistance genes; R, rifampicin resistance genes.

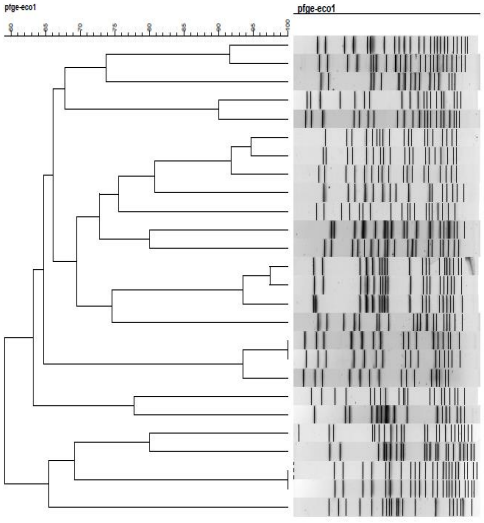

Isolate number #
F
M
L
C
S
T
I
R
fosA
fosA3
fosA4
fosA5
mdf(A)
mph(A)
mph(E)
msr(E)
erm(B)
ere(A)
lnu(F)
catB3
catA1
catA2
cmlA1
floR
sul1
sul2
sul3
tet(A)
tet(B)
tet(D)
tet(41)
dfrA1
dfrA12
dfrA14
dfrA15
dfrA17
dfrA27
arr-2
arr-3
E. coli
EC1
EC2
EC3
EC4
EC5
EC6
EC7
EC8
EC9
EC10
EC11
EC12
EC13
EC14
EC15
EC16
EC17
EC18
EC19
EC20
EC21
EC22
EC23
EC24
EC25
EC26
EC27
Klebsiella spp.
KP1
KP2
KP3
KP4
KP5
KP6
KP7
KP8
KP9
KP10
KP11
KQ1
KP12
KP13
KP14
KP15
KP16
KP17
KP18
KP19
KP20
KP21
KP22
KP23
KP24
E. hormaechei
EH1
EH2
EH3
EH4
EH5
C. freundii
CF1
CF2
CF3
M. morganii
MM1
MM2
MM3
S. marcescens
SM1
SM2

3.3. *E. coli* MLST typing, Serotyping, and PFGE analysis

PFGE and *in silico* MLST based on seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA*, and *recA*) were used to investigate the genetic relatedness of the *E. coli* (n=27) isolates. A total of 12 different STs were identified. ST410 (n=5) of serotypes O8-like:H9-like and H21-like (n=4; n=1) and ST167 (n=5) of serotypes O89-like:H5-like and O89-like:H9-like (n=4; n=1) were the most prevalent. The next common MLST types were ST648 (n=4) and ST354 (n=3) of serotypes O45:H6-like, ST405 (n=3) of serotypes O102-like and O102-like:H6-like (n=2; n=1) and seven singletons.

According to the combination of marker genes, seven phylogroups (A, B1, B2, C, D, E and F) were assigned to the *E. coli* isolates. The distribution of the studied *E. coli* isolates according to their phylogroups obtained from Goseqit was as follows: six (22.2 %) belonged to group A, four (14.8 %) group B1, five (18.5%) group C, four (14.8%) group D, and eight (29.6 %) group F.

A total of 19 different pulsotypes were identified with one isolate (EC17) being untypeable, even after applying a secondary enzyme. Isolates having same STs clustered together based on PFGE except for one *E. coli* ST410 as well as ST648 and ST167 that clustered into two distinct groups. All of the isolates typed with ≥90% PFGE similarity patterns had identical STs, phylogroups, and serotypes. However, no significant clonal relatedness was observed between the isolates (Figure 1).



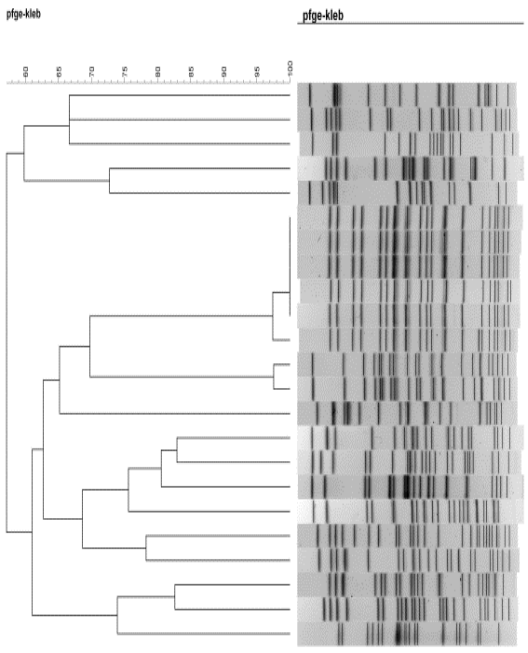
PT	Isolate#	Gender	Specimen	Year	Phylogroup	Serotype	ST	Carbapenamase genes
1	EC23	F	SP	2018	F	O45:H6-like	ST648	*bla* OXA-244
2	EC27	M	UR	2019	F	O45:H6-like	ST648	*bla* NDM-1
3	EC26	M	FL	2019	D	O1-like:H15-like	ST38	*bla* OXA-48
4	EC25	F	UR	2018	D	O102-like	ST405	*bla* NDM-5
5	EC2	F	UR	2016	D	O102-like	ST405	-
6	EC9	F	UR	2017	C	O8-like:H9-like	ST410	*bla* OXA-181
6	EC10	F	UR	2017	C	O8-like:H9-like	ST410	*bla* OXA-181
6	EC11	F	SP	2017	C	O8-like:H9-like	ST410	*bla* OXA-181
7	EC12	M	WD	2017	C	H21-like	ST410	*bla* OXA-181
8	EC14	F	FL	2017	B1	H5-like	ST940	*bla* NDM-5
9	EC4	F	DTA	2016	B1	O100-like:H12-like	ST205	*bla* OXA-181
10	EC5	F	UR	2016	C	O8-like:H9-like	ST410	*bla* OXA-181
11	EC21	F	UR	2018	A	O89-like:H5-like	ST167	*bla* NDM-19
11	EC22	F	UR	2018	A	O89-like:H5-like	ST167	*bla* NDM-19
11	EC20	F	UR	2018	A	O89-like:H5-like	ST167	*bla* NDM-19
12	EC1	F	BL	2016	F	O11-like:H25-like	ST457	*bla* OXA-48
13	EC7	M	BL	2016	F	O45:H6-like	ST354	*bla* OXA-48
13	EC8	F	UR	2017	F	O45:H6-like	ST354	*bla* OXA-48
13	EC3	F	BL	2016	F	O45:H6-like	ST354	*bla* OXA-48
14	EC24	F	SP	2018	A	O9-like:H10	ST46	*bla* NDM-5, *bla* OXA-181
15	EC13	M	WD	2017	B1	O9:H30-like	ST8717	*bla* OXA-48
16	EC16	F	UR	2017	A	O89-like:H5-like	ST167	*bla* NDM-5
17	EC19	M	BL	2018	A	O89-like:H9-like	ST167	*bla* NDM-5
18	EC15	M	FL	2017	F	O45:H6-like	ST648	*bla* OXA-244
18	EC6	F	UR	2016	F	O45:H6-like	ST648	*bla* OXA-244
19	EC18	F	UR	2018	B1	O109-like:H51	ST224	*bla* NDM-1
NT	EC17	M	FL	2017	D	O102-like:H6-like	ST405	-

Figure 1: PFGE profiles, phylogenetic groups, serotypes, sequence types (STs) of the 27 *E. coli* isolates. Dendogram generated by BioNumerics software version 7.6.1 showing the relationship of the isolates based on their banding patterns. The restriction enzyme used was *Xba*I. The pulsotype, isolate number #, gender, specimen type, collection year, phylogroup, serotype, ST, and detected carabapenemases are listed after each PFGE pattern. Isolates sharing same ST were color coded. NT: not typeable; PT: pulsotype; UR, urine; SP, sputum; BL, blood; WD, wound; FL, fluid; DTA, deep tracheal aspirate.

3.4. *K. pneumoniae* MLST typing, K-typing, and PFGE analysis

PFGE and *in silico* MLST were also used to investigate the genetic relatedness of the *K. pneumoniae* isolates (KP1-KP24; n=24). A total of 14 STs were identified of which ST16 was the most prevalent (25%; n=6), followed by ST147 (12.5%; n=3), ST15 (8.3%; n=2), ST17 (8.3%; n=2), ST45 (8.3%; n=2) and nine singletons. Additionally, the K-type was determined using Kaptive which analyzes the entire *cps* locus of each isolate. Fifteen K-types were identified. K51 (25%; 6/24) was the most common, followed by K102 (12.5%; 3/24), K17 (8.3%; 2/24), K64 (8.3%; 2/24), and 11 distinct K-types.

A total of 17 different pulsotypes were identified with one isolate (KP1) being untypeable, even after applying a secondary enzyme. ST15 and ST45 did not cluster together based on their pulsotypes. One isolate belonging to ST392 clustered with ST147. It is noteworthy that all six isolates of ST16 shared ≥ 95% PFGE similarity patterns and having the same pulsotype (PT6), similar K-types (K51), carbapenem and β-lactam resistance profiles (*bla*$_{\text{NDM-7}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{SHV-26}}$). Additionally, two isolates of ST17 also shared ≥ 95% PFGE similarity patterns with similar K-types (K102), plasmidic profiles, and commonly harboring *bla*$_{\text{OXA-48}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-14b}}$, and *bla*$_{\text{SHV-94}}$. PFGE (Figure 2).



PT	Isolate#	Gender	Specimen	Year	ST	K-type	Carbapenemase genes
1	KP19	F	WD	2018	ST1065	K35	*bla* $_{\text{OXA-48}}$
2	KP10	F	BL	2017	ST307	K102	*bla* $_{\text{OXA-48}}$
3	KP7	F	UR	2017	ST45	K24	*bla* $_{\text{OXA-181}}$
4	KP3	M	DTA	2016	ST394	K3	*bla* $_{\text{NDM-1}}$
5	KP9	F	WD	2017	ST870	K17	*bla* $_{\text{OXA-48}}$
6	KP14	M	DTA	2018	ST16	K51	*bla* $_{\text{NDM-7}}$
6	KP15	F	UR	2018	ST16	K51	*bla* $_{\text{NDM-7}}$
6	KP16	F	UR	2018	ST16	K51	*bla* $_{\text{NDM-7}}$
6	KP6	F	UR	2017	ST16	K51	*bla* $_{\text{NDM-7}}$
6	KP13	M	DTA	2018	ST16	K51	*bla* $_{\text{NDM-7}}$
6	KP8	M	WD	2017	ST16	K51	*bla* $_{\text{NDM-7}}$
7	KP20	F	DTA	2018	ST17	K102	*bla* $_{\text{OXA-48}}$
7	KP22	F	WD	2018	ST17	K102	*bla* $_{\text{OXA-48}}$
8	KP17	M	UR	2018	ST101	K17	*bla* $_{\text{OXA-48}}$
9	KP4	M	WD	2016	ST147	K10	-
10	KP23	M	WD	2019	ST147	K64	*bla* $_{\text{NDM-1}}$
11	KP2	M	WD	2016	ST392	K27	*bla* $_{\text{OXA-48}}$
12	KP5	F	UR	2017	ST147	K64	*bla* $_{\text{NDM-5;}}$ *bla* $_{\text{OXA-48}}$
13	KP11	M	WD	2017	ST15	K2	*bla* $_{\text{NDM-1}}$
14	KP12	F	UR	2017	ST45	K62	-
15	KP18	F	UR	2018	ST1844	K54	-
16	KP24	M	SP	2019	ST15	K48	*bla* $_{\text{OXA-48}}$
17	KP21	F	UR	2018	ST35	K110	*bla* $_{\text{OXA-48}}$
NT	KP1	F	UR	2016	ST2923	K117	*bla* $_{\text{OXA-48}}$

Figure 2: PFGE profiles, pulsotypes, sequence types (STs), and K-types of the 24 sequenced *K. pneumoniae*. Dendogram generated by BioNumerics software version 7.6.1 showing the relationship of the isolates based on their banding patterns. The restriction enzyme used was *Xba*I. Pulsotype, isolate number #, gender, specimen type, collection year, MLST: ST, K-type, and detected carabapenemases are listed after each

PFGE pattern. Isolates sharing same ST were color coded. NT: not typeable; PT: pulsotype; UR, urine; SP, sputum; BL, blood; WD, wound; DTA, deep tracheal aspirate.

3.5. Pan-genome analysis

3.5.1. *E. coli* Isolates

Roary was used to construct the pan genomes and identify the core and accessory genes of the *E. coli* isolates. There were 3, 258 protein coding sequences in the core genome which are present in all *E. coli* isolates. Furthermore, 2, 499 of the protein coding sequences were part of the shell genes which were detected in 15 – 95% of the isolates (Figure 3). However, the genes present in less than 15% of the isolates comprised 9, 348 of the protein coding sequences. The commonly shared core genome of 64.8kb in size was mainly composed of cell shape-determining proteins, DNA replication and repair proteins, flagellar biosynthesis protein and motility protein, chemotaxis proteins, LPS-assembly protein, transcriptional regulators, activators, and repressors, Vitamin B12 transporter. Noticeably, the accessory genome included type IV secretion system proteins (*ptlE*, *ptlH*, *virB11*, *virB9*), multi-drug resistance protein (MexA/B), prophage integrases (IntS and IntA), CRISPR/Cas system genes, β-lactamases, insertion sequences, and transposases (Figure 4). Isolates with similar STs, phylogroups and serotypes were grouped together. *E. coli* typed as ST410, phylogroup C, and $bla_{\text{OXA-181}}$ carriers clustered together, and shared highly similar set of accessory genes. Additionally, ST648 and ST354 shared similar accessory genes. On the other hand, EC18 was the only isolate harboring the bla_{LEN12} among the *E. coli* isolates in addition to IS3 family transposase (IS*1400*, IS*Ec52*, and IS*Kpn1*), multidrug efflux RND

transporter permease subunit OqxB25, and Tn3 family transposase IS*Shes11* in its accessory genome.

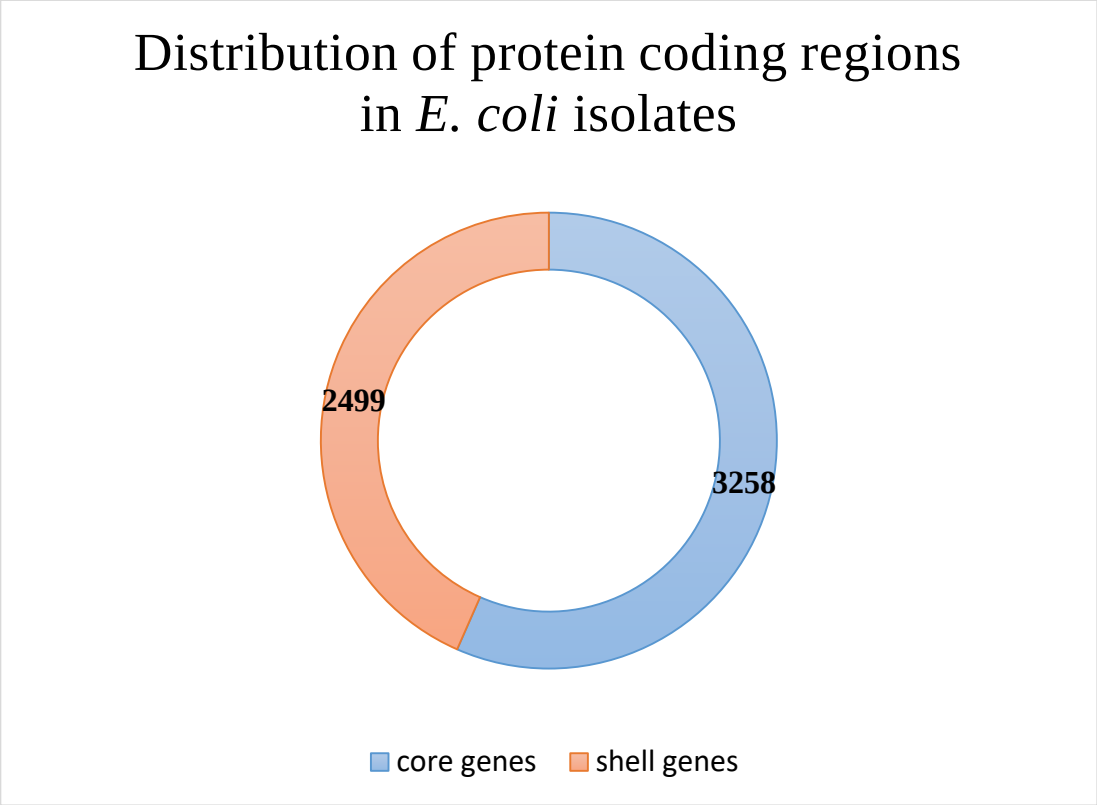


Figure 3: Circular diagram presenting the distribution of protein coding regions *in E. coli* isolates.

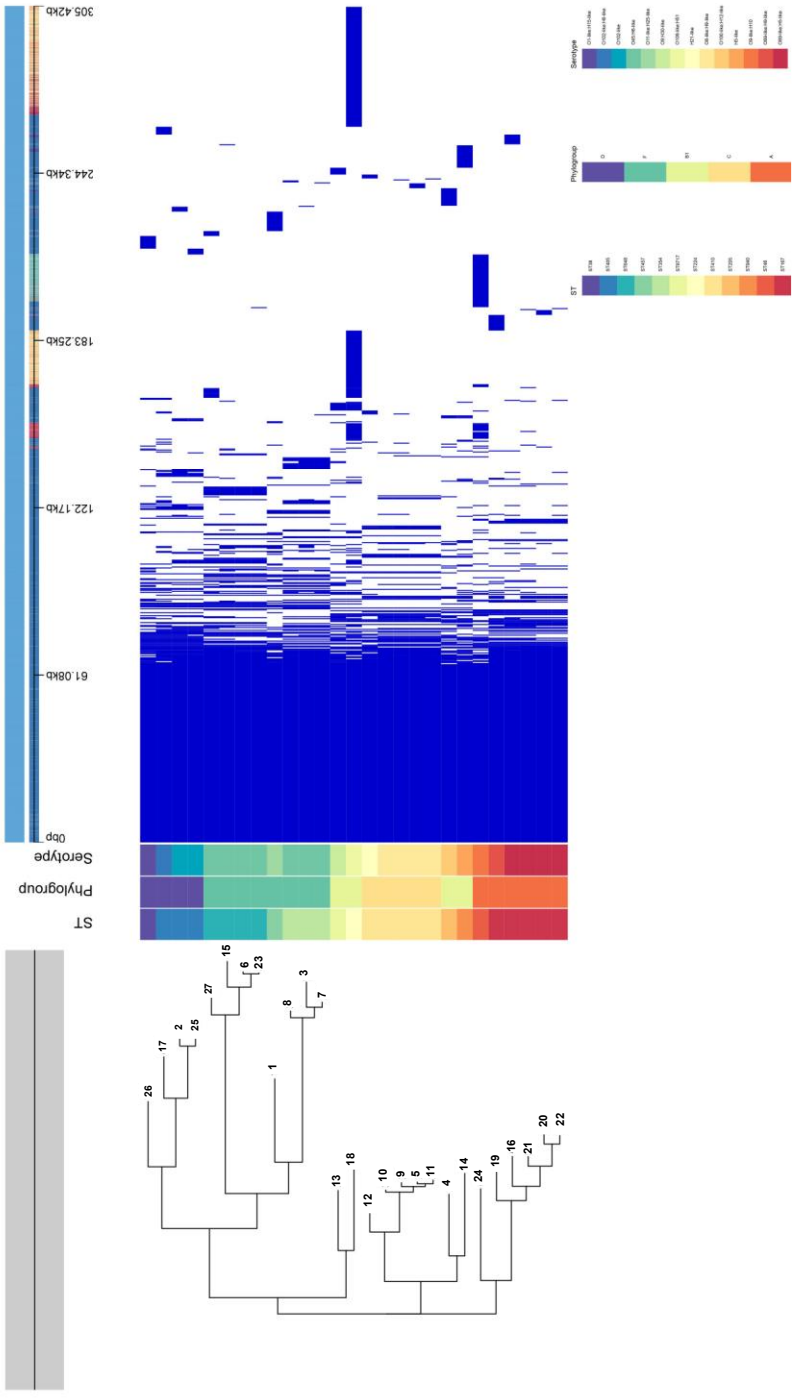


Figure 4: Pan genome analysis of the coding regions in *E. coli* isolates performed using Roary and showing the presence/absence matrix, ST, serotypes, and phylogroups. The maximum likelihood phylogenetic tree was constructed based on the core genomes (blue

block on the left) and accessory genome (separate blue blocks on the right) and showed the relatedness among the isolates.

3.5.2. *K. pneumoniae* Isolates

Pan-genome analysis was also performed to differentiate between the core and accessory genes of *K. pneumoniae* isolates. A total of 11118 genes are present with 4185 protein coding sequences constituting the core. However, 1917 protein coding sequences, which were detected in 15-95% of the isolates, constituted the shell. The cloud genes, which is made up of 4922 protein coding sequence, is identified in less than 15% of the isolates (Figure 5). The core genome, around 83.7kb in size, is shared among the *K. pneumoniae* isolates and mainly includes inner and outer membrane proteins, acid stress proteins, cell division proteins, cellulose biosynthesis proteins, efflux pump membrane transporters, fimbria adhesion protein, flagellar regulators, heat shock proteins, transcriptional regulators and repressors, proteins linked to metabolic activities, iron uptake system components, transposases, phage shock proteins, type II secretion system proteins, and vitamin binding proteins. Remarkably, the accessory genes were linked to acid shock proteins, aminoglycoside resistance genes, toxin-antitoxins systems (Ccd A, Hig A, PemI), β-lactamases, bleomycin resistance protein, IS1 and IS3 family transposases, multi-drug resistance proteins (AcrB/F, EmrA/B, MdtC/E/H/K/N), phage shock proteins (A,B,C,D), phage integrases (IntA, IntS), Tn3 family transposase, type IV secretion system protein (*virB4, virB11, virB8*, *virB9*, *ptlH, ptlE*). *K. pneumoniae* with ST16 and K51 are clustered together and shared highly identical accessory genomes compared to other isolates, which is also the same for ST17 isolates (Figure 6).

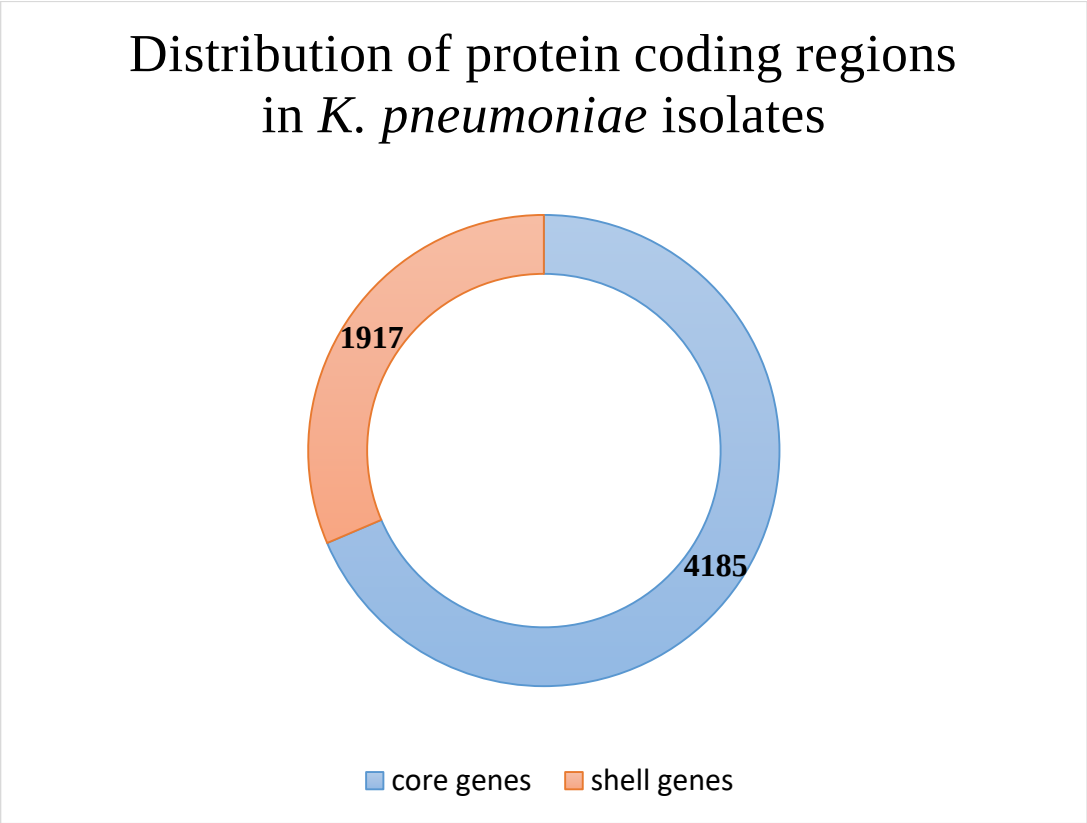


Figure 5: Circular diagram presenting the distribution of protein coding regions in *K. pneumoniae* isolates.

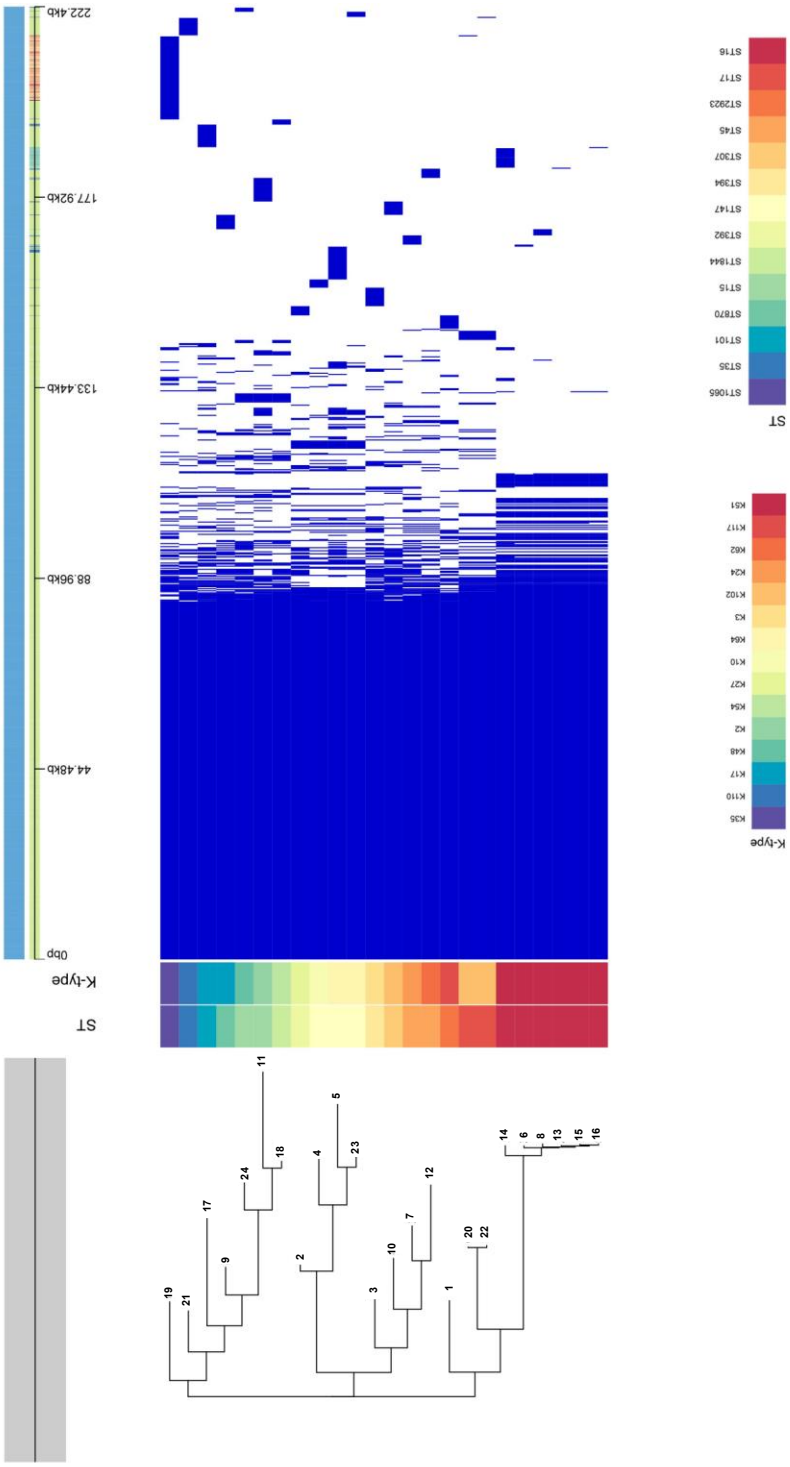


Figure 6: Pan genome analysis for the coding regions in *K. pneumoniae* isolates performed using Roary and presenting the presence/absence matrix, ST, and K-types. The maximum likelihood phylogenetic tree was constructed based on the core genomes

(blue block on the left) and accessory genome (separate blue blocks on the right) and show relatedness among the isolates.

3.6. Plasmid Typing

The combined results from PCR-based replicon typing (PBRT) and PlasmidFinder v.2.1. were analyzed to identify the incompatibility groups of the plasmids. A variety of plasmid incompatibility groups as well as Col-type plasmids were detected. IncFIB (K) (43.1%; n=28) was the most prevalent type, followed by IncFIA (40.0%; n=26), IncL (35.4%; n=23), IncX3 (32.3%; n=21), IncI1 (32.3%; n=21), IncFIIK (29.2%; n=19) and IncFIB (AP001918) (24.6%; n=16). Among the ten detected Col plasmids, Col440I (46.2%; n=30) was the most common, followed by Col(BS512) (24.6%; n=16), Col(MG828) (16.9% ; n=11), and ColRNAI (16.9% ; n=11) (Figure 7).

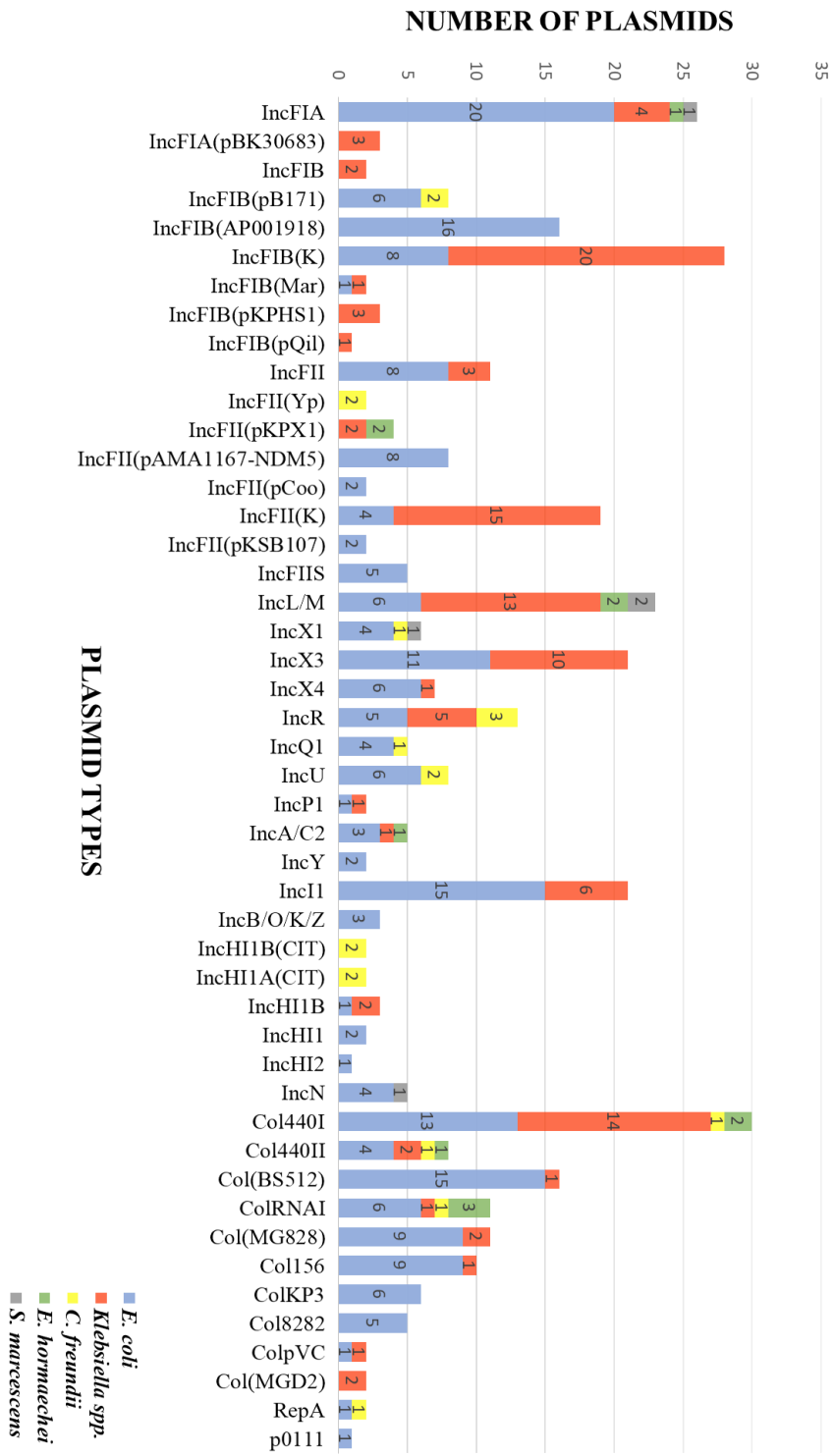


Figure 7: Incompatibility groups and Col plasmids present in the 65 sequenced isolates based on PlasmidFinder and PBRT results.

3.7. Plasmids profiling

3.7.1. *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{OXA-181}}$ carrying plasmids

pOXA-181_X3 was extracted from EC11. Plasmid analysis revealed that it is 51,479 bp in size and had a conserved plasmid backbone consisting of the replication initiation site and the conjugal transfer proteins (Figure 8). pOXA-181_X3 belongs to IncX3 group and harbors *bla*$_{\text{OXA-181}}$ along with the quinolone resistance gene qnrS1 in a composite transposon flanked by IS*26*. Surrounding *bla*$_{\text{OXA-181}}$ are IS*Kpn19* (upstream) and Tn*3*-like transposons IS*3000* -IS*Ec63* followed by IS*26* (downstream). However, qnrS1 was localized between a truncated IS2 insertion sequence and Tn3-like resolvase. BLAST analysis showed that the plasmid was identical to several plasmids including the E. coli plasmid pAMA1167-OXA-181 (CP024806.1) and the *K. pneumoniae* plasmid pBC947-OXA-181 (MK412920.1) (100% sequence identity) reported from Egypt and the United Arab Emirates, respectively.

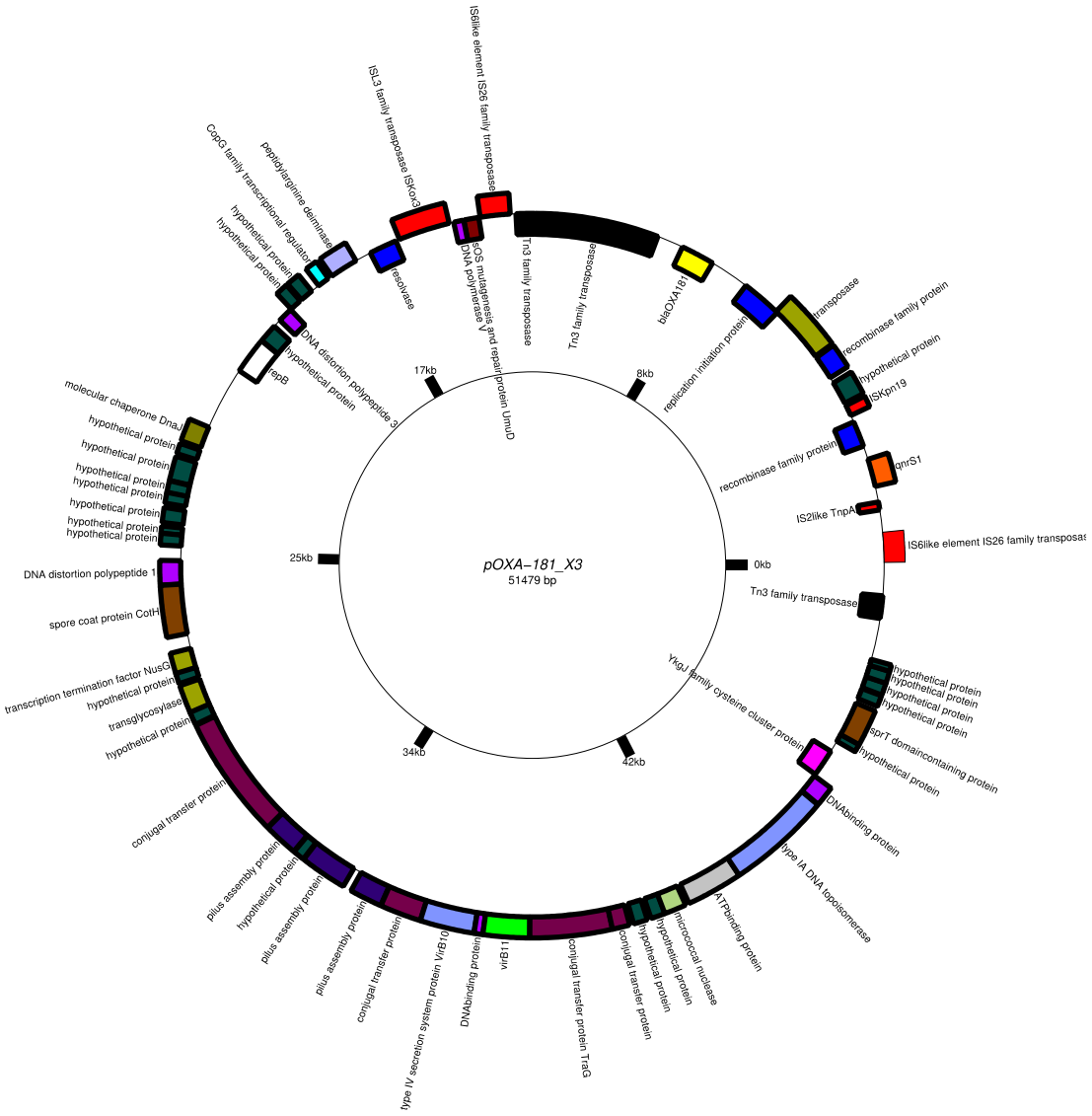


Figure 8: Overview of pOXA-181_X3. Complete sequence of the 51,479 bp plasmid, fully annotated. *bla*$_{\text{OXA-181}}$ is colored in yellow, *qnrS1* gene is colored in orange, IncX3 *repB* gene colored in white. *bla*$_{\text{OXA-181}}$ is flanked by IS*3000*-IS*Ec63*-IS*26* in the downstream region and IS*Kpn19* in the upstream region.

The majority of the isolates carrying *bla*$_{\text{OXA-48}}$ were positive for the IncL replicon. The isolates also harboured the three backbone genes, *parA*, *repA* and *traU*, which are common features of an IncL pOXA-48 plasmid. Contigs with the *bla*$_{\text{OXA-48}}$ gene were

BLASTed against a known complete plasmid pOXA-48 (JN626286, 61.8 kb) and were found to be 97-99% identical.

3.7.2. *bla*$_{NDM}$ carrying plasmids

bla$_{NDM-7}$ was found on a conjugative IncX3 plasmid in six *K. pneumoniae* isolates. The genetic environment surrounding the *bla*$_{NDM-7}$ is comprised of genes encoding partitioning (*parB*), plasmid maintenance (*topB*), replication (*repB*), transfer proteins (*tax*) and conjugation (*vir*) which represent the typical backbone of an IncX plasmid (Figure 9). *bla*$_{NDM-7}$ was preceded by the insertion sequence IS*5* in the upstream region and followed by the bleomycin resistance gene, putative phosphoribosyl-anthranilate isomerase encoding genes, the oxidoreductase DsbC superfamily protein, and the insertion sequence IS*26* (*ble*$_{MBL}$-*trpF*-*dsbC*-IS*26*) in the downstream region. The contig carrying *bla*$_{NDM-7}$ in the six studied isolates was analysed using BLAST and showed a 99.97% identity (100% coverage) to several previously reported plasmids such as pJN05NDM7 plasmid (MH523639) from an *E. coli* isolated in China, pEC25_NDM-7 (CP035125) from an *E. coli* strain isolated in China, pHN4109c (MK088485) from an *E. coli* isolated from chicken samples in Pakistan, p14ARS_MMH0055-5 (LR697126) from a *K. pneumoniae* isolated in Philippines, and pEco70745_2 (CP023260) from an *E. coli* strain isolated in Sweden.

Figure 9: Overview of pNDM-7_X3. Complete sequence of the 45, 249 bp plasmid, fully annotated. *bla*$_{\text{NDM-7}}$ is colored in yellow, IncX3 *repB* gene colored in light grey, *parB* gene colored in white, *topB* gene colored in orange, and *vir* genes colored in green. *bla*$_{\text{NDM-7}}$ is flanked by the insertion sequence IS*5* upstream and *ble*$_{\text{MBL}}$-*trpF*-*dsbC*-IS*26* downstream.

bla$_{\text{NDM-19}}$ carrying plasmids were classified as IncX3 plasmid and showed high similarity (99.99-100%) to pHN4109c (MK088485.1) from an *E. coli* isolate in Pakistan.

Plasmid analysis revealed identical genetic environment between *bla*$_{NDM-19}$ and previously described *bla*$_{NDM-7}$ genes. Two insertion sequences (IS*Aba125* and IS*5*) were detected upstream of *bla*$_{NDM-19}$, while the following structure *ble*$_{MBL}$*-trpF-dsbC* and a copy of an IS*26* insertion element were downstream from *bla*$_{NDM-19}$ (Figure 10).

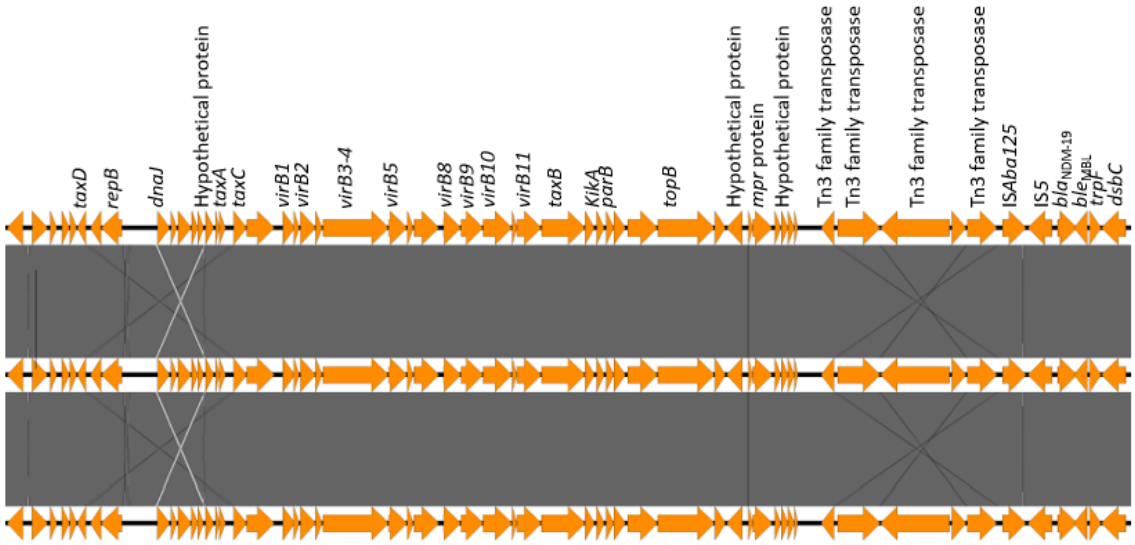


Figure 10: Comparison of the genetic environment of *bla*$_{NDM-19}$ carried on an IncX3 plasmid. *bla*$_{NDM-19}$ was carried by an IS*Aba125*-IS*5*-*bla*$_{NDM-19}$-*ble*$_{MBL}$*-trpF-dsbC*-IS*26* genetic structure.

Plasmid analysis also revealed in this study that *bla*$_{NDM-5}$ was carried on plasmids with different replicon groups including InF and IncX3. A BLASTN search against NCBI revealed that the plasmids carrying *bla*$_{NDM-5}$ were closest to plasmid p91_NDM5 (accession no. MN007141.1) (94% query coverage and 99% identity) and plasmid pAMA1167-NDM-5 (NZ_CP024805.1) (89% query coverage and 99% identity). They belonged to the IncF type plasmid including IncFIA/IncFII replicon groups and shared

the same backbone genes including plasmid replication genes (*repB*) in addition to partition (*parA*) and conjugal transfer genes (*tra* locus). These plasmids also carried multiple resistance genes including *aadA2* (aminoglycoside resistance), *ble* (bleomycin resistance), *dfrA12* (trimethoprim resistance), and *sul1* (sulphonamide resistance) that were part of a class 1 integron sequence (Figure 11). The genetic environment of *bla*$_{NDM-5}$ showed that it is located in a highly conserved region delineated by two copies of IS*26* and composed of a putative composite transposon also containing an IS*CR1* element and a class 1 integron integrase with the *intI1* gene truncated by one of the IS*26* copy in the downstream region. The plasmids also showed high sequence similarity to other plasmids (identity >90%) based on BLAST analysis such as pM309-NDM5 (accession no. AP018833) and p1ESCUMpO83_CORR (accession no. CP033159.1).

Other *bla*$_{NDM-5}$ harboring plasmids had an IncX3 replicon with contigs of 98-100% coverage and 99.9–100% identity to the 46,161 bp reference IncX3 plasmids pNDM5_020001 (CP032424), pGDQ8D112M-NDM (MK628734), and pNDM5_SCNJ06 (MN865121.1) isolated from China. IS*5* family transposase was located upstream of *bla*$_{NDM-5}$ along with ble, trpF, tat genes, and IS*26* downstream from bla$_{NDM-5}$.

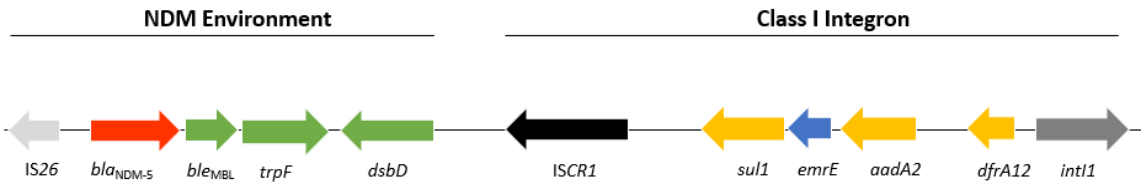


Figure 11: Genetic environment of *bla*$_{NDM-5}$ allocated on IncF plasmid. IS*26* (light grey) is found upstream of *bla*$_{NDM-5}$ (orange), while IS*CR1* element (black), resistance genes *sul1*, *aadA2*, *dfrA1* (yellow), and *intI1* (dark grey) are found downstream.

Plasmids with different replicons were found to harbor *bla*$_{NDM-1}$ including: IncF, IncL/M, and IncA/C$_2$. Contigs containing *bla*$_{NDM-1}$ align to plasmid pK516_NDM1 (NZ_CP022350.1) with a 99% identity and plasmid pKp_Goe_629-2 (NZ_CP018366.1) with a 100% identity that belonged to IncF type plasmid containing IncFII and IncFIB replicons. The plasmids also co-harbor two antibiotic resistance genes: sulfonamide *sul1* and aminoglycoside *rmtC* resistance genes. The genomic environment of *bla*$_{NDM-1}$ was similar to that reported in several *bla*$_{NDM-1}$-carrying plasmids in *Enterobacteriaceae*. It contains the core conserved region including *bla*$_{NDM-1}$ and *ble*$_{MBL}$ which is preceded by ΔIS*Aba125*- *rmtC* and followed by the highly conserved region including *trpF-dsbC-cutA-groES-groEL* (Figure 12). The plasmid also showed high identity to other plasmids including pRJF866 (KF732966.1) (100% coverage and 99% identity) and pKp199-2 (NZ_CP035537.1) (89% coverage and 100% identity).

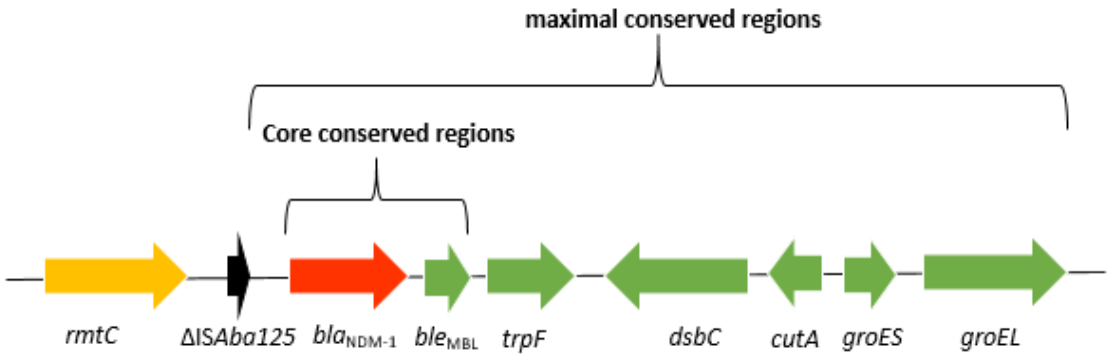


Figure 12: Genetic environment of *bla*$_{NDM-1}$ allocated on IncF plasmid. The genetic element carrying *bla*$_{NDM-1}$ was as follows: *rmtC*-ΔIS*Aba125*- *bla*$_{NDM-1}$- *ble*$_{MBL}$- *trpF-dsbC-cutA-groES-groEL*.

bla$_{NDM-1}$ harboring plasmids in *E. hormaechei* isolates of ST114 aligned to plasmids pLAU_ENC1 (MN688131.1) and pLAU_ENM30_NDM1 (MN792917.1) with a 100% similarity and coverage. The carbapenemase gene was carried on an IncFII replicon. IS*Aba125*-IS*3000* was found upstream of *bla*$_{NDM-1}$, while *ble*$_{MBL}$ gene that encodes bleomycin resistance and Tn*5403*, a Tn3-like transposon, were found downstream (Figure 13).

Figure 13: Genetic environment of *bla*$_{NDM-1}$ allocated on IncFII plasmid. The genetic element carrying *bla*$_{NDM-1}$ was as follows: Tn*3000*-IS*Aba125*- *bla*$_{NDM-1}$- *ble*$_{MBL}$- *trpF*-*dsbD*- Tn*5403*.

bla$_{NDM-1}$ was also detected on an IncFIB(pQil)-type plasmid that encoded multiple resistance genes including aminoglycoside and fluoroquinolone resistance genes (*aac(6')-Ib-cr*, *aph(3')-VI*, *qnrS1*), β-lactam resistance genes (*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$), sulphonamide resistance genes *sul1* , phenicol (*catB3*) and rifampicin (*arr-3*) resistance genes.

IncA/C$_2$, the broad-host-range plasmid, was also found to carry *bla*$_{NDM-1}$ and the class C β-lactamase gene, *bla*$_{CMY-6}$. The contig containing *bla*$_{NDM-1}$ was highly similar (higher than 99%) to the following 140Kbp plasmids p1605752AC2 (NZ_CP022126.1) and pNDM-US (NZ_CP006661.1) where a truncated version of Tn*125* carrying *bla*$_{NDM-1}$ was identified. ISAba125 was truncated by the insertion of ISKpn14 in the upstream region. The following structure *ble*$_{MBL}$-*trpF*-*dsbC*-*cutA*- groES-groEL was found downstream of

the bla_{NDM-1} gene. rhs gene, located beside the heat shock chaperone cluster groES-groEL, was truncated by an insertion sequence (Figure 14).

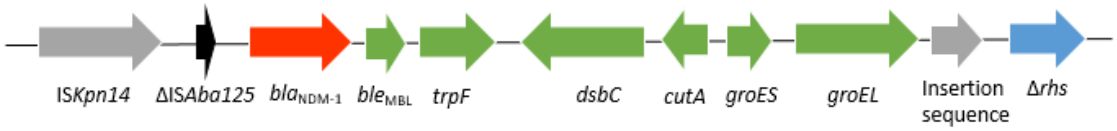


Figure 14: Genetic environment of *bla*$_{NDM-1}$ allocated on IncA/C_2 plasmid. The genetic element carrying *bla*$_{NDM-1}$ was as follows: IS*Kpn14*-ΔIS*Aba125*- *bla*$_{NDM-1}$- *ble*$_{MBL}$- *trpF*-*dsbC*-*cutA*-*groES*-*groEL*-IS-Δ*rhs*

Another contig was also highly similar (>99% identity) to pNDM-OM (JX988621.1) from *K. pneumoniae* in Oman and pNDM-HK (HQ451074.1) from *E. coli* in Hong-Kong that belonged to IncL/M type plasmid. *bla*$_{NDM-1}$ was part of a Tn*1548*-like element that flanked by IS*26* and contained multiple resistance determinants including *armA* aminoglycoside resistance gene, *sul1* sulphonamide resistance gene, *mph(E)* and *msr(E)* macrolide resistance genes, and the beta-lactamase gene *bla*$_{DHA-1}$ (Figure 15).

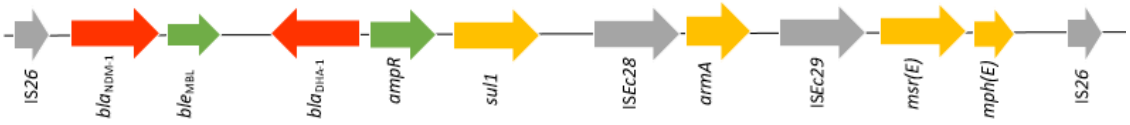


Figure 15: Genetic environment of bla$_{NDM-1}$ encoded on a Tn*1548*-like element. β-lactamases genes are colored in orange, resistance genes are colored in yellow, and insertion sequences are colored in grey.

Chapter Four

Discussion

In the past decade, we witnessed a worldwide surge in ESBL producing and carbapenem-resistant bacteria. The appearance of carbapenem-resistant *Enterobacteriaceae* in Lebanon and adjacent countries is a threat to public health (El-Herte, Kanj, Matar, & Araj, 2012). The extensive spread and acquisition of carbapenemases among different bacterial species are mediated by large conjugative plasmids. A wide variety of incompatibility groups were revealed to be associated with carbapenem resistance (Nordmann, Naas, & Poirel, 2011; Kopotsa, Sekyere, & Mbelle, 2019). In this study, we did a whole-genome based molecular characterization of 65 *Enterobacteriaceae* isolates collected from a large tertiary hospital in Lebanon, to study the resistance profiles, genes, plasmids and other mobile elements contributing to the spread of resistance determinants.

The Middle Eastern region is acknowledged as endemic to carbapenemase-producing *Enterobacteriaceae* (CPE), mainly involving OXA-48-like and NDM-like carbapenemases (Touati & Mairi, 2020). In this study, OXA-48 was the most prevalent carbapenemase detected where 33.8% (n=22) of the isolates were positive for the $bla_{\text{OXA-48}}$ gene. Global genomic surveillance and clinical study reports previously proposed that OXA-48-like carbapenemases are endemic in several parts of the world and are regularly spreading in non-endemic areas causing nosocomial outbreaks (Pitout, Peirano, Kock, Strydom, & Matsumura, 2019). In 2012 however, 73% of clinical *E. coli* isolates collected

from Lebanon were depicted as producers of OXA-48 (Beyrouthy et al., 2014). We showed in this study that the *bla*$_{\text{OXA-48}}$ was mainly linked to what was previously identified as a highly transmissible, 50-80 kb average sized, broad host range IncL plasmid (Poirel, Bonnin, & Nordmann, 2011; Kopotsa, Sekyere, & Mbelle, 2019). *bla*$_{\text{OXA-48}}$ is commonly associated with the IncL with the possibility of spreading a single epidemic plasmid among various enterobacterial species (Poirel, Potron, & Nordmann, 2012). All twenty-two (33.8%) OXA-48 producing isolates were ESBL and/or AmpC producers and were additionally resistant to non-β-lactam antibiotics. ST354 *E. coli* isolates detected in a Lebanese hospital were positive for *bla*$_{\text{OXA-48}}$ carbapenemase gene and *bla*$_{\text{CTX-M-24}}$ (Dagher et al., 2018) and this was found to be compatible with our results where three ST354 *E. coli* isolates (EC3, EC7, and EC8) also carried the *bla*$_{\text{OXA-48}}$, *bla*$_{\text{CTX-M-24,}}$ and at the same time resistant to ciprofloxacin. ST354 lineage have been described to colonize the gastrointestinal tract of companion animals and were associated with extraintestinal infections in both humans and pets (Guo et al., 2015), highlighting the importance of this clone in transmitting the multi-drug resistant strains between humans and dogs.

OXA-181, the second most globally known OXA-48 variant, was detected in one *K. pneumoniae* and seven *E. coli* isolates analyzed in this study. OXA-181 differs from OXA-48 by four amino acid substitutions and has an identical carbapenemase activity (Poirel, Potron, & Nordmann, 2012). It was first detected in India in 2007 (Castanheira et al., 2010) followed by its sporadic emergence in various countries (Poirel, Potron, & Nordmann, 2012). Bitar et al. (2018) reported the first case of OXA-181-producing *E. coli* in Lebanon (Bitar, Dagher, Salloum, Araj, & Tokajian, 2018). Among the *E. coli* isolates harboring *bla*$_{\text{OXA-181}}$ (n=7), 71.4% (n=5) belonged to the most common high-risk global

ST410 clone (Roer et al., 2018). Three OXA-181 producing *E. coli* (EC9,EC10,EC11) in this study harbored *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-2}}$, *aac(6')-Ib-cr*, and *qnrS* genes which was consistent with that previously detected in *E. coli* carrying same resistance genes from Myanmar (Aung et al., 2018). The IncX3 plasmid was another important common finding in the isolates that were positive for the *bla*$_{\text{OXA-181}}$ in this study. The genetic environment surrounding the *bla*$_{\text{OXA-181}}$ gene in the extracted pOXA-181_X3 (Figure 8) was highly identical to previously studied plasmids including the pBC947-OXA-181 (MK412920.1) and pAMA1167-OXA-181(CP024806.1) (Mouftah et al., 2019; Overballe-Petersen et al., 2018). *bla*$_{\text{OXA-181-}}$ in *E. coli* was previously found to be carried on the self-transmissible IncX3 plasmid type. Further supporting the role of this plasmid and mobile elements in general in the rapid dissemination of *bla*$_{\text{OXA-181}}$ and other resistance determinants (Bitar, Dagher, Salloum, Araj, & Tokajian, 2018; Qin, Cheng, Wang, Feng, & Liu, 2018)

Interestingly, EC24 co-harbored a *bla*$_{\text{OXA-181}}$, *bla*$_{\text{NDM-5}}$, and *bla*$_{\text{CTX-M-15}}$, and which was consistent with previous findings that showed the association of the *bla*$_{\text{OXA-181}}$ with other carbapenemase genes such as the *bla*$_{\text{NDM-1,}}$ *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{VIM-5}}$ (Baek et al., 2019).The co-existence of *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{NDM-5}}$ was first detected in *K. pneumonia*e (Balm et al., 2013), and then later in *E. coli* (Ahn et al., 2019). Gamal et al. (2016) showed that *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{CTX-M-15}}$ were both carried on an IncF plasmid, while the *bla*$_{\text{OXA-181}}$ was mainly detected on IncX3 plasmids (Gamal, Fernández-Martínez, El-Defrawy, Ocampo-Sosa, & Martínez-Martínez, 2016). *E. coli* isolates co-harboring NDM and OXA-48-like carbapenemases were also reported in Italy (Monaco, Mento, Cuscino, Conaldi, & Douradinha, 2018), Myanmar (Aung et al., 2018), and Iran (Solgi et al., 2017).

Several NDM variants were identified in this study with *bla*$_{NDM-1}$ being the most common (16.9%; n=11), and being detected on plasmids with different replicon types including: IncF, IncL/M, and IncA/C_2. Previously, *bla*$_{NDM}$ was detected along with bleomycin resistance gene *ble*$_{MBL}$ on a chimeric structure on IncF plasmids (Poirel et al., 2012). *bla*$_{NDM-1}$ was found previously to be on Tn*125* and flanked by two upstream IS*Aba125*. IS*Aba125*, which acts as a promoter for the gene, was originally identified in *Acinetobacter spp*. and later acquired by *Enterobacteriaceae* (Poirel et al., 2014; Qu et al., 2015). In this study the *bla*$_{NDM-1}$ was detected on a multi-replicon IncF type plasmid containing additionally IncFII and IncFIB replicons, *sul1*, and *rmtC* antibiotic resistance determinants and a truncated version of IS*Aba125*, which was consistent to a previous report from China (Zheng et al., 2017; Qu et al., 2015). Having the *bla*$_{NDM-1}$ on a multi-replicon plasmid is linked to a broader host range and hence higher transmissibility (Villa, García-Fernández, Fortini, & Carattoli, 2010). IncF plasmids are usually recognized to harbor several different replicons of which IncFIA, IncFII, and IncFIB are the most common (Carattoli, 2013). Furthermore, *bla*$_{NDM-1}$ detected in *E. hormaechei* isolates that belonged to ST114 were found on an IncFII plasmid with *bla*$_{NDM-1}$-*ble*$_{MBL}$ genes shown to be delineated by an upstream IS*Aba125* and a downstream a Tn3-like transposon, Tn*5403* (Figure 13). IncFII plasmds were highly similar to plasmids pLAU_ENC1 (MN688131.1) and pLAU_ENM30_NDM1 (MN792917.1) from *E. hormaechei* in Lebanon and plasmid pkpn-235-BG (KT852336.1) from *K. pneumoniae* in Bulgaria (Kostyanev et al., 2016). It is noteworthy that in this study two *E. hormaechei* were typed as ST114, which is an international *Enterobacter* clone present in both human and companion pets (Izdebski et al., 2014; Haenni et al., 2016), and are linked to various

carbapenemases (VIM-1, OXA-48, and NDM-1) (Guillard et al., 2015). ST114 was also associated with hospital outbreaks in the United States (Kanamori et al., 2016), and was detected in different countries including Greece , Morocco, Serbia, and Kuwait (Peirano et al., 2018).

NDM-1 carbapenemase genes were detected on different plasmid types belonging to multiple Inc groups (N, X3, A/C, L/M, R , F, T) (Marquez-Ortiz et al., 2017). *bla*$_{\text{NDM-1}}$ in our study was detected on the broad-host-range IncA/C_2 plasmid where ISAba125 located upstream of bla$_{\text{NDM-1}}$ was truncated by ISKpn14 element and *rhs* located downstream was truncated by an insertion sequence. It also showed a similar genetic environment to previously reported IncA/C_2 plasmids found in *Enterobacteriaceae* (Sartor et al., 2014; Carattoli, Villa, Poirel, Bonnin, & Nordmann, 2011). Additionally, *bla*$_{\text{CMY-6}}$ was detected on the IncA/C_2 plasmid with the *bla*$_{\text{NDM-1}}$ carbapenemase in this study, which was consistent with other studies with both being frequently detected on broad-host-range plasmids (Man et al., 2018).

Twenty-four worldwide NDM variants were so far detected. In this study, *bla*$_{\text{NDM-5}}$ carbapenemase was seen on a multi-replicon IncF type plasmid having the IncFII and IncFIA replicons. We found it to be highly similar (>90%) to IncF plasmids detected in Denmark and Myanmar (Sugawara et al., 2018; Overballe-Petersen et al., 2018). In addition, the facilitated dissemination of *bla*$_{\text{NDM-5}}$ was linked to its mobility through different plasmids including IncN, IncX3, and IncF (Li et al., 2018). *bla*$_{\text{NDM-5}}$ detected in EC14 and EC19 was linked to IncX3 plasmid showing high sequence identity to pNDM5_020001 (CP032424) and pGDQ8D112M-NDM (MK628734) isolated from China (Feng et al., 2019; Ma et al., 2020). NDM-5 however, differs from NDM-1 by two

amino acid substitutions (Val88Leu and Met154Leu), and was reported for the first time in an *E. coli* isolate in 2011. The isolate was recovered from a patient in the United Kingdom and had a high hydrolytic activity against both extended-spectrum cephalosporins and carbapenems (Hornsey, Phee, & Wareham, 2011). On the other hand, the first detection of NDM-5 in Lebanon was in 2019 from *K. pneumoniae* (Nawfal Dagher, Azar, Al-Bayssari, Chamieh & Rolain, 2019).

bla$_{\text{NDM-7}}$ another important NDM variant, has two amino acid substitution at positions Asp-130-Asn and Met-154-Leu compared to *bla*$_{\text{NDM-1}}$ and was first reported in *E. coli* recovered from Germany in 2013 (Gottig, Hamprecht, Christ, Kempf, & Wichelhaus, 2013). *bla*$_{\text{NDM-7}}$ isolates showed an increase in carbapenemase activity compared to the ones that were NDM-1 positive (Cuzon, Bonnin, & Nordmann, 2013). We had six *K. pneumoniae* (KP6, KP8, KP13, KP14, KP15, and KP16) isolates that were positive for *bla*$_{\text{NDM-7}}$ and all had the sequence type ST16. ST16 was previously shown to be involved in the dissemination of ESBLs, NDM-5, and NDM-1 carbapenemase genes in Denmark (Hammerum et al., 2015) and Italy (Avolio, Vignaroli, Crapis, & Camporese, 2017). In addition, outbreaks associated with CTX-M-15 producing *K. pneumoniae* belonging to ST16 were also reported in New Zealand (Lester, et al., 2011) and Sweden (Lytsy et al., 2008). Arabaghian et al. (2019) previously detected in *K. pneumoniae* ST16 recovered from Lebanon the *bla*$_{\text{NDM-7}}$ gene on an IncX3 plasmid. The *K. pneumoniae* isolates harboring *bla*$_{\text{NDM-7}}$ in this study, had the IncX3 plasmid (pNDM-7_X3) that was highly similar (>99%) to plasmids isolated from China, Sweden, Pakistan, and Philippines (Figure 9). The genetic environment surrounding the *bla*$_{\text{NDM-7}}$ was comprised, as was also previously reported, of genes representing the typical backbone of an IncX plasmid

including genes encoding partitioning (*parB*), plasmid maintenance (*topB*), replication (*repB*), transfer proteins (*tax*) and conjugation (*virB1-virB11*) (Bitar, Dagher, Salloum, Araj, & Tokajian, 2018). *bla*$_{\text{NDM-7}}$ was carried on an IS*5*-*bla*$_{\text{NDM-7}}$- *ble*$_{\text{MBL}}$-*trpF*-*dsbC*-IS*26* genetic element and was consistent with Hao et al. (2018) findings.

bla$_{\text{NDM-19}}$ detected in *E. coli* (EC20, EC21, and EC22) was also among the carbapenemase resistance determinants in this study. The isolates were typed as ST167. ST167 was linked to the global spread of *bla*$_{\text{NDM}}$ in human, animals, and food (Baloch et al., 2019). This is the first detection of *bla*$_{\text{NDM-19}}$ in Lebanon. The gene was first seen in *K. pneumoniae* isolated from China in 2018 (Liu et al., 2019), and then in *E. coli* recovered from Switzerland in 2019 (Mancini, Keller, Greiner, Bruderer, & Imkamp, 2019). The main difference between NDM-19 and NDM-7 is the outcome of one mutation (substitution) at position A233V (Liu et al., 2019). NDM-19 expression exhibited a decrease in susceptibility to carbapenems and cephalosporins under zinc restricted conditions (Mancini, Keller, Greiner, Bruderer, & Imkamp, 2019). The IncX3 plasmids carrying the *bla*$_{\text{NDM-19}}$ showed complete sequence similarity to a *bla*$_{\text{NDM-7}}$ carrying plasmid pHN4109c (MK088485.1) with the exception of the point mutation (Baloch et al., 2019). It is very likely that IncX3 plasmids harboring distinct *bla*$_{\text{NDM}}$ variants have emerged from the same ancestral plasmid with point mutations being introduced with time (Hao, Shao, Bai, & Jin, 2018). *bla*$_{\text{NDM-1}}$ mutations along with the transmissibility of the IncX3 plasmid were linked to the dispersal of multiple NDM variants (Liu et al., 2019).

MLST and PFGE were conducted to examine the clonal relatedness among the isolates. PFGE results showed that isolates belonging to the same ST for both *E. coli* and *K. pneumoniae* clustered together, yielding nineteen and seventeen pulsotypes,

respectively. PFGE clustering of six *K. pneumoniae* ST16 harboring $bla_{\text{NDM-7}}$ on IncX3 plasmids suggested clonal dissemination. Similar case was seen in the PFGE clustering of four *E. coli* ST410 and three ST167 harboring $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-19}}$ on IncX3 plasmids, respectively. A wide variety of STs were detected among the 65 understudied *Enterobacteriaceae* isolates. The majority of the *E. coli* isolates belonged to either ST410 (n=5), ST167 (n=5), ST648 (n=4), or ST405 (n=3). The mentioned STs represent multi-drug resistant high-risk global clones that contribute to the worldwide dissemination of multi-drug resistance among *Enterobacteriaceae* (Mathers, Peirano, & Pitout, 2015). ST410 is a worldwide known lineage previously reported in Europe, North America, South America, Asia, and Africa and is linked to cross-sectorial transmission between humans, animals, and water sources (Roer et al., 2018). ST405 and ST648 are extra-intestinal pathogenic *E. coli* (ExPEC), which cause urinary tract infections and sepsis and promote the spread of CTX-M-producing *E. coli* (Paulshus et al., 2019). ST405, belonging to phylogroup D, was detected in several countries such as the United States (Tian et al., 2011), Japan (Matsumura et al., 2013), and Lebanon (Dagher et al., 2018), and was linked to the spread of $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr* (Coque et al., 2008). ST648 on the other hand, linked again to the spread of $bla_{\text{CTX–M–15,}}$ was recovered from humans, birds, and companion animals shedding the light on zoonosis (Liu, Thungrat, & Boothe, 2016).

On the other hand, most *K. pneumoniae* in this study belonged to either ST16 (n=6) or ST147 (n=3). ST147, is an international high-risk extensively drug resistant (XDR) clone of *K. pneumoniae* (Navon-Venezia, Kondratyeva, & Carattoli, 2017), and associated with regional and inter-hospital outbreaks detected in the Middle East, North America, and Germany (Pitout, Peirano, Kock, Strydom, & Matsumura, 2019).

The most commonly detected ESBL gene in this study was *bla*$_{\text{CTX-M-15}}$ (n=38/65; 58.5%). CTX-M-15 was the most frequent ESBL identified in Lebanon retrieved from humans and animals (Moubareck et al., 2005). The facilitated spread of *bla*$_{\text{CTX-M-15}}$ from one organism to another is linked to its presence on a broad host range IncF plasmids (Villa, García-Fernández, Fortini, & Carattoli, 2010). Additionally, ESBL types (*bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$), AmpC β-lactamases (*bla*$_{\text{CMY}}$ and *bla*$_{\text{DHA}}$), quinolones and aminoglycoside resistance determinants were also found on IncF plasmids (Carattoli, 2011). *bla*$_{\text{CTX-M-15}}$ was also found to be co-transmitted with carbapenemase genes such as *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ (Cantón, González-Alba, & Galán, 2012) as seen in most of the isolates in this study. Moreover, all of the *bla*$_{\text{CTX-M-15}}$ genes except one were co-carried with other ESBL genes (*bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$) highlighting the clinical importance of the transmissibility of the relevant mobile elements.

Among the AmpC β-lactamases, *bla*$_{\text{CMY-2}}$ (10.8%; n=7) was the most prevalent, followed by *bla*$_{\text{CMY-42}}$ (9.2%; n=6). CMY-42 differs from CMY-2 by substituting Valine with Serine at position 211 which resulted in a reduced susceptibility to third and fourth generation cephalosporins (Hentschke et al., 2011). The mentioned AmpC enzymes were previously detected on IncI plasmids (Feng et al., 2015; Lorme et al., 2018) and was consistent with our results.

To our knowledge, this is one of the largest genome-based studies in the region revealing the tremendous heterogeneity, plasmid content and diverse resistance determinants circulating in the country. The identification of international high-risk carbapenem-resistant *Enterobacteriaceae* clones pose a significant threat to patients and healthcare systems in Lebanon. The observed plethora of plasmid-encoded resistance

genes shed light over the importance of implementing corrective measures to mitigate the spread of the carbapenem resistant *Enterobacteriaceae* isolates.

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