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Molecular Characterization of Carbapenem Resistant *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* Isolated from Lebanon

By

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Molecular Characterization of Carbapenem Resistant *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* Isolated from Lebanon

Harout K. Arabaghian

ABSTRACT

Klebsiella pneumoniae is a Gram-negative rod, mostly known for causing nosocomial infections in the immunocompromised, the elderly, and the neonates. In the last decade, the emergence and spread of both extensively drug resistant (XDR) and hypervirulent *K. pneumoniae* (hvKP) clones have rendered the species a major public health concern. Whole-genome sequencing (WGS) was used for the molecular characterization of 32 carbapenem-resistant *K. pneumoniae* (CRKP) and two carbapenem-resistant *K. quasipneumoniae*. Resistance was assessed by performing antibiotic susceptibility testing (AST) and PCRs for the most common extended-spectrum β-lactamase and carbapenemase encoding genes. Virulence was studied by sequencing the *wzi* gene and determinants confirmed *in silico*. PCR-based replicon typing (PBRT) was performed to identify plasmid incompatibility groups. Genetic relatedness of the isolates was determined by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and whole-genome based SNPs analysis. All of the isolates were non-susceptible to at least one of the three carbapenems tested, with 100% (34/34) of the isolates being non-susceptible to ertapenem, 85.3% (29/34) to imipenem, and 70.6%

(24/34) to meropenem. This phenotypic resistance was found to be due to either *bla*$_{\text{OXA-48}}$ (61.8%; 21/34) or *bla*$_{\text{NDM-1}}$ (14.7%; 5/34) or *bla*$_{\text{NDM-7}}$ (2.9%; 1/34), or the coupling of ESBLs and outer membrane porin loss or modification (20.6%; 7/34). IncL and IncFIIK were the most common replicons detected (91.2%; 31/34). Twenty and 21 different capsular types (K-types) and sequence types (STs) were detected, respectively. Thus our results reveal a very high level of diversity in the *K. pneumoniae* population circulating in Lebanon. We also report the presence of carbapenem-resistant *K. quasipneumoniae*, the circulation of multi-replicon plasmids harboring NDM type enzymes, and the convergence of extensively drug resistant and hypervirulent features in Lebanon.

Key Words: *Klebsiella pneumoniae*, NDM, *Klebsiella quasipneumoniae*, carbapenems, WGS, SNPs, OXA-48.

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LIST OF ABBREVIATIONS

AmpC: Class C β-lactamase

API: Analytical profile index

ARGs: Antimicrobial resistance genes

Asn: Asparagine

Asp: Aspartic acid

AST: Antibiotic susceptibility testing

AUBMC: American University of Beirut Medical Center

CARD: Comprehensive antibiotic resistance database

CDC: Centers for Disease Control and Prevention

cKP: Classical *K. pneumoniae*

CPS: Capsular polysaccharide

CRKP: Carbapenem-resistant *K. pneumoniae*

CTX-M: Resistance to cefotaxime

DLV: Double locus variant

DTA: Deep throat aspirate

ESBL: Extended-spectrum β-lactamases

HGT: Horizontal gene transfer

hvKP: Hypervirulent *K. pneumoniae*

ICU: Intensive care unit

IMP: a metallo-β-lactamase in *Pseudomonas aeruginosa*

Inc: Incompatibility

IS: Insertion Sequence

KP: *K. pneumoniae*

KPC: *K. pneumoniae* carbapenemase

K-type: Capsular type

KQ: *K. quasipneumoniae*

Leu: Leucine

LPS: Lipopolysaccharide

MDR: Multi-drug resistant

Met: Methionine

MLST: Multi-locus sequence typing

NDM: New Delhi metallo-β-lactamase

NJ: Neighbor-joining

OXA: Oxacillin hydrolyzing-β-lactamase

OmpK: Outer membrane porin protein

PBRT: PCR-based replicon typing

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

PLACNET: Plasmid Constellation Network

PT: Pulsotype

RAST: Rapid Annotation using Subsystem Technology

SHV: Sulfhydryl variable

SLV: Single locus variant

SMART: Study for Monitoring Antimicrobial Resistance Trends

ST: Sequence type

TEM: Temoneria β-lactamase

TSA: Tryptic soy agar

UK: United Kingdom

VF: Virulence factor

VIM: Verona integron-encoded metallo- β-lactamase

WGS: Whole genome sequencing

wg: Whole genome

WHO: World Health Organization

XDR: Extensively drug resistant

Chapter One

Introduction

1.1. Overview of *Klebsiella pneumoniae*

Klebsiella pneumoniae is an encapsulated, non-motile, Gram-negative, rod shaped bacterium first described in 1882 by Friedlander (Shon, Bajwa, & Russo, 2013; Zhao et al., 2010). *Klebsiella* spp. are ubiquitously found in nature and have two major habitats (Podschun & Ullmann, 1998). The first being the environment including: sewage, soil, and plants (Podschun & Ullmann, 1998), and the second being the mucosal surfaces of mammals including: humans, swine, and horses (Podschun & Ullmann, 1998).

K. pneumoniae is considered to be an opportunistic pathogen due to its presence in many organs asymptomatically as part of the human microbiota (Holt et al., 2015). Namely, it can colonize the nose, throat, or the gastrointestinal tract of healthy individuals (Holt et al., 2015; Zhao et al., 2010). In recent years, *K. pneumoniae* was singled out as an urgent threat to human health by international healthcare organizations including the U.S. Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (The et al., 2015). This was mainly due to the increasing isolation rate of carbapenem-resistant *K. pneumoniae* (CRKP), it serving as a reservoir for antimicrobial resistance genes (ARGs) and the growing resistance to all currently available therapeutic agents (The et al., 2015; Woodford et al., 2004).

1.2. Epidemiology

K. pneumoniae infections account for up to 8% of all nosocomial bacterial infections affecting neonates, the elderly and the immunocompromised and 3% of epidemic outbreaks with mortality rates reaching up to 40% (Chen et al., 2004; Holt et al., 2015; Podschun & Ullmann, 1998). In general, *K. pneumoniae* carrier rates reach as high as 77% in hospitalized patients' stools and 44% on their hands (Podschun & Ullmann, 1998). *K. pneumoniae* is responsible for 7-14% of pneumonia, 4-15% of bacteremia, 2-4% of wound infections, 4-17% of nosocomial infections in intensive care units, 6-17 % of urinary tract infections, and is the second most common cause of Gram-negative bacteremia (Bialek-Davenet et al., 2015; Holt et al., 2015; Y. M. Liu et al., 2014; Zhao et al., 2010). It can also cause high numbers of serious community-acquired infections with 66% of infected individuals were found to suffer from alcoholism (Holt et al., 2015). It was also identified as the most common etiologic agent of primary liver abscess, which is a complication seen in young healthy individuals and diabetic patients (Ko et al., 2002; Sarno, McGillivary, Sherratt, Actis, & Tolmasky, 2002; Struve et al., 2015).

1.3. Classic *K. pneumoniae*

K. pneumoniae strains involved in nosocomial infections are referred to as classic *K. pneumoniae* (cKP) (Pomakova et al., 2012). It is considered as an important bacterial pathogen in humans with hospital-associated infections, in fact it is one of the top 8 hospital pathogens and a member of the ESKAPE group of pathogens, known for causing two-thirds of all nosocomial infections (P. Liu et al., 2012; Pendleton, Gorman, & Gilmore, 2013). cKP is linked to the majority of infections caused by *K. pneumoniae*

in the western part of the world and is known for its high notoriety due to its increased ability to acquire ARGs (Shon et al., 2013). It is known that the ability of cKP to acquire ARGs is a direct result of positive pressure exerted by antibiotics usage in the clinical setting (Pomakova et al., 2012).

1.4. Hypervirulent *K. pneumoniae*

A rapidly spreading clinical pathotype known as hypervirulent *K. pneumoniae* (hvKP) is involved in community-acquired infections. hvKP was first described two centuries ago with initial reports emerging from the Asian Pacific Rim. It affects healthy young individuals lacking any disease with the defining manifestation being pyogenic liver abscesses (P. Liu et al., 2012; Pomakova et al., 2012). One of the many striking features of hvKP is its ability to undergo metastasis from the primary site of infection into distant sites including lungs, joints, kidneys, and others, observed in as much as 80% of the cases (Pomakova et al., 2012). This is unusual for enteric Gram-negative rods yet it is a common phenomenon detected in *Staphylococcus aureus* and *Streptoccocci* (Shon et al., 2013).

The hypervirulent phenotype of hvKP is due to the overproduction of capsule molecules hence the hypermucoviscous phenotype observed (Pomakova et al., 2012). hvKP also produces more iron-acquisition molecules compared to cKP, is more resistant to human serum, and is more resistant to antimicrobial peptides and neutrophil-mediated bactericidal activity; hence the increased virulence compared to that of cKP (P. Liu et al., 2012; Pomakova et al., 2012; Shankar et al., 2017; Shon et al., 2013). This high level of invasiveness was usually coupled with low levels of resistance, until recently where

the convergence of hypervirulent and highly resistant phenotypes took place (Gu et al., 2018; Pomakova et al., 2012).

1.5. Transmission

K. pneumoniae infections are spread upon exposure to the bacteria via the respiratory tract or the circulatory tract (Gottesman, Agmon, Shwartz, & Dan, 2008). Although *Klebsiella* spreads very rapidly and easily, infections cannot occur through the air (Gottesman et al., 2008). In hospitals, *Klebsiella* infections are spread through person-to-person contact by contaminated hands of patients or even employees, with hand contamination by hospital staff being a major contributor (Abuzaid & Amyes, 2015). Moreover, patients who are on catheters, ventilators, or have surgery wounds are highly prone to catching *Klebsiella* infections, in fact, about 50% of outbreaks in neonatal ICUs are caused by contaminated instruments (Abuzaid & Amyes, 2015).

1.6. Virulence factors

1.6.1. Capsular polysaccharide

Despite the significance of *K. pneumoniae* in the medical field, little was known about its pathogenicity and virulence features. Upon the emergence of the hvKP pathotype, invasiveness and virulence of *K. pneumoniae* gained significant attention. One of the most discussed virulence factor is the capsular polysaccharide (CPS) (Chen et al., 2004; Podschun & Ullmann, 1998). CPS is found on the outer layer of the pathogen as a fibrillous structure and composes the outer capsule of the bacterial cell (Chen et al., 2004; Podschun & Ullmann, 1998; Shon et al., 2013). This capsule is essential to the *Klebsiella* virulence (Podschun & Ullmann, 1998). Besides being involved in resistance

against phagocytosis, the capsule also protects the bacteria from being killed by bactericidal factors circulating in the serum (Chen et al., 2004; Sarno et al., 2002).

CPS is encoded by many genes, such as *wzi, wza, wzv,* or *wzc*, all of which are conserved and make up the *cps* mosaic locus (Pan et al., 2015). Sequencing of the CPS genes could be used to type *K. pneumoniae* into 79 different K-types (Pan et al., 2015; Podschun & Ullmann, 1998). Capsular types K1, K2, K5, and others are generally associated with increased virulence and are the most common capsular types detected in hvKP isolates (Breurec et al., 2016; Podschun & Ullmann, 1998). These capsular types are known for their lack of mannose containing repetitive units in their CPS molecules (Podschun & Ullmann, 1998). Since these repetitive units can easily be detected by surface lectin of macrophages and lead to opsonin-independent phagocytosis, the above mentioned capsular types have the ability to escape these macrophages thus showing higher degrees of virulence (Podschun & Ullmann, 1998).

1.6.2. Lipopolysaccharide

The lipopolysaccharide (LPS) is another important virulence feature common to Gram negative organisms (Podschun & Ullmann, 1998). LPS which contains lipid A, a core region, and a O-polysaccharide antigen is involved in resistance against complement-mediated killing and serum bactericidal proteins (Chen et al., 2004; Sarno et al., 2002). In pathogenic *K. pneumoniae*, serum resistance is carried out by either of the two hypothesized mechanisms involving LPS and CPS. Either the thick CPS layer masks the underlying LPS preventing the activation of the complement system (Podschun & Ullmann, 1998), or the long O side chain of LPS protruding through the thick CPS layer activates the complement, however due to steric hindrance, the complement protein gets fixed far away from the bacterial cell membrane, preventing the

formation of the membrane attack complex and bacterial killing (Podschun & Ullmann, 1998).

Besides being involved in virulence, LPS has also some implications in resistance. Recent data has shown that the lipid A moiety of LPS can undergo chemical modifications leading to resistance to colistin, one of the last resort antimicrobial agents currently used to treat highly resistant *K. pneumoniae* isolates (Leung et al., 2017). Among the many modifications, 4-amino-4-deoxyl-I-arabinose addition to lipid A seems to be one of the most common ones (Leung et al., 2017).

1.6.3. Iron acquisition systems

Iron acquisition systems are essential for bacterial cells to acquire iron; an element used by bacterial cells for growth and replication (Podschun & Ullmann, 1998; Shon et al., 2013). Since a human host has numerous iron-sequestering proteins that function in withholding iron from invading pathogens, bacteria have to produce siderophores showing higher iron-binding capabilities, enabling them to acquire iron within the host (Struve et al., 2015). This ability is crucial for establishing an infection and is implied in the virulence potential of a strain (Gomez-Simmonds et al., 2016). Strains having numerous iron-acquisition systems are known for their increased virulence, and hvKP are known for producing multiple siderophores (Podschun & Ullmann, 1998; Shon et al., 2013). Iron is essential for growth, so siderophores can also play an important role in noninvasive cKP infections (Gomez-Simmonds et al., 2016).

Four siderophores have been described in *K. pneumoniae* and include aerobactin, enterobactin, salmochelin, and yersiniabactin (Podschun & Ullmann, 1998; Shon et al., 2013). Aerobactin is the dominant siderophore of hvKP, it is commonly detected in hvKP strains rather than cKP strains, and directly contributes to bacterial virulence

(Podschun & Ullmann, 1998; Russo, Olson, MacDonald, Beanan, & Davidson, 2015; Shon et al., 2013). Aerobactin, encoded by *iucABCD,* is known for its increased effectiveness because it is recyclable after each transport (Shon et al., 2013). Moreover, functional siderophore systems require a specific surface receptor, and for aerobactin, the receptor is encoded by *iutA* and is intrinsically expressed in *K. pneumoniae* (Shon et al., 2013). As such, *K. pneumoniae* strains lacking the actual siderophore genes can benefit from siderophores secreted exogenously from adjacent strains especially during coinfections (Breurec et al., 2016; Shon et al., 2013). Additionally, unlike most other siderophore systems, which have host transferrin as the main source of iron, the main iron source of aerobactin is the host cell (Shon et al., 2013). Consequently, strains possessing aerobactin and other siderophores will have access to more than one source of iron, leading to their enhanced growth and virulence as seen in hvKP infections (Shon et al., 2013).

Enterobactin on the other hand, is one of the most common siderophores comprising the main iron uptake system of many enterobacteria (Shon et al., 2013). Encoded by *entB,* enterobactin does not contribute to bacterial virulence. It is known to be the siderophore with the highest affinity for ferric iron and an effective competitor of host transferrin bound iron molecules (Gomez-Simmonds et al., 2016; Russo et al., 2015; Shon et al., 2013). Consequently, enterobactin is crucial under iron limiting conditions (Breurec et al., 2016).

Salmochelin, is another siderophore encoded by the *iroBCDEN* gene cluster and has originated in *Salmonella* species before being disseminated into other bacteria (Müller, Valdebenito, & Hantke, 2009). Salmochelin is poorly studied in *K. pneumoniae* and is commonly associated with hvKP (Shon et al., 2013). Finally, yersiniabactin, first

described in *Yersinia* species, is among the most commonly detected virulence associated factors in hvKP and has a direct implication in virulence (Breurec et al., 2016; Lawlor, O'Connor, & Miller, 2007). Yersiniabactin is encoded by the Yersinia pathogenicity island, which encompasses many genes including *ybt, irp1, irp2, fyu,* and *kfu,* and can easily be transferred between species through horizontal gene transfer (HGT) (Breurec et al., 2016; Shon et al., 2013).

1.6.4. Pili

Adhesion molecules known as pili or fimbriae are important for bacterial colonization and attachment to mucosal surfaces (Gerlach, Clegg, & Allen, 1989; Podschun & Ullmann, 1998). *K. pneumoniae* is known for expressing two predominant types of fimbriae, the type 1 and the type 3 (Schembri, Blom, Krogfelt, & Klemm, 2005). Both of these filamentous structures are composed of a protein called pilin (Podschun & Ullmann, 1998).

Type 1 pili are referred to as common pili and are involved in binding bacterial cells to epithelial cells of the respiratory, intestinal or urogenital tracts, or to mucus (Podschun & Ullmann, 1998; Sarno et al., 2002). Consequently, type 1 pili are associated with urinary tract infections, pyelonephritis, and pneumonia (Podschun & Ullmann, 1998). Type 1 pili undergo phase variation, involving the *fim* family of genes (Podschun & Ullmann, 1998). Since they are only involved in bacterial colonization and adhesion and not in subsequent steps of pathogenesis, *K. pneumoniae* is capable of switching off the expression of these genes and prevent the establishment of a host immune response once invasion has taken place (Podschun & Ullmann, 1998). In contrast, type 3 fimbriae, referred to as mannose-resistant *Klebsiella*-like hemagglutinin, are encoded by the *mrk* family of genes and are rarely detected in *Enterobacteriaceae* (Hornick, Allen, Horn, &

Clegg, 1992). Little is known about the contribution of these pili to virulence and pathogenesis, yet they are known for being involved in adhesion to basolateral surfaces and components of basement membranes (Allen, Gerlach, & Clegg, 1991; Podschun & Ullmann, 1998).

1.6.5. Other factors of invasiveness

Other virulence features usually restricted to hvKP include the *allS* gene which enables the organism to metabolize allantoin (Struve et al., 2015). Moreover, the hypermucoviscous phenotype, commonly detected in hvKP, is linked to *rmpA/2* (Shon et al., 2013). These genes are commonly associated with both aerobactin and salmochelin on a large virulence plasmid of 180 to 220 kb in size and have never been detected in cKP isolates (Podschun & Ullmann, 1998; Russo et al., 2015; Shon et al., 2013).

Just like the presence of VFs strictly associated with hvKP, many other are known to be exclusive for cKP, the pathotype responsible for hospital associated infections. These include genes involved in the resistance to many commonly used antiseptics such as the *cepA* and *qacEΔ1* genes (Abuzaid & Amyes, 2015).

1.7. Carbapenem resistance

The clinical importance of *K. pneumoniae* lies in its ability to easily acquire resistance genes and become multidrug resistant (MDR) or extensively-drug resistant (XDR), or even totally-drug resistant (Abuzaid & Amyes, 2015; Magiorakos et al., 2012). MDR *K. pneumoniae* have emerged due to a selective pressure exerted by the inappropriate use of antibiotics (D. Wang et al., 2014). The most common MDR *K. pneumoniae* isolates are the ones classified as being extended-spectrum β-lactamases (ESBL) producing *K. pneumoniae.* ESBLs are capable of hydrolyzing later generation of

cephalosporins and have caused increased incidences of morbidity and mortality (Abuzaid & Amyes, 2015; Al-Marzooq, Yusof, & Tay, 2015; Jiang et al., 2010). Consequently carbapenems were used to treat ESBL-producing *K. pneumoniae* infections (D. Wang et al., 2014). But due to the widespread dissemination of resistance genes, ESBL-producing isolates became highly prevalent, and the overuse of carbapenems created a positive pressure triggering resistance against these agents (Krishnappa, Marie, & Sheikh, 2015; Tokajian, Eisen, Jospin, Farra, & Coil, 2015). It is important to note that, this was also how *K. pneumoniae* became resistant to the most primitive of antibiotics such as penicillins, followed by resistance to aminoglycosides, beta-lactams, fluoroquinolones and in the last decade to carbapenems and the remaining last resort antibiotic agents available (Lombardi et al., 2015).

Carbapenems were once the last resort for the treatment of MDR *K. pneumoniae* until 2001 when the first outbreaks caused by carbapenem-resistant *K. pneumoniae* (CRKP) were reported in North Carolina (Arend, Toledo, Pilonetto, & Tuon, 2015; Gaëlle Cuzon et al., 2010; Lombardi et al., 2015). And since then, they have been frequently identified in multiple nosocomial infections throughout Europe and the Middle East (Gaëlle Cuzon et al., 2010). Carbapenem resistance can arise either through the dissemination of carbapenemases located on mobile genetic elements or through other mechanisms which will be mentioned below.

One of the most prevalent CRKP clones worldwide is classified as multilocus sequence type (ST) ST258 and its derivative ST11 (Brinkworth et al., 2015; Gaëlle Cuzon et al., 2010). These have been previously reported in the Middle East (Brinkworth et al., 2015; Gaëlle Cuzon et al., 2010; Kitchel et al., 2009). Other common CRKP found in the

Middle East include the ST278 and ST14 which are known to carry the *bla*$_{NDM-1}$ gene (Lerner et al., 2016).

These organisms are resistant to all beta-lactams and often to other important therapeutic agents (Brinkworth et al., 2015). Unfortunately, outcomes of CRKP infections are poor, especially if the infections are caused by highly resistant epidemic clones (Khong et al., 2016).

1.7.1. Types of carbapenemases

The most frequently encountered enzymes involved in carbapenem resistance are the Ambler class A KPCs, class B metallo-beta-lactamases (VIM, IMP, OR NDM-1) and the class D OXA-type enzymes (OXA-48-like) (Salloum, Arabaghian, Alousi, Abboud, & Tokajian, 2017). These enzymes are capable of hydrolyzing the highly protected beta-lactam ring of carbapenem antibiotics (Krishnappa et al., 2015). Of importance to Lebanon and this study, the relevant carbapenemases will be discussed below.

Since its first description in late 2009, New Delhi metallo-β-lactamase 1 (NDM-1) has become established as a major public health threat (Karlowsky et al., 2017). NDM-1 was epicentral to India and the countries surrounding it but has shown considerable international dissemination and endemic potential (Karlowsky et al., 2017; Nordmann, Cuzon, & Naas, 2009). As a result, secondary reservoirs currently include the Arabian Peninsula and Northern Africa (Lixandru et al., 2015; Tzouvelekis, Markogiannakis, Psichogiou, Tassios, & Daikos, 2012). NDM-1 has become one of the most clinically important carbapenemases as it efficiently hydrolyses a broad range of β-lactams, including penicillins, cephalosporins, and carbapenems, but spares monobactams such as

aztreonam. As compared with NDM-1, other variants especially, NDM-4, NDM-5, and NDM-7 possess increased activity towards carbapenems (Giske et al., 2012; Nordmann et al., 2009).

bla$_{\text{OXA-48}}$, first identified in *K. pneumoniae* in Turkey, showed geographic confinement to the region until 2008, but worldwide accounts showing its global dissemination were later reported and secondary reservoirs established, which include North African countries and the Middle East (Lixandru et al., 2015; Nordmann et al., 2009; Tzouvelekis et al., 2012). Class D β-lactamases, also named OXAs, include more than 400 enzymes, among which only some variants exhibit carbapenemase activity (mainly OXA-48 family detected in *Enterobacteriaceae*) (Nordmann et al., 2009). Notably, the OXA-48 family possesses weak carbapenemase activity and this does not confer high-level resistance to carbapenems if it is not associated with other factors, such as permeability defects (Lixandru et al., 2015). Additionally, unlike other carbapenemases, OXA-48 like enzymes lack a cephalosporinase activity (Nordmann et al., 2009).

1.7.2. Plasmids

The emergence of carbapenem resistance was facilitated by the conjugational transmission of antibiotic resistance genes located on bacterial mobile genetic elements, such as plasmids, introns, and transposons, across bacterial species and genera (Krishnappa et al., 2015; D. Wang et al., 2014; Ying et al., 2015). Most of these antibiotic resistance genes are allocated on plasmids (Jiang et al., 2010). These are transferred from one organism to the other via HGT. Moreover, these genes are usually coupled with many other antibiotic resistance determinants located on the same plasmid and transferred together from one cell to the other, thus they present an unfortunate therapeutic outcome in cases of severe infections. One such example is the above

mentioned *bla*$_{\text{NDM-1}}$ gene commonly coupled with a huge number of other resistance determinants (The et al., 2015). However, a limitation of HGT is plasmid incompatibility (Inc) which can be defined by the failure of two co-resident plasmids to get transmitted in a single host cell in the lack of external selection (Ying et al., 2015). This phenomenon is mainly due to sharing of one or more elements involved in plasmid replication and partitioning, thus incompatible plasmids belong to the same incompatibility group and can be divided into different types (Ying et al., 2015). IncF plasmids are one of the most common plasmid types associated with the dissemination of resistance determinants in *Enterobacteriaceae* and IncFIIK is the most common plasmid replicon found in *Klebsiella* species (Al-Marzooq et al., 2015). Other common plasmid replicons include the IncR and IncM (Al-Marzooq et al., 2015; Compain et al., 2014). Most of these plasmids range from 3 Kb to 270 Kb in size and carry varying genetic determinants for MDR against aminoglycosides, β-lactams and other antimicrobial classes (Zhao et al., 2010). One of the most common plasmids harboring multiple resistance determinants are the ones which harbor an NDM type carbapenemase (Villa, García-Fernández, Fortini, & Carattoli, 2010). Moreover, these plasmids are commonly multi-replicon in nature and have a broader host range. This manifestation is alarming as it further facilitates their widespread dissemination (Villa et al., 2010). On the other hand, *bla*$_{\text{OXA-48}}$ harboring plasmids usually possess the IncL/M or IncA/C groups and do not harbor multiple resistance determinants on the same plasmid (Zowawi et al., 2015).

1.7.3. Other mechanisms of carbapenem resistance

In Gram-negative organisms, carbapenem resistance can also be due to mechanisms other than carbapenemase production (Krishnappa et al., 2015). First of

such mechanisms is the loss or modification of porin channels, such as OmpK35, OmpK36 or others, coupled with the upregulation of β-lactamase production such as ESBLs of the OXA, TEM, SHV, or CTX-M-type, or AmpC β-lactamases such as DHA-type enzymes (Baroud et al., 2012; Martínez-Martínez et al., 1999; Nordmann, Dortet, & Poirel, 2012). Porin modifications include mutations which may lead to either the premature truncation of the protein leading to complete loss or an ineffective porin (Baroud et al., 2012; Krishnappa et al., 2015).

The second possible mechanism which may lead to carbapenem resistance in carbapenemase non-producing *K. pneumoniae* is the upregulation of efflux pumps leading to the active export of carbapenems entering the bacterial cell (Baroud et al., 2012). Genes involved in this mechanism include AcrAB and OqxAb, but are less common than the first mechanism described above (Nordmann et al., 2012).

1.8. Treatment

Currently, only few antimicrobial agents exist to treat patients with a CRKP infection. The most commonly used ones are colistin and tigecycline. Although resistance is very rare, there have been many reports of colistin or tigecycline resistant CRKP (Lerner et al., 2016; Weterings et al., 2015). To a lesser extent, fosfomycin is used in combination with doxycycline and meropenem in the treatment of CRKP (Kyle, Stollings, White, Noto, & Wheeler, 2015; Lerner et al., 2016). Unfortunately, the increase of CRKP had led to an increase in resistance to these last resort agents.

Chapter Two

Materials and Methods

2.1. Bacterial isolate collection

A total of 32 *K. pneumoniae* and two *K. quasipneumoniae* isolates collected from 2012 to 2015 were provided by the American University of Beirut Medical Center (AUBMC) and were designated as KP1-KP32 and KQ1-KQ2, respectively. AUBMC is a 350-bed tertiary care teaching hospital. The isolates were collected from various body sites such as urine, skin wounds including one from a C-section, among others, as part of the hospital's routine clinical processing (Table 1). The mean patients' age was 58 ± 21 years old, with a range of 18 to 87 years with a 1:1 male to female ratio. Initial species identification was performed based using the API 20E system (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions.

Table 1. Isolate information including type of specimen, patients' sex and age, isolation year and NCBI accession numbers.

Name	Source	Sex	Age	Year	Accession #
KP1	DTA	M	75	2012	NIBY00000000
KP2	C Section Wound	F	38	2012	NIBZ00000000
KP3	Urine	F	18	2013	NICA00000000
KP4	Urine	F	87	2013	NICB00000000
KP5	DTA	M	67	2013	NICC00000000
KP6	Cyst	M	20	2013	NICD00000000
KP7	Drain Site	F	83	2013	NICE00000000
KP8	Drain Site	F	83	2013	NICF00000000
KP9	Urine	F	63	2013	NICG00000000
KP10	Urine	F	71	2013	NICH00000000
KP11	Lung Swab	M	69	2013	NICI00000000
KP12	DTA	F	79	2013	NIFL00000000
KP13	DTA	F	79	2013	NIFM00000000
KP14	Urine	M	72	2013	NICJ00000000
KP15	Urine	M	73	2013	NICK00000000

KP16	Unknown	F	82	2013	NICL00000000
KP17	Urine	F	33	2013	NICM00000000
KP18	DTA	M	59	2013	NICN00000000
KP19	Urine	M	68	2013	NICO00000000
KP20	Leg Tissue	M	22	2013	NICP00000000
KP21	Incision Site Swab	M	32	2014	NICQ00000000
KP22	Skin	F	50	2014	NICR00000000
KP23	Skin	M	64	2014	NICU00000000
KP24	Peripheral	M	54	2015	NICV00000000
KP25	Urine	F	33	2015	NICW00000000
KP26	Wound	F	74	2015	NICX00000000
KP27	Urine	M	30	2015	NICY00000000
KP28	Urine	M	74	2015	NICZ00000000
KP29	Skin	M	59	2015	NWBX00000000
KP30	Urine	F	57	2013	NWBY00000000
KP31	Skin	M	62	2015	NWBZ00000000
KP32	Urine	M	30	2015	NWCA00000000
KQ1	Isolate	F	36	2014	NICS00000000
KQ2	Pus	F	79	2014	NICT00000000

2.2. String Test

A phenotypic screening test called the string test was used to assess the hypermucoviscosity of bacterial colonies. The test was performed on fresh bacterial colonies, as previously described (Shon et al., 2013). A positive result was assigned in cases where a viscous string was formed with at least five mm in length upon stretching a bacterial colony on an agar plate using an inoculation loop.

2.3. Antimicrobial susceptibility testing

AST was performed by the disk diffusion technique on Mueller-Hinton agar. It included a panel of 26 antibiotic disks belonging to 15 categories. The tested drug categories included: aminoglycosides (amikacin and gentamicin), phosphonic acids (fosfomycin), polymyxins (colistin), glycylcyclines (tigecycline), fluoroquinolones (ciprofloxacin, norfloxacin, levofloxacin, and ofloxacin), nitrofurans (nitrofurantoin), penicillin

(ampicillin), penicillin with a β-lactamase inhibitor (augmentin and tazobactam), monobactams (aztreonam), cephamycin (cefoxitin), non-extended and extended-spectrum cephalosporins (cefuroxime, ceftazidime, cefamandole, cefotaxime, cefixime, and cefepime), tetracycline, folate pathway inhibitors (trimethoprim) and carbapenems (ertapenem, imipenem, and meropenem). The obtained data was interpreted according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017) and (Galani et al., 2008) (2008).

2.4. Bacterial DNA extraction

Bacterial DNA extraction was performed from fresh bacterial colonies grown on TSA plates using the Nucleospin® Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instructions.

2.5. PCR amplification and sequencing of the *wzi* gene

wzi gene typing was performed to determine the capsular type of the isolates. PCR amplification and sequencing was performed using *wzi*-TR and *wzi*-TF primers as previously described (Brisse et al., 2013) (Table 2). The Institute Pasteur database was used to assign the K-types (http://bigsdb.pasteur.fr/klebsiella). K-types that could not be differentiated solely based on the *wzi* gene or had no K-types associated with the allelic variant were re-analyzed using the publically available Kaptive online tool, which analyses the entire *cps* locus and assigns capsular locus types (Wyres et al., 2016) (https://github.com/katholt/Kaptive).

2.6. Detection of resistance genes by PCR assays

The most common ESBLs and carbapenemases encoding genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{IMP}, bla_{VIM}, bla_{KPC}, $bla_{\text{NDM-1}}$, and $bla_{\text{OXA-48-like}}$, were amplified as previously described

(Elumalai, Muthu, Selvam, & Ramesh, 2014; Li et al., 2012; Lixandru et al., 2015; Pérez-Pérez & Hanson, 2002; Voets, Fluit, Scharringa, Cohen Stuart, & Leverstein-van Hall, 2011; D. Wang et al., 2014) (Table 2).

Table 2. Resistance genes targeted by PCR assays, their corresponding primer sequences and amplicon sizes.

Target	Primer	Sequence 5'-3'	Size
wzi	wzi-F	GTGCCGCGAGCGCTTTCTATCTTGGTATTCC	580 bp
	wzi-R	GAGAGCCACTGGTTCCAGAA[C or T]TT[C or G]ACCGC	
bla$_{SHV}$	SHV-F	GGTTATGCGTTATATTCGCC	865 bp
	SHV-R	TTAGCGTTGCCAGTGCTC	
bla$_{CTX-M}$	CTX-M-F	ATGTGCAGYACCAGTAARGT	593 bp
	CTX-M-R	TGGGTRAARTARGTSACCAGA	
bla$_{IMP}$	IMP-F	GGAATAGAGTGGCTTAATTCTC	624 bp
	IMP-R	CCAAACCACTACGTTATC	
bla$_{VIM}$	VIM-F	AGTGGTGAGTATCCGACAG	212 bp
	VIM-R	TCAATCTCCGCGAGAAG	
bla$_{KPC}$	KPC-F	ATGTCACTGTATCGCCGTC	882 bp
	KPC-R	TTACTGCCCGTTGACGCC	
bla$_{NDM-1}$	NDM-1-F	TGCATTGATGCTGAGCGGGTG	621 bp
	NDM-1-R	ATCACGATCATGCTGGCCTTG	
bla$_{OXA-48-like}$	O48-GDM-F	CCAAGCATTTTTACCCGCATCKACC	389 bp
	O48-GDM-R	GYTTGACCATACGCTGRCTGCG	

2.7. Plasmid replicon typing

Plasmid typing was performed using the DIATHEVA PBRT kit (Diatheva, Fano, Italy) through a PCR-based replicon detection consisting of eight multiplex PCR assays for the amplification of 25 replicons found in the family *Enterobacteriaceae*: A/C, B/O, FIA, FIB, FIB-M, FIC, FII, FIIK, FIIS, HI1, HI2, HIB-M, I1, I2, K, L/M, N, P, R, T, U, W, X1, X2, and Y. Positive controls were included for all reactions. PCR reactions were performed following the manufacturer's instructions and visualized on a 2.5% agarose gel stained with ethidium bromide.

2.8. Multilocus sequence typing (MLST)

MLST was performed following the previously described scheme for seven housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB* and *tonB*) using primers with universal sequencing tails. Genes were sequenced using the universal oF and oR primer pair (Diancourt, Passet, Verhoef, Grimont, & Brisse, 2005). STs were assigned using the Institute Pasteur database. Novel STs were submitted to the curator and assigned with new designations (www.pasteur.fr/mlst)**.**

2.9. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using the XbaI restriction enzyme (ThermoScientific, Waltham, MA, USA). The universal laboratory standard *Salmonella enterica subsp. enterica serovar Braenderup* (ATCC® BAA664™) was used as a reference. Electrophoresis was carried out using the Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA) with a run time of 12 h and switch time of 5–40 s (https://www.cdc.gov/pulsenet/) according to the standard PulseNet protocol (http://www.pulsenetinternational.org). Gels were stained with ethidium bromide. For samples showing identical pulsotypes (PT) or were untypable by XbaI, PFGE was repeated using AvrII (ThermoScientific, Waltham, MA, USA).

PFGE fingerprints were analyzed with the BioNumerics software version 7.6.1 (Applied Maths, Belgium), with profiles assigned as different PTs if a difference of three or more bands was detected. Clustering was accomplished using the BioNumerics software through dice correlation coefficients with an optimization of 1% and tolerance of 1%.

2.10. Whole-genome sequencing

Genomic libraries were constructed using the Nextera XT DNA library preparation kit with dual indexing (Illumina, San Diego, CA, USA). The libraries were sequenced on an Illumina MiSeq with 250 bp x 2 read length. 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).

2.11. Genome assembly and annotation

The assembled genomes were annotated using the RAST online server (http://rast.nmpdr.org). The Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017) and ResFinder 3.0 (Zankari et al., 2012) available on the Center for Genomic Epidemiology website (www.genomicepidemiology.org) were used to determine the presence of resistance genes. The presence of putative virulence factors (VFs) was screened using the VF scheme available on http://bigsdb.pasteur.fr. *in silico* STs were determined using the MLST 1.8 server (Larsen et al., 2012). The presence of plasmids in the genomic sequences was determined using PlasmidFinder 1.3 (Carattoli et al., 2014). The Plasmid Constellation Network (PLACNETw) was used to identify and analyze plasmids starting from raw reads by creating a network of contig interactions (Lanza et al., 2014).

2.12. Comparative genome analysis

Plasmid sequences were extracted and aligned with corresponding reference strains using BioNumerics software version 7.6.1(Applied Maths, Sint-Martens-Latem, Belgium).

2.13. wgSNP-based phylogenetic analysis

The 34 genomes were aligned using a comparative approach with eight other complete genomes of previously described strains with the following accession numbers; MGH63 (accession # JJNB00000000), KGM-IMP126 (accession # LJOI00000000), HK787 (accession # NZ_CP006738.1), MGH78578 (accession # CP000647.1), KPN01 (accession # CP012987.1), Kp_Goe_149473 (accession # CP018686.1), LAU-KP1 (accession # AYQE00000000.1), KP_Z4175 (accession # LVCD01000000). Mapped against the reference genome *K. pneumoniae* strain KP_ST11_OXA-48 (accession # JNHB00000000.1) using the Burrows–Wheeler Aligner (BWA). SNP-calling was done by mapping the paired-end reads of the genomes on BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium). All insertions and deletions were excluded. And a neighbor-joining (NJ) tree based on categorical differences was drawn in BioNumerics by using the wgSNP data as input. The tree was rooted with the genome sequence of *Streptococcus pneumoniae* MGAS315 (accession # AE014074.1).

Chapter Three

Results

3.1. Isolates typing

MLST and K-typing were performed for sequence based molecular typing of the isolates. A total of 21 different STs including, ST15 (14.7%; 5/34), ST16 (11.8%; 4/34), ST45 (8.8%; 3/34), ST2720 (5.9%; 2/34), ST1799 (5.9%; 2/34), ST147 (5.9%; 2/34), and singlets (2.9%; 1/34) were detected (Figure 1). Two new MLST allelic profiles were detected and curated, and new STs were assigned. The allelic profile of ST2719 was 2-1-151-87-7-1-12, while for ST2720 it was 42-22-26-96-86-20-51 as an allelic profile (Figure 2).

wzi gene sequencing identified thirteen different K-types. 14.7% (5/34) of the isolates were identified as K15/K17/K50/K51/K52, which was the most prevalent, while 26.5% (9/34) were not linked to any K-type. Consequently, for the isolates that were untypable or not differentiable solely by *wzi* sequencing, Kaptive was used to extract capsular locus types from the whole-genome data. Using Kaptive, 20 different K-types were inferred from the entire *cps* locus and were used throughout the manuscript.

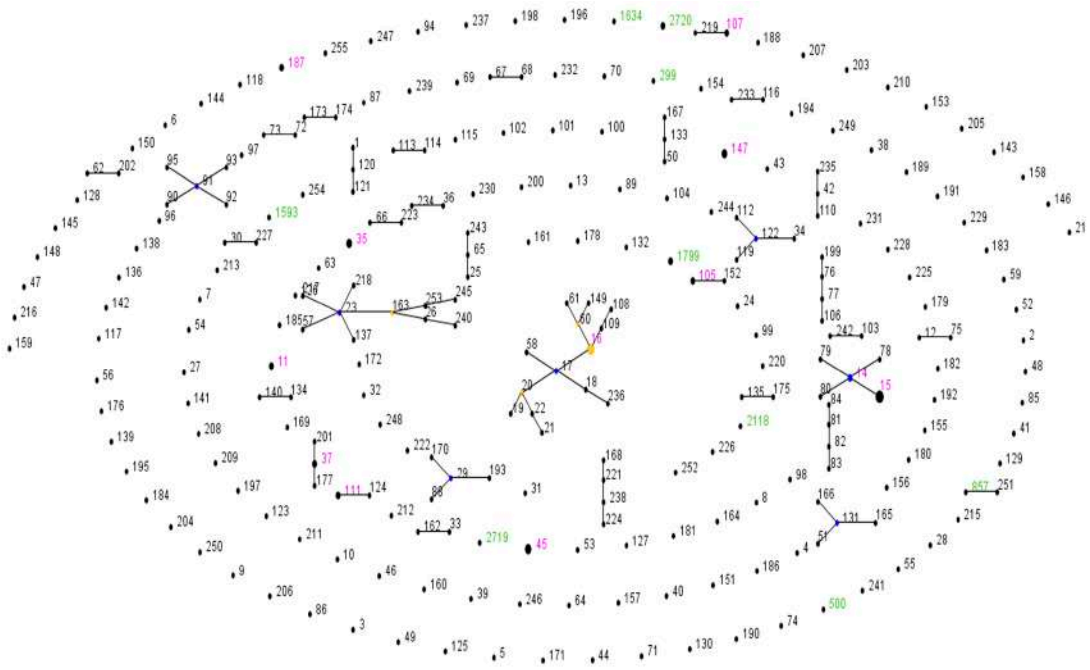


Figure 1: A population snapshot of all detected STs and their relatedness generated using eBURST V3. Detected STs and their relatedness with other global STs.

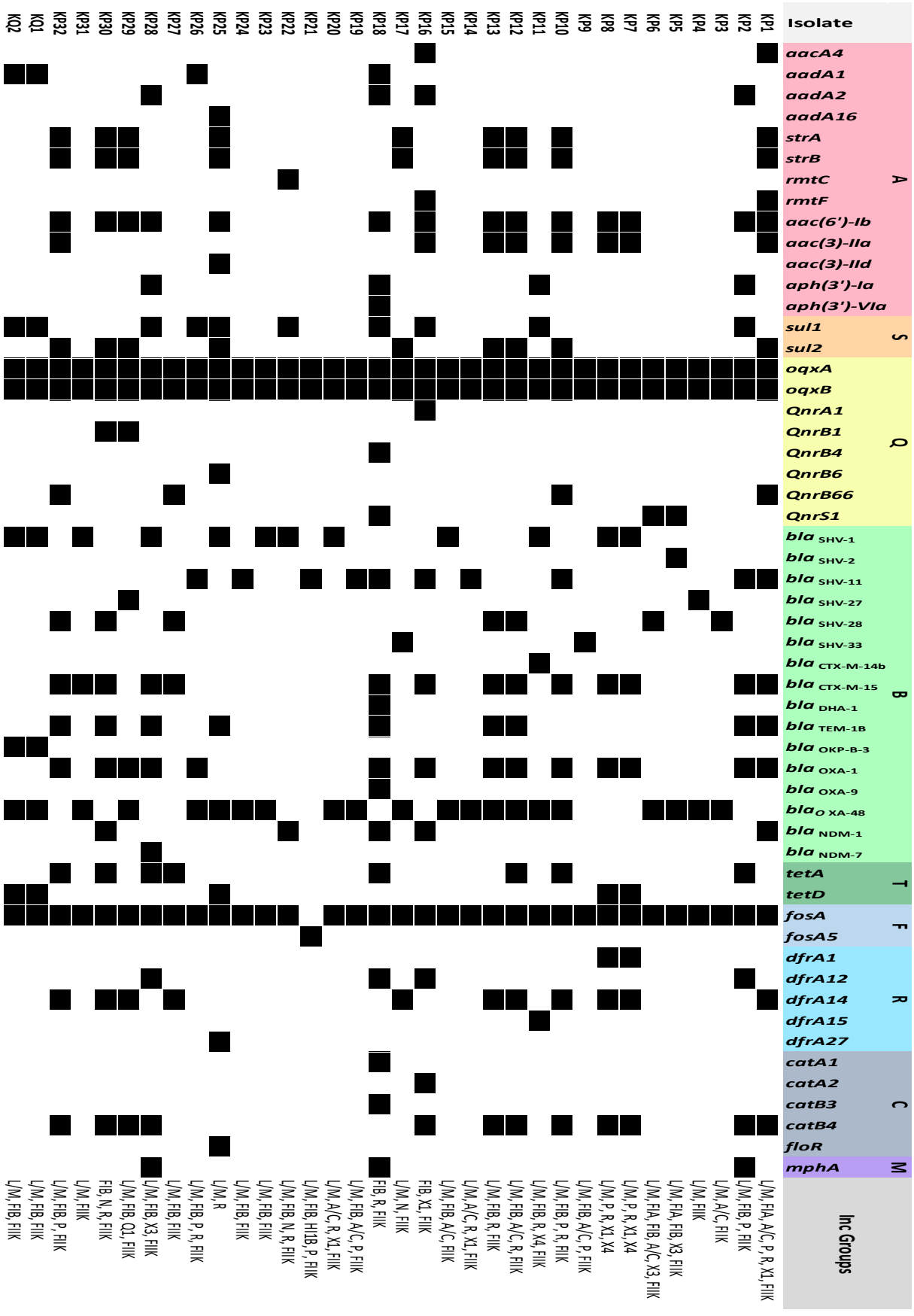

Isolate
A
S
Q
B
T
F
R
C
M
aacA4
aadA1
aadA2
aadA16
strA
strB
rmtC
rmtF
aac(6')-Ib
aac(3)-IIa
aac(3)-IId
aph(3')-Ia
aph(3')-VIa
sul1
sul2
oqxA
oqxB
QnrA1
QnrB1
QnrB4
QnrB6
QnrB66
QnrS1
bla SHV-1
bla SHV-2
bla SHV-11
bla SHV-27
bla SHV-28
bla SHV-33
bla CTX-M-14b
bla CTX-M-15
bla DHA-1
bla TEM-1B
bla OKP-B-3
bla OXA-1
bla OXA-9
bla OXA-48
bla NDM-1
bla NDM-7
tetA
tetD
fosA
fosA5
dfrA1
dfrA12
dfrA14
dfrA15
dfrA27
catA1
catA2
catB3
catB4
floR
mphA
KQ2
KQ1
KP32
KP31
KP30
KP29
KP28
KP27
KP26
KP25
KP24
KP23
KP22
KP21
KP20
KP19
KP18
KP17
KP16
KP15
KP14
KP13
KP12
KP11
KP10
KP9
KP8
KP7
KP6
KP5
KP4
KP3
KP2
KP1
Inc Groups
L/M, FIB, FIIK
L/M, FIB, FIIK
L/M, FIB, P, FIIK
FIB, N, R, FIIK
L/M, FIIK
L/M, FIB, X3, FIIK
L/M, FIB, Q1, FIIK
L/M, FIB, FIIK
L/M, FIB, P, R, FIIK
L/M, R
L/M, FIB, FIIK
L/M, FIB, FIIK
L/M, FIB, N, R, FIIK
L/M, FIB, H1B, P, FIIK
L/M, A/C, R, X1, FIIK
L/M, FIB, A/C, P, R, FIIK
FIB, R, FIIK
L/M, N, FIIK
FIB, X1, FIIK
L/M, FIB, A/C, FIIK
L/M, A/C, R, X1, FIIK
L/M, FIB, R, FIIK
L/M, FIB, A/C, R, FIIK
L/M, FIB, R, X4, FIIK
L/M, FIB, P, R, FIIK
L/M, FIB, A/C, P, FIIK
L/M, P, R, X1, X4
L/M, P, R, X1, X4
L/M, FIA, FIB, A/C, X3, FIIK
L/M, FIA, FIB, X3, FIIK
L/M, FIIK
L/M, A/C, FIIK
L/M, FIB, P, FIIK
L/M, FIA, A/C, P, R, X1, FIIK

Figure 2: Antibiotic resistance genes and incompatibility groups detected by PCR, PBRT, and *in silico*. Classes of antibiotic resistance genes are labeled in the following way: A, aminoglycoside resistance genes; S, sulfonamide resistance genes; Q, quinolone resistance genes; B, β-lactam resistance genes; T, tetracycline resistance genes; F, fosfomyocin resistance genes; R, trimethoprim resistance genes; C, chloramphenicol resistance genes; M, macrolide resistance genes.

3.2. Susceptibility profiling

Isolate selection criterion was the non-susceptibility to at least one of the three clinically tested carbapenems, namely imipenem, ertapenem, or meropenem. All of the isolates were non-susceptible to ertapenem, 85.3% (29/34) to imipenem, and 70.6% (24/34) to meropenem (Table 3-4).

Regarding last resort antibiotics used in the treatment of CRKP, 32.3% (11/34) of the isolates were intermediate to colistin. Lowest observed resistances were for amikacin and fosfomycin with 14.7% (5/34) and 8.8% (3/34) of the isolates being resistant, respectively, whereas 23.5% (8/34) of the isolates were non-susceptible to tigecycline.

Almost 23% (8/34) of the isolates were identified as XDRs (KP1, KP2, KP7, KP16, KP27, KP28, KP30, and KP32). An XDR organism is defined as being non-susceptible (which includes resistant and intermediate phenotypes) to at least one antibiotic agent in all but two or fewer of the categories chosen (Magiorakos et al., 2012). All of the above-mentioned XDR isolates remained susceptible to two drug categories with the exception of KP30, which remained susceptible only to phosphonic acids.

Tables 3 and 4 further elucidate the resistance patterns for all of the isolates against the tested antimicrobial categories and agents.

Table 3: Antimicrobial susceptibility testing results for some of the tested antibiotics against all of the isolates.

Isolate	Aminoglycosides		Penicillin	Penicillin + b-lactamase inhibitor		Monobactam	Non-extended Cephalosporin	Extended-spectrum Cephalosporin					Cephamycin
								2nd generation Cephalosporins	4th generation Cephalosporins	3rd generation Cephalosporins			
	Amikacin	Gentamicin	Ampicillin	Augmentin	Tazobactam	Aztreonam	Cefuroxime	Cefamandole	Cefepime	Ceftazidime	Cefotaxime	Ceftixime	Cefoxitin
KP1	R	R	R	R	R	R	R	R	R	R	R	R	R
KP2	S	S	R	I	I	R	R	R	R	R	R	R	R
KP3	S	S	R	R	R	R	R	R	S	R	R	R	S
KP4	S	S	R	R	R	S	I	I	S	S	S	S	S
KP5	S	S	R	R	R	R	R	R	S	R	R	S	S
KP6	S	S	R	R	R	R	R	R	R	R	R	R	S
KP7	S	R	R	I	I	R	R	R	R	R	R	R	R
KP8	S	R	R	I	R	R	R	R	R	R	R	R	I
KP9	S	S	R	R	R	S	I	I	S	S	S	S	S
KP10	S	R	R	R	R	R	R	R	S	R	R	R	S
KP11	S	S	R	R	R	I	R	R	I	S	R	R	R
KP12	S	R	R	R	R	R	R	R	R	R	R	R	S
KP13	S	R	R	R	R	R	R	R	R	R	R	R	S
KP14	S	S	R	R	R	S	I	I	S	S	I	S	S
KP15	S	S	R	R	R	S	R	R	S	S	R	S	R
KP16	R	R	R	R	R	R	R	R	R	R	R	R	R
KP17	S	S	R	R	R	R	R	R	R	R	R	R	R
KP18	S	S	R	R	S	R	R	R	R	R	R	R	R
KP19	S	S	R	R	R	S	I	I	S	S	S	S	S
KP20	R	R	R	R	R	R	R	R	S	R	R	R	R
KP21	S	R	R	I	S	R	R	R	R	R	R	S	S
KP22	R	R	R	R	R	S	R	R	R	R	R	R	R
KP23	S	S	R	R	R	S	S	S	S	S	S	S	S
KP24	S	S	R	R	R	S	I	I	S	S	I	S	S
KP25	S	R	R	R	R	S	S	S	S	S	S	S	S
KP26	S	S	R	R	R	S	S	S	S	S	I	S	S
KP27	S	S	R	R	S	R	R	R	R	R	R	R	R
KP28	S	S	R	R	R	R	R	R	R	R	R	R	R
KP29	S	S	R	R	R	S	S	S	S	S	S	S	S
KP30	R	R	R	R	R	R	R	R	R	R	R	R	R
KP31	S	S	R	R	R	S	S	S	S	S	S	S	S
KP32	S	R	R	I	I	R	R	R	R	R	R	R	R
KQ1	S	S	R	R	R	S	S	S	S	S	S	S	S
KQ2	S	S	R	R	R	S	S	S	S	S	S	S	S

Table 4: Antimicrobial susceptibility testing results for some of the tested antibiotics against all of the isolates.

Isolate	Fluoroquinolones				Nitrofurans	Tetracycline	Folate pathway inhibitors	Carbapenems			Phosphonic acids	Polymyxins	Glycyclines
	Ciprofloxacin	Levofloxacin	Norfloxacin	Ofloxacin	Nitrofurantoin	Tetracycline	Trimethoprim	Ertapenem	Imipenem	Meropenem	Fosfomycin	Colistin	Tigecycline
KP1	R	R	R	R	R	R	R	R	R	R	S	S	I
KP2	R	R	R	R	R	R	R	R	S	S	I	S	I
KP3	R	R	R	R	R	R	R	R	R	I	S	S	S
KP4	S	S	S	S	R	S	S	R	R	I	S	S	S
KP5	I	I	S	R	I	S	S	R	R	I	S	I	S
KP6	S	S	R	S	R	S	S	I	I	I	S	I	S
KP7	R	R	R	R	R	R	R	R	S	I	R	S	S
KP8	R	R	R	R	I	R	R	R	S	S	S	S	S
KP9	S	S	S	S	S	S	S	R	I	S	R	S	S
KP10	I	I	I	I	S	R	R	I	I	S	S	S	S
KP11	S	S	S	S	S	R	R	R	I	S	S	S	I
KP12	R	R	R	R	S	R	R	R	R	S	S	S	S
KP13	R	R	R	R	S	S	R	R	R	I	S	S	S
KP14	S	S	S	S	R	S	S	R	R	R	S	S	S
KP15	S	S	S	S	R	S	S	R	R	R	R	I	I
KP16	R	R	R	R	R	R	R	R	R	R	S	I	S
KP17	S	S	S	S	R	S	R	R	R	R	S	I	S
KP18	R	R	R	R	R	R	R	R	R	R	S	I	S
KP19	S	S	S	S	I	S	S	R	R	R	S	S	S
KP20	R	R	S	S	R	R	R	R	R	S	S	S	S
KP21	I	I	S	S	R	R	R	I	S	S	S	S	S
KP22	S	S	S	S	R	S	S	R	R	R	S	S	S
KP23	S	S	S	S	S	S	S	R	R	I	S	S	S
KP24	S	S	S	S	S	S	S	R	R	I	S	I	S
KP25	S	S	S	S	S	R	R	I	R	S	S	S	S
KP26	S	S	S	S	R	S	S	R	R	I	S	S	S
KP27	R	R	R	R	R	R	R	R	R	I	S	I	R
KP28	R	R	R	R	R	R	R	R	R	R	S	I	I
KP29	I	S	S	S	S	S	R	R	R	R	S	I	S
KP30	R	R	R	R	R	R	R	R	R	S	S	I	I
KP31	S	S	S	S	R	S	S	R	R	I	S	S	S
KP32	R	R	R	R	R	R	R	R	S	I	S	S	R
KQ1	S	S	S	S	S	R	S	I	I	I	S	S	S
KQ2	S	S	S	S	S	R	S	R	R	I	S	S	S

3.3. Resistance genes

The AST results were supported by PCR based ARGs detection and ResFinder. Individual PCRs and *in silico* analysis revealed the presence of 54 distinct genes. Each gene conferred resistance to one of the nine antibacterial categories including aminoglycosides, sulfonamides, quinolones, β-lactams, tetracyclines, fosfomycin, trimethoprim, chloramphenicol, and macrolides (Figure 2).

Macrolides was the only category where a single gene, *mphA*, was detected and it was present in 8.8% (3/34) of the isolates (KP2, KP18, and KP28). KP2, KP18, and KP28 had at least one resistant determinant belonging to each one of the nine listed categories. For all remaining antimicrobial categories, multiple genes were detected.

Core chromosomal genes, *oqxA* and *oqxB*, which confer low-level resistance to quinolones, were detected in all of the isolates, whereas 32.4% (11/34) of the isolates had at least one additional quinolone resistance determinant with *qnrB66* being the most prominent (11.8%; 4/34). KP18 however, had two additional quinolone resistance determinants, *qnrB4* and *qnrS1*.

Another core chromosomal resistant determinant, *fosA*, which confers low-level resistance to fosfomycin, was detected in 97.1% (33/34) of the isolates. It is noteworthy that KP21 (2.9%; 1/34) harbored the *fosA5* variant.

Genes conferring resistance to β-lactams showed the highest level of variability. β-lactams exhibited 16 different genes and variants. Carbapenemases were encountered in 79.4% (27/34) of the isolates, harboring one of the three identified enzymes. $bla_{\text{OXA-48}}$ was the most common (61.2%; 21/34), followed by $bla_{\text{NDM-1}}$ (14.7%; 5/34), and $bla_{\text{NDM-7}}$ (2.9%; 1/34). ESBLs were the most common β-lactamase category detected. Five

variants of *bla*$_{\text{SHV-1, 2, 11, 27, 28, 33}}$ were detected in this study. *bla*$_{\text{CTX-M-15}}$ was the most common ESBL variant, and was seen in 41.2% (14/34) of the isolates. Additionally, a chromosomally-encoded β-lactamase, *bla*$_{\text{OKP-B-3}}$, was found in both *K. quasipneumoniae* isolates (KQ1 and KQ2).

Thirteen different aminoglycosides resistance genes and their variants were detected. The most common was *aac(6')-Ib* and was found in 41.2% (14/34) of the isolates.

Five different gene variants encoding for both chloramphenicol and trimethoprim resistance were detected. *catB4* (35.3%; 12/34) and *dfrA14* (32.4%; 11/34) were the most common for each of the categories, respectively. In contrast, sulfonamides and tetracyclines showed very little variability with only two variants being detected for each: *sul1* (29.4%; 10/34) and *tetA* (23.5%; 8/34) were the most common for each category, respectively.

Figure 2 further elucidates the different combinations of ARGs detected in each of the 34 isolates.

3.4. Incompatibility profiling

PCR-based replicon typing (PBRT) and PlasmidFinder 1.3 successfully identified 13 Inc groups (Figure 2). By using both of these methods, a complete information regarding Inc groups was depicted; groups that were not covered by the PBRT kit, such as IncX4, were detected by PlasmidFinder, whereas many others missed in the *in silico* analysis of the draft genomes were detected by the PBRT kit.

At least three Inc groups were identified in the majority of the tested isolates (88.2%; 30/34). KP1 had seven Inc groups, which was the highest. PLACNETw revealed the one

big multi-replicon plasmid, carrying IncFIIK, IncFIA, and IncR, and three smaller plasmids (Figure 3).

The most commonly identified Inc groups were IncFIIK and IncL/M, each present in 91.2% (31/34) of the isolates, followed by IncFIB (70.1%; 24/34), IncR (41.2%; 14/34), IncP (29.4%; 10/34), IncA/C (26.5%; 9/34), and IncX1 (17.6%; 6/34). Many other Inc groups were also detected, but with much lower prevalence (Figure 2).

IncX3 carrying the *bla*$_{\text{NDM-7}}$ gene was of interest, consequently its genetic environment was studied in details (Figure 4).

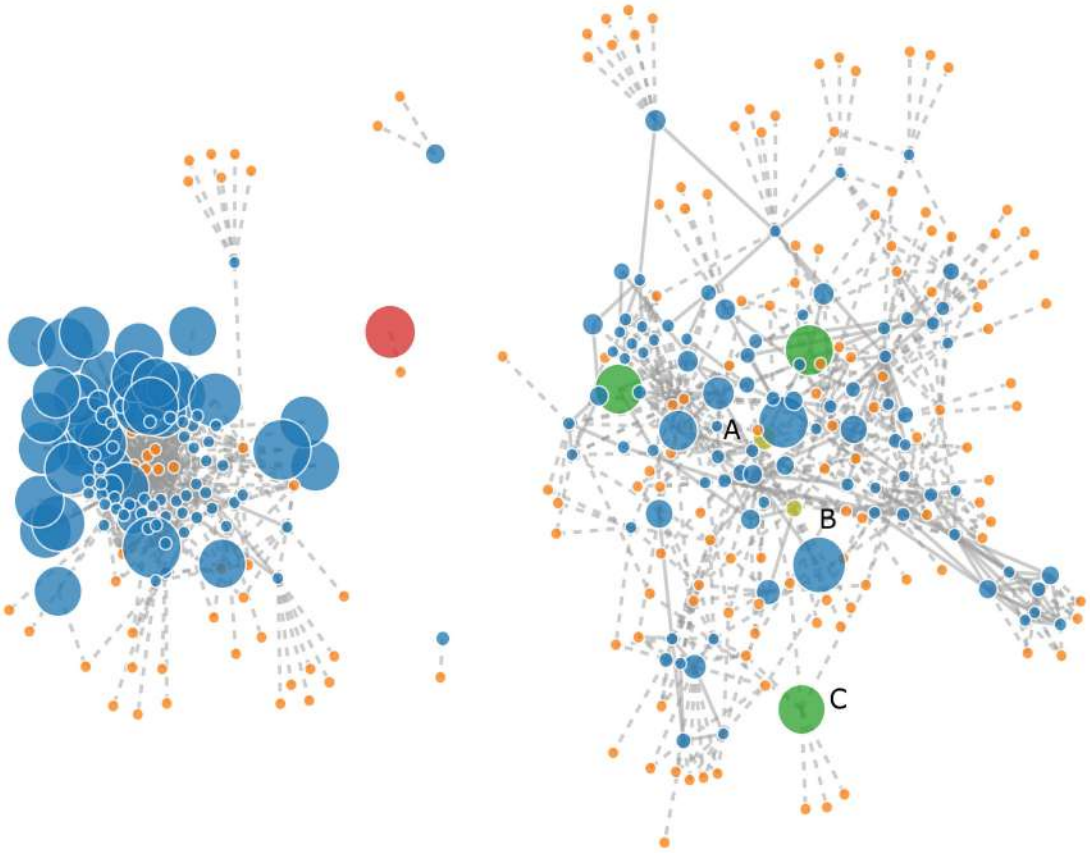


Figure 3: A schematic representation of the KP1 genome using PLACNETw. KP1 contained a single big multi-replicon plasmid (with three replicons A, B, and C) and three smaller plasmids. A: IncFIA, B: IncR, C: IncFIIK, green circle: relaxase and replication initiator site; red circle: relaxase; yellow circle: replication intitiator site; blue circle: contigs.

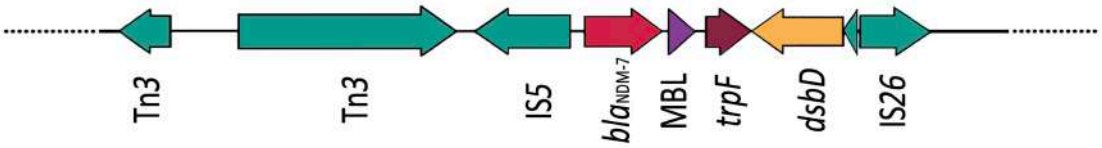


Figure 4: Genetic environment of bla_{NDM-7} allocated on an IncX3 plasmid in KP28. bla_{NDM-7} was carried by an IS*5*-bla_{NDM-7}-ble_{MBL}-*trpF*-*dsbD*-IS*26* genetic element.

3.5. Virulence factors

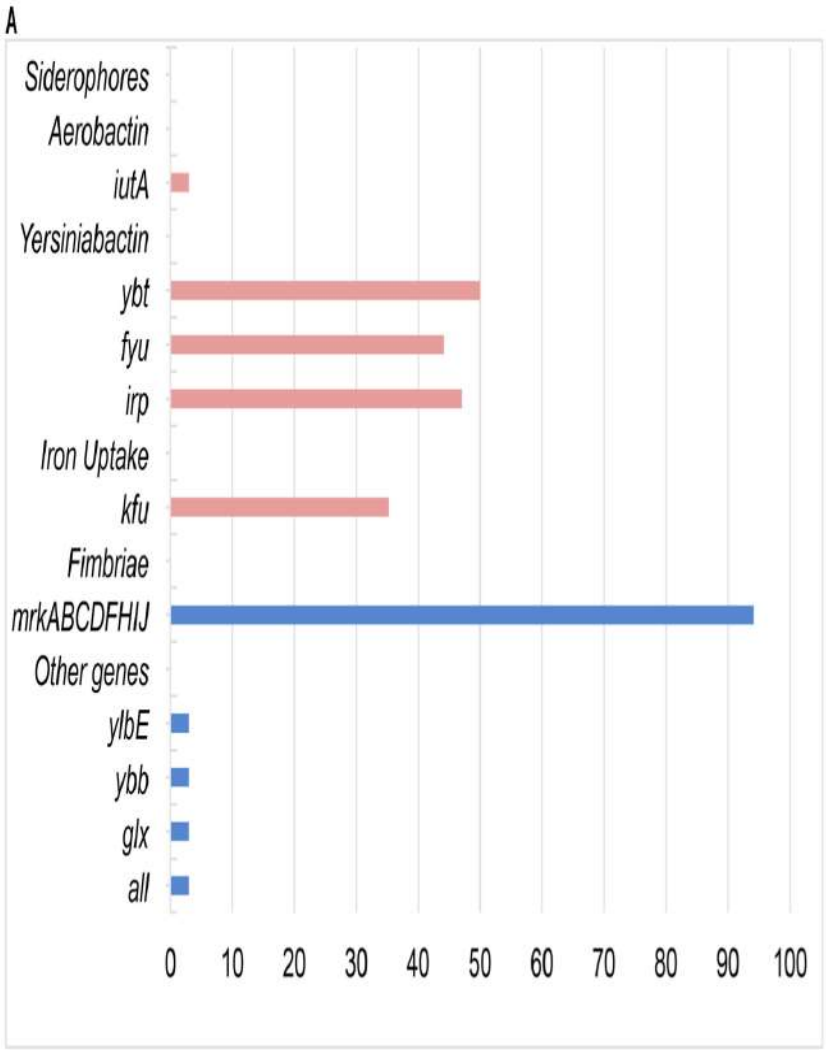
Fimbriae encoding genes *mrkABCDFHIJ* were the most common virulence associated factor detected in 94.1% (32/34) of the isolates. As for yersiniabactin siderophores, encoded by *ybt*, *fyu,* and *irp*, at least one was detected in 56% (19/34) of the isolates. The *kfu* iron uptake system and *iutA* aerobactin siderophore were found in 35% (12/34) and 2.9% (1/34) of the isolates, respectively. *all,* the activator of the allantoin regulon, *glx*, *ybb*, and *ylbE,* were only detected in KP25. The highest number of VFs was detected in KP5 and KP6 isolates having 26 factors each (Table 5).

Isolates identified as *K. quasipneumoniae*, KQ1 and KQ2, had similar repertoire of VFs having only *mrkABCDFHIJ w*ith no detected siderophores.

The convergence of VFs and ARGs was assessed (Figure 5) to detect newly emerging *K. pneumoniae* clones. The analysis showed that 23.5% (8/34) of the isolates (KP1, KP2, KP10, KP13, KP25, KP28, KP29, and KP32) possessed 17-21 VFs with 13-18 ARGs. Of interest, was one XDR isolate, KP32, which was recovered from the urine of a 30-year old male, had a positive hypermucoviscous phenotype, and was designated as hvKP. The isolate was negative for *rmpA* and *rmpA2* genes, regulators of the hypermucoviscous phenotype in *K. pneumoniae* (Y. M. Liu et al., 2014).

Table 5: The prevalence of all virulence determinants searched in the isolates.

Isolate	# of VFs	Siderophore						Iron uptake	Hypermucoviscous		LPS	Adhesins and Biofilm			Tellurite resistance	Colibactin	Porins (Loss of porins=> CR-KP)		Others			
		Yersiniabactin			Aerobactin	Salmochelin	Enterobactin					Fimbriae		Pili (Twitching motility)								
		ybt	*fyu*	*irp*	*iucABCD*	*iutA*	*iro*	*ent*	*kfu*	*rmpA*	*rmpA2*	*waa*	*fim*	*mrkABCDFHIJ*	*pil*	*ter*	*pks*	*ompK35*	*ompK36*	*all*	*glx*	*ybb*	*ybtE*
KP1	18																						
KP2	19																						
KP3	17																						
KP4	6																						
KP5	26																						
KP6	26																						
KP7	8																						
KP8	8																						
KP9	22																						
KP10	17																						
KP11	18																						
KP12	21																						
KP13	21																						
KP14	5																						
KP15	11																						
KP16	1																						
KP17	24																						
KP18	8																						
KP19	11																						
KP20	16																						
KP21	15																						
KP22	4																						
KP23	18																						
KP24	10																						
KP25	17																						
KP26	15																						
KP27	21																						
KP28	19																						
KP29	19																						
KP30	10																						
KP31	6																						
KP32	21																						
KQ1	5																						
KQ2	5																						


A
Siderophores
Aerobactin
iutA
Yersiniabactin
ybt
fyu
irp
Iron Uptake
kfu
Fimbriae
mrkABCDFHIJ
Other genes
ylbE
ybb
glx
all
0
10
20
30
40
50
60
70
80
90
100

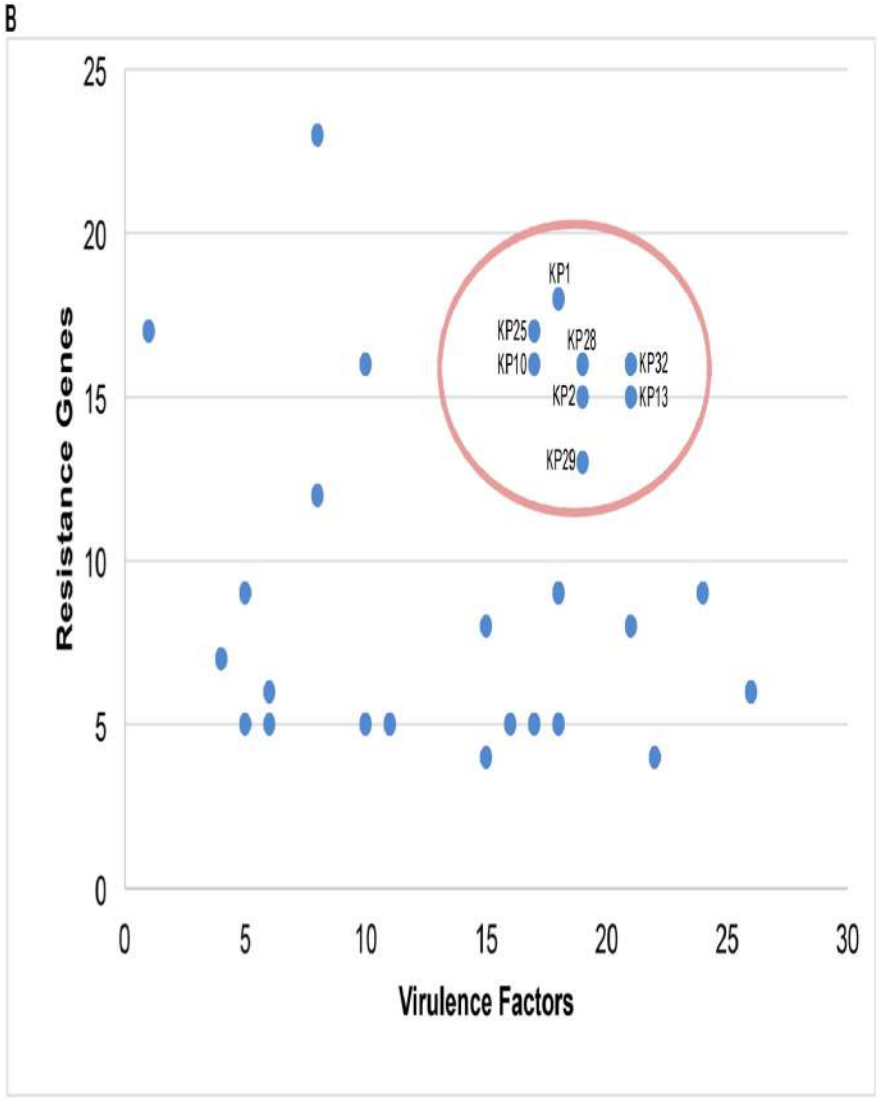

B
Resistance Genes
Virulence Factors
0
5
10
15
20
25
30
KP1
KP25
KP10
KP28
KP32
KP2
KP13
KP29

Figure 5: Prevalence of relevant virulence factors detected and the total number of virulence factors in a relationship with the total number of resistance genes. (**A**) Relative prevalence of the important virulence determinants. (**B**) Blue dots representing one or more isolate with a specific number of VFs and ARGs. Encircled are a group of emerging isolates with a high number of both VFs and ARGs.

3.6. Molecular characterization of the STs and K-types

Regarding the relationship of STs versus the K-types, important β-lactamase genes, incompatibility groups, and virulence features, which highlights the level of diversity, no significant correlations were illucidated. Table 6 depicts the relative relationship of each ST and the remaining listed criteria.

Table 6: Molecular characterization and characteristics of all STs. 21 detected STs and their molecular features including K-locus, Inc groups, β-lactamase genes, and selected virulence features.

ST	K-locus	Inc Groups	β-Lactam Resistance Genes	Virulence Factors
ST15 (n=5)	K24 (n=1), K112 (n=4)	A/C (n=2),FIB (n=5), FIIK (n=5), L/M (n=5), P (n=1), R (n=2)	*bla* $_{SHV-28}$ (n=5), *bla* $_{CTX-M-15}$ (n=4), *bla* $_{TEM-1B}$ (n=3), *bla* $_{OXA-1}$ (n=3), *bla* $_{OXA-48}$ (n=3)	*ybt* (n=5),*fyu* (n=4), *irp* (n=5), *kfu* (n=4), *mrkABCDFHIJ* (n=5)
ST16 (n=4)	K51 (n=4)	FIB (n=2), FIIK (n=2), L/M (n=4), P (n=3), R (n=2), X1 (n=2), X3 (n=1), X4 (n=2)	*bla* $_{SHV-11}$ (n=3), *bla* $_{SHV-11}$ (n=1), *bla* $_{CTX-M-15}$ (n=4), *bla* $_{TEM-1B}$ (n=2), *bla* $_{OXA-1}$ (n=4), *bla* $_{NDM-7}$ (n=1)	*ybt* (n=2),*fyu* (n=2), *irp* (n=2), *mrkABCDFHIJ* (n=4)
ST45 (n=3)	K24 (n=2), K62 (n=1)	FIB (n=3), FIIK (n=3), Q1 (n=1), L/M (n=3), R (n=1), X4 (n=1)	*bla* $_{SHV-1}$ (n=2), *bla* $_{SHV-27}$ (n=1), *bla* $_{CTX-M-14b}$ (n=1), *bla* $_{OXA-1}$ (n=1), *bla* $_{OXA-48}$ (n=3)	*ybt* (n=3),*fyu* (n=3), *irp* (n=3), *mrkABCDFHIJ* (n=3)
ST35 (n=2)	K105 (n=1), K12 (n=1)	A/C (n=1), FIB (n=1), FIIK (n=2), L/M (n=2), N (n=1), P (n=1)	*bla* $_{CTX-M-14b}$ (n=2), *bla* $_{OXA-48}$ (n=1)	*ybt* (n=1),*fyu* (n=1), *irp* (n=1), *kfu* (n=2), *mrkABCDFHIJ* (n=2)
ST147 (n=2)	K64 (n=2)	A/C (n=1), FIA (n=1), FIB (n=1), FIIK (n=2), L/M (n=1), P (n=1), R (n=1), X1 (n=2)	*bla* $_{SHV-11}$ (n=2), *bla* $_{CTX-M-15}$ (n=2), *bla* $_{TEM-1B}$ (n=1), *bla* $_{OXA-1}$ (n=2), *bla* $_{NDM-7}$ (n=2)	*ybt* (n=1),*fyu* (n=1), *irp* (n=1), *iutA* (n=1), *mrkABCDFHIJ* (n=1)
ST1799 (n=2)	K16 (n=2)	A/C (n=1), L/M (n=2), FIA (n=2), FIB (n=2), FIIK (n=2), X3 (n=2)	*bla* $_{SHV-28}$ (n=1), *bla* $_{LEN12}$ (n=1), *bla* $_{OXA-48}$ (n=2)	*ybt* (n=2),*fyu* (n=2), *irp* (n=2), *kfu* (n=2), *mrkABCDFHIJ* (n=2)
ST2720 (n=2)	K159 (n=2)	L/M (n=2), FIB (n=2), FIIK (n=2)	*bla* $_{SHV-1}$ (n=2), *bla* $_{OKP-B-3}$ (n=2), *bla* $_{OXA-48}$ (n=2)	*mrkABCDFHIJ* (n=2)
ST11 (n=1)	K105	FIB, FIIK, R	*bla* $_{SHV-11}$, *bla* $_{CTX-M-15}$, *bla* $_{DHA-1}$, *bla* $_{TEM-1B}$, *bla* $_{OXA-1}$, *bla* $_{OXA-9}$,	*mrkABCDFHIJ*
ST14 (n=1)	K2	FIB, FIIK, N, R	*bla* $_{SHV-28}$, *bla* $_{CTX-M-15}$, *bla* $_{TEM-1B}$, *bla* $_{OXA-1}$, *bla* $_{NDM-1}$	*kfu, mrkABCDFHIJ*
ST37 (n=1)	K15	A/C, FIB, FIIK, L/M, P	*bla* $_{SHV-11}$, *bla* $_{OXA-48}$	*ybt, mrkABCDFHIJ*
ST105 (n=1)	K102	A/C, FIIK, L/M, R, X1	*bla* $_{SHV-1}$, *bla* $_{OXA-48}$	*ybt, fyu, mrkABCDFHIJ*
ST107 (n=1)	K103	A/C, FIB, FIIK, L/M,	*bla* $_{SHV-1}$, *bla* $_{OXA-48}$	*kfu, mrkABCDFHIJ*
ST111 (n=1)	K63	FIB, FIIK, L/M, P, R	*bla* $_{SHV-11}$, *bla* $_{CTX-M-15}$, *bla* $_{OXA-1}$, *bla* $_{OXA-48}$	*ybt, irp, mrkABCDFHIJ*
ST187 (n=1)	K10	FIB, FIIK, L/M, P, R	*bla* $_{SHV-11}$, *bla* $_{OXA-1}$, *bla* $_{OXA-48}$	*ybt, fyu, mrkABCDFHIJ*
ST299 (n=1)	K7	FIB, FIIK, L/M, N, R	*bla* $_{SHV-1}$, *bla* $_{NDM-1}$	*mrkABCDFHIJ*
ST500 (n=1)	K34	FIB, FIIK, L/M	*bla* $_{SHV-11}$, *bla* $_{OXA-48}$	*kfu, mrkABCDFHIJ*
ST857 (n=1)	K13	FIIK, L/M	*bla* $_{SHV-11}$, *bla* $_{CTX-M-15}$, *bla* $_{OXA-48}$	*mrkABCDFHIJ*
ST1593 (n=1)	K137	HI1B, FIB, FIIK, L/M, P	*bla* $_{SHV-11}$, *bla* $_{OXA-48}$	*ybt, fyu, irp, mrkABCDFHIJ*
ST1634 (n=1)	K151	L/M, R	*bla* $_{SHV-1}$,*bla* $_{TEM-1B}$, *bla* $_{OXA-48}$	*kfu, all, gtr, ybb, ybtE*
ST2118 (n=1)	K46	A/C, FIIK, L/M, R, X1	*bla* $_{SHV-11}$, *bla* $_{OXA-48}$	*mrkABCDFHIJ*
ST2719 (n=1)	K81	FIIK, L/M	*bla* $_{SHV-27}$, *bla* $_{OXA-48}$	*mrkABCDFHIJ*

3.7. PFGE

By performing PFGE, a total of 25 different PTs were identified (Figure 6). Both *K. quasipneumoniae* isolates (KQ1 and KQ2) clustered among a clade of seven *K. pneumoniae* isolates. Isolates having identical STs, or single, or double locus variants, SLVs or DLVs, respectively, were closely related and grouped under the same branch in the PFGE dendogram, with the exception of KP30 (ST14), a SLV of ST15, which was most closely associated with KP15 (ST107). All of the isolates typed with ≥80% PFGE similarity patterns had identical STs and K-types; thus the obtained PFGE banding patterns were in agreement with the MLST and capsular typing results. It is noteworthy that KP31 had a distinct PFGE pattern from all the other isolates, while KP14 was untypable, even after using a secondary enzyme, AvrII.

Isolate Pulsotype gapA infB mdh pgi phoE rpoB tonB ST K-type Source
KP10 1 2 1 5 1 17 4 42 111 K63 Urine
KP22 2 2 10 1 1 56 24 31 299 K7 Skin
KP18 3 3 3 1 1 1 1 4 11 K105 DTA
KP25 4 1 6 1 26 12 25 9 1634 K151 Urine
KP1 5 3 4 6 1 7 4 38 147 K64 DTA
KP16 6 3 4 6 1 7 4 38 147 K64 Unknown
KP13 7 1 1 1 1 1 1 1 15 K112 DTA
KP32 7 1 1 1 1 1 1 1 15 K112 Urine
KP12 7 1 1 1 1 1 1 1 15 K112 DTA
KP27 7 1 1 1 1 1 1 1 15 K112 Urine
KP3 8 1 1 1 1 1 1 1 15 K24 Urine
KP24 9 2 6 1 72 4 4 64 500 K34 Peripheral
KP19 10 2 9 2 1 13 1 16 37 K15 Urine
KP26 11 23 31 1 1 9 4 23 187 K10 Wound
KP15 12 2 1 2 17 27 1 39 107 K103 Urine
KP30 13 1 6 1 1 1 1 1 14 K2 Urine
KP20 14 2 3 2 1 1 4 18 105 K102 Leg tissue
KP7 15 2 1 2 1 4 4 4 16 K51 Drain Site
KP8 15 2 1 2 1 4 4 4 16 K51 Drain Site
KP2 15 2 1 2 1 4 4 4 16 K51 C-section wound
KP28 15 2 1 2 1 4 4 4 16 K51 Urine
KP5 16 2 1 1 1 3 1 34 1799 K16 DTA
KP6 16 2 1 1 1 3 1 34 1799 K16 Cyst
KP4 17 2 1 151 87 7 1 12 2719 K81 Urine
KP21 18 4 1 11 1 9 4 59 1583 K137 Incision site swab
KQ1 19 42 22 26 96 86 20 51 2720 K159 Isolate
KQ2 19 42 22 26 96 86 20 51 2720 K159 Pus
KP11 20 2 1 1 6 7 1 12 45 K24 Lung swab
KP29 21 2 1 1 6 7 1 12 45 K24 Skin
KP23 22 2 1 1 6 7 1 12 45 K62 Skin
KP9 23 2 1 2 1 10 1 19 35 K105 Urine
KP17 24 2 1 2 1 10 1 19 35 K16 Urine
KP31 25 2 35 2 35 56 24 19 857 K13 Skin
100
90
80
70
60
50
40

Figure 6: PFGE profiles, PT, seven-gene MLST profiles, ST, K-type, and source of the sequenced isolates. Dendogram generated by BioNumerics software version 7.6.1 showing the relationship of the isolates based on their banding patterns generated by XbaI restriction digestion.

3.8. wgSNP-based phylogenetic diversity and analysis

wgSNP-based phylogenetic typing of 43 *Klebsiella* reference strains reflected a close association between STs and K-types (Figure 7). The majority (94.1%; 32/34) of the isolates understudy was grouped into eight major clusters with at least 80% similarity (Clusters I-VIII) and in agreement with MLST and capsular typing, except clusters III and IV; had variable STs or K-types. The two identified *K. quasipneumoniae* (KQ1 and KQ2) clustered separately with *K. quasipneumoniae* KP-Z4175 (accession # LVCD01000000) with 30% similarity compared to the 40 remaining *K. pneumoniae* isolates. The nine reference genomes represented diverse STs (ST11, ST14, ST15, and ST147), K-types (K2), harbored variable resistance genes such as $bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$, $bla_{\text{NDM-7}}$, and $bla_{\text{OXA-48}}$, and were distributed among the major clusters.

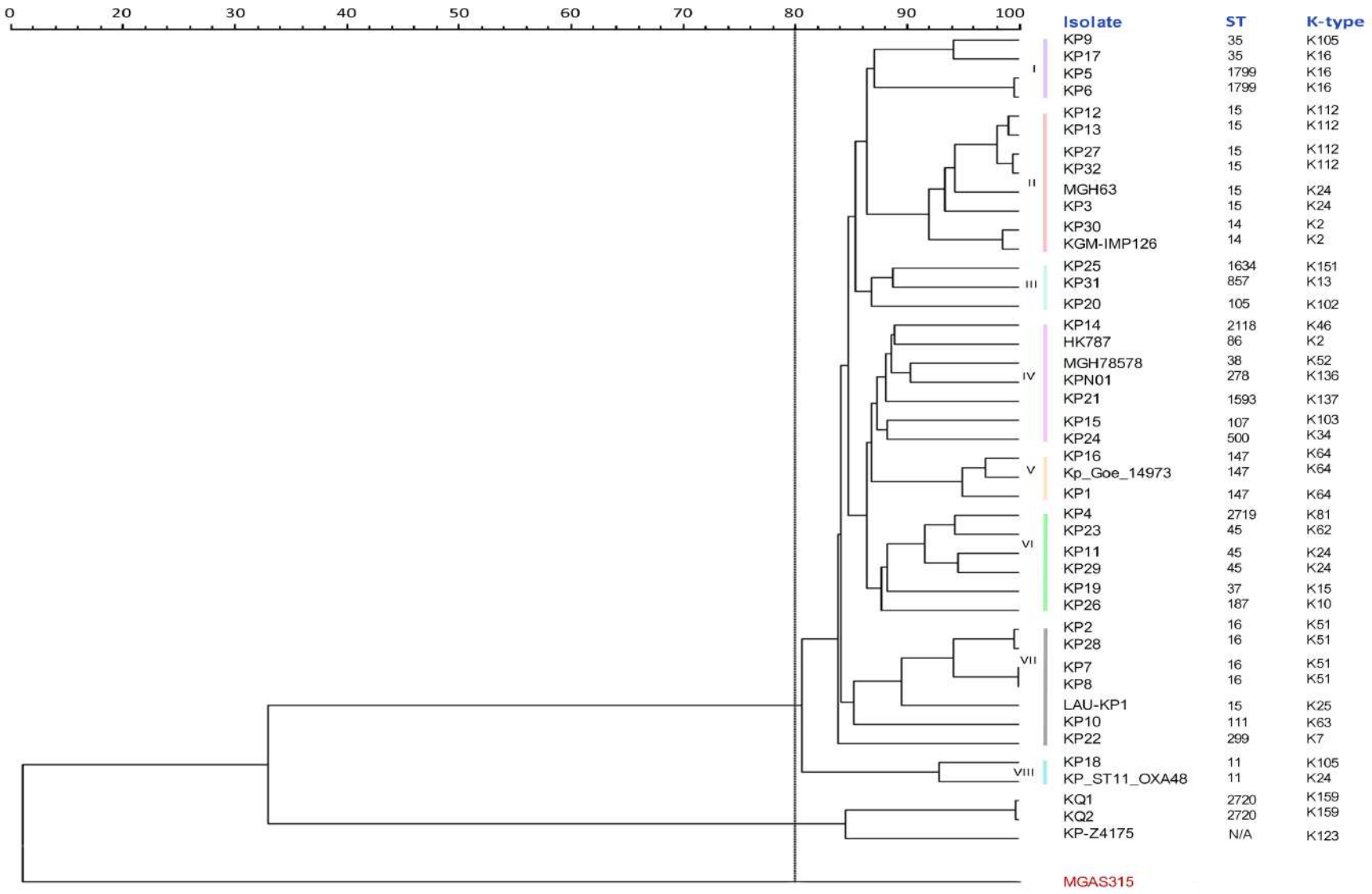

0
20
30
40
50
60
70
80
90
100
Isolate
ST
K-type
KP9 35 K105
KP17 35 K16
KP5 1799 K16
KP6 1799 K16
KP12 15 K112
KP13 15 K112
KP27 15 K112
KP32 15 K112
MGH63 15 K24
KP3 15 K24
KP30 14 K2
KGM-IMP126 14 K2
KP25 1634 K151
KP31 857 K13
KP20 105 K102
KP14 2118 K46
HK787 86 K2
MGH78578 38 K52
KPN01 278 K136
KP21 1593 K137
KP15 107 K103
KP24 500 K34
KP16 147 K64
Kp_Goe_14973 147 K64
KP1 147 K64
KP4 2719 K81
KP23 45 K62
KP11 45 K24
KP29 45 K24
KP19 37 K15
KP26 187 K10
KP2 16 K51
KP28 16 K51
KP7 16 K51
KP8 16 K51
LAU-KP1 15 K25
KP10 111 K63
KP22 299 K7
KP18 11 K105
KP_ST11_OXA48 11 K24
KQ1 2720 K159
KQ2 2720 K159
KP-Z4175 N/A K123
MGAS315
I
II
III
IV
V
VI
VII
VIII

Figure 7: wgSNPs-based phylogenetic tree generated using BioNumerics. Nine reference genomes were included with the 34 sequenced genomes and compared. These references presented a diverse population and had different sets of ARGs, STs, and virulence characteristics.

Chapter Four

Discussion

One of the major players sustaining the successful worldwide spread of carbapenemase encoding genes in *Enterobacteriaceae* is their transferability via plasmids. Consequently, the increase in carbapenem resistance was largely driven by self-conjugative plasmids. Many of these plasmids that harbor a carbapenemase gene also carry multiple resistance determinants (Nordmann et al., 2009; The et al., 2015).

In this study, a significant variability was observed in resistance and plasmid Inc groups even among identical STs and K-types. None of the ARGs could be associated with a specific Inc group, with the exception of $bla_{\text{OXA-48}}$, which was confined to IncL/M, and the $bla_{\text{NDM-1,}}$ to IncF replicons.

$bla_{\text{OXA-48}}$ (61.8%; 21/34) and bla_{NDM} (17.6%; 6/34) were the only two detected carbapenemase encoding genes. Such a finding is in accordance with previous reports, where both of these types were found to be the major carbapenemase encoding genes detected in the Middle East (Gomez-Simmonds et al., 2016). In fact, according to the Global Surveillance Program, SMART, all CRKP isolates collected from Lebanon between 2008-2014 were $bla_{\text{OXA-48}}$ positive (Karlowsky et al., 2017). Yet five of the NDM producing isolates were discovered during that period. The observed diversity in OXA-48 producing isolates suggested either their prolonged prevalence in a hospital setting or the presence of a constant source introducing new clones into the Lebanese population (Zowawi et al., 2015).

The highest detected Inc types in one isolate were seven, KP1, presented as one big multi-replicon and three smaller plasmids (Figure 3). The multi-replicon plasmid had

three replicons, IncFIIK, IncFIA, and IncR. Multi-replicon plasmids have been increasingly detected with IncF plasmids being the most common type showing this feature, and plasmids with three distinct replicons, IncFIIK, IncR, and IncFII, were previously described by Coelho et al. (Coelho et al., 2010; Stoesser et al., 2014). Additionally, IncFIIK and IncFIA are commonly associated on the same conjugative plasmid (Stoesser et al., 2014). It is noteworthy that the multi-replicon plasmid carried a *bla*$_{\text{NDM-1}}$ gene. This multi-replicon status, which entails broader host range replication, is alarming as it further facilitates their widespread dissemination (Villa et al., 2010).

The IncX group of plasmids is relatively poorly studied compared to other Inc types (Burmølle, Norman, Sørensen, & Hansen, 2012). IncX1 was detected in 14.7% (5/34), IncX3 in 8.8% (3/34), and IncX4 in 8.8% (3/34) of all isolates and distributed among many STs and K-types. IncX1 and IncX4 in both KP7 and KP8 did not appear to carry previously described typical constituents of an IncX plasmid. Alignment with the IncX plasmid pOLA52 (accession # NC_010378.1) revealed plasmid-encoded type IV secretion system proteins in both.

The IncX4 plasmid harboring the *bla*$_{\text{CTX-M-14b}}$ gene was found in KP11 (ST45). The detection of a functional and intact *bla*$_{\text{CTX-M-14b}}$ explains the resistance of KP11 to 2$^{\text{nd}}$, 3$^{\text{rd}}$, 4$^{\text{th}}$, and non-extended cephalosporins, with the exception of ceftazidime; a phenotype that was not seen in the remaining ST45 isolates, which were lacking the *bla*$_{\text{CTX-M-14b}}$ gene. The detected plasmid was highly similar to plasmid pSAM7 isolated from the *E. coli* ECSAM7 strain (accession # NC_022105.1), similarly carrying the *bla*$_{\text{CTX-M-14b}}$ gene. Moreover, this is the first description of *bla*$_{\text{CTX-M-14b}}$ in Lebanon. It was first described in the UK and associated with the pSAM7 plasmid (Lo et al., 2014; Stokes et al., 2013). The latter was implicated in the dissemination of *bla*$_{\text{CTX-M-14b}}$

between different species of bacteria in humans and animals, without compromising the fitness burden of the bacterial host (Lo et al., 2014; Stokes et al., 2013).

On the other hand, KP28 was the only isolate simultaneously positive for an IncX3 replicon and the *bla*$_{\text{NDM-7}}$ gene. This is the first report of IncX3 carrying *bla*$_{\text{NDM-7}}$ in Lebanon. To date 20 NDM allele types were reported, with *bla*$_{\text{NDM-7}}$ being recently described (Z. Liu et al., 2018). This variant has only two amino acid substitutions at positions Asp-130-Asn and Met-154-Leu (Gaelle Cuzon, Bonnin, & Nordmann, 2013). *bla*$_{\text{NDM-7}}$ is known for showing higher hydrolysis efficiency against carbapenems as compared to its ancestor *bla*$_{\text{NDM-1}}$, and often co-exists with other antibiotic resistance genes on the same IncX3 type conjugative plasmid (Gaelle Cuzon et al., 2013; L. Wang et al., 2016). Unlike the other two IncX plasmids detected, IncX1 and IncX4, the analysis of the genetic environment of IncX3 in KP28 revealed a typical backbone of an IncX plasmid consisting of genes encoding replication (*pir* and *bis*), partitioning (*par*), maintenance (*topB* and *hns*), and conjugal transfer (*pil* and *tax*) (Figure 4) (Norman, Hansen, She, & Sørensen, 2008). *bla*$_{\text{NDM-7}}$ was carried by an IS*5*-*bla*$_{\text{NDM-7}}$-*ble*$_{\text{MBL}}$-*trpF*-*dsbD*-IS*26* genetic element. This structure lacked IS*Aba125* as opposed to previous reports (Ho et al., 2012). IncX3 carrying *bla*$_{\text{NDM-7}}$ was previously reported in *Enterbacteriacae* in China, India, and other areas (Paul, Garg, & Bhattacharjee, 2017; L. Wang et al., 2016). The ability of the IncX3 plasmid carrying *bla*$_{\text{NDM-7}}$ to persist even after withdrawal of antibiotic pressure is a major public health concern underscoring its ability to rapidly disseminate within community and hospital settings (Paul et al., 2017). The remaining two IncX3 plasmids, which carried type IV secretion proteins PtlH, VirB1, Virb4, and VirB9019, were detected in KP5 and KP6. These IncX3 plasmids did not harbor any antibiotic resistance determinants, unlike their closely matched *K.*

pneumoniae strain BIC-1 plasmid pBIC-1b (accession # NZ_CP022575.1), which carried a *bla*$_{SHV-12}$.

The genetic environment of the *bla*$_{NDM-1}$ gene was also investigated, which in all isolates was carried on an IncF replicon in a chimeric structure with *ble*$_{MBL}$ encoding bleomycin resistance (Poirel et al., 2012). The *bla*$_{NDM}$-*ble*$_{MBL}$ operon along with some neighboring genes has originated in *Acinetobacter spp*. before being acquired by *Enterobacteriaceae*. It is usually present on Tn*125* bracketed by two IS*Aba125* (Poirel et al., 2014). The downstream IS*Aba125* was detected in all of the isolates, whereas the upstream IS*Aba125* was absent due to a Tn*3* insertion. This was also previously observed in *K. pneumoniae* and other *Enterobacteriaceae* (Nordmann et al., 2009; Salloum et al., 2017). Additionally, the *bla*$_{NDM-1}$ gene detected in KP18 (ST11) was allocated on a multi-replicon plasmid possessing IncFIIK, IncFIB, and IncR replicons. KP18 harbored 23 different ARGs and these replicons were the only ones detected in it. This suggested the presence of a single big plasmid carrying a considerable number of ARGs. The detection of *bla*$_{NDM-1}$ on such a multi-replicon plasmid was previously reported in Nepal with an IncFIIK/IncFIB plasmid contributing to its dissemination (Stoesser et al., 2014). Moreover, IncF plasmids are known for carrying more than one replicon per plasmid (IncFII, IncFIA, and IncFIB being the most common) (Carattoli, 2013). On the other hand, plasmids with an IncR replicon within *K. pneumoniae* are commonly associated with a variety of ARGs such as *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{DHA-1}$, *bla*$_{OXA-1}$, *qnrS1, aac(6')-Ib,* and *qnrB4* (Rodrigues, Machado, Ramos, Peixe, & Novais, 2014), all of which were detected in KP18. Moreover, being characterized as ST11, a SLV of ST258, KP18 has a superior epidemic potential. ST11 was previously responsible for the global success of CTX-M-15 enzymes in *K. pneumoniae* (Williamson et al., 2012). The detection of a

number of resistance determinants along with *bla*$_{\text{NDM-1}}$ on a single multi-replicon plasmid within an epidemic clone of CRKP is highly concerning and poses a major therapeutic and epidemiological concern.

The absence of carbapenemase genes with phenotypic resistance to carbapenem antibiotics was detected in seven isolates (KP2, KP7-9, KP21, KP27, and KP32). This phenotype can be explained by the presence of an ESBL coupled with porin loss or modification (Martin & Bachman, 2018). KP2, KP7-9 all carried multiple β-lactamases and mutated versions of the outer membrane porins OmpK35 and OmpK36 with at least seven and 13 mutations found in each, respectively. These modifications did not lead to the premature truncation of the proteins, but may have contributed to porin modification leading to carbapenem resistance. As for KP21, it carried a *bla*$_{\text{SHV-11}}$ ESBL and a truncated OmpK36 porin. The truncation was due to a premature stop codon in the *ompK36* gene. Similarly, KP27 and KP32, two of the ST15 isolates, possessed *bla*$_{\text{SHV-28}}$ and *bla*$_{\text{CTX-M-15}}$ ESBLs coupled with a truncated OmpK36 porin, also due to a premature stop codon in the *ompK36* gene. As for the *ompK35* in KP21, KP27, and KP32, it contained multiple mutations. Interestingly, OmpK36-modified ST15 CRKP clones were previously linked to an outbreak in Portugal (Novais et al., 2012). Also, *K. pneumoniae* ST15 containing both *bla*$_{\text{SHV-28}}$ and *bla*$_{\text{CTX-M-15}}$ was described as an epidemic clone in the Copenhagen region (Nielsen et al., 2011).

VFs in *K. pneumoniae* include several siderophore systems such as yersiniabactin, aerobactin, colibactin, salmochelin, and microcin, *rmpA* and *rmpA2,* encoding regulators of the mucoid phenotype activating capsule biosynthesis, *kfuABC,* encoding a ferric uptake system, and *kvgAS,* encoding a two-component regulator that functions in countering free radical stresses and detecting iron-limiting conditions (Holt et al., 2015).

Given the scarce amounts of free iron present in the host, pathogens like *K. pneumoniae* have to use siderophores, which have a higher affinity for iron than the host transport proteins (Holt et al., 2015). In half of the sequenced isolates, the most prevalent VFs were yersiniabactin siderophores encoded by *ybt*, *irp1*, *irp2*, and *fyuA* genes, which together form the *Yersinia* high-pathogenicity island (HPI). 35% of all isolates (12/34) possessed *kfu*, which is strongly associated with highly virulent and invasive KP clones (Holt et al., 2015). Moreover, the presence of aerobactin is always associated with a hypermucoviscous capsule, yet KP16 encoding the aerobactin siderophore was not hypermucoviscous (Miethke & Marahiel, 2007). KP25 carried a unique repertoire of VFs that included *kfu*, *all, glx*, *ybb,* and *ylbE*. Interestingly, the identification of *kfu* and *all* in KP25 with the capsular type K151, is a novel finding being previously reported only in strains having the K1 capsular type (Luo, Wang, Ye, & Yang, 2014). On the other hand, *all* is a relatively rare virulence gene found in organisms metabolizing allantoin to obtain carbon and nitrogen from the environment (Vogels & Van der Drift, 1976). The metabolism of allantoin appears to be upregulated in hvKP (Chou et al., 2004). Moreover, *glx*, *ybb*, and *ylbE* are associated with the *all* operon in hypermucoviscous *K. quasipneumoniae***,** yet KP25 was not hypermucoviscous (Arena et al., 2015).

The coexistence of a high number of VFs and ARGs in 23.5% (8/34) of the isolates (KP1, KP2, KP10, KP13, KP25, KP28, KP29, and KP32) belonging to various STs (ST15, ST16, ST45, ST147, ST111, and ST1634) is an alarming phenomenon that could indicate the potential of an emerging population of untreatable invasive *K. pneumoniae.* Most of these STs are internationally distributed and associated with carbapenem resistance (Bathoorn, Rossen, Lokate, Friedrich, & Hammerum, 2015; Poirel et al.,

2012; Salloum et al., 2017; uz Zaman et al., 2014). This poses a major threat on the global dissemination of high-risk XDR clones such as KP1, KP2, and KP28, and KP32, which was both hypervirulent and XDR.

Interestingly, out of the eight detected XDR isolates, four carried an NDM-type carbapenemase (KP1, KP16, KP28, and KP30), none harbored *bla*$_{\text{OXA-48}}$ and four did not have a carbapenemase-encoding gene (KP2, KP7, KP27, and KP32). KP30, possessing *bla*$_{\text{NDM-1}}$ remained susceptible to a single drug category (Phosphonic acids) as opposed to the other XDRs that remained susceptible to two drug categories (Magiorakos et al., 2012). KP16 also had a *bla*$_{\text{NDM-1}}$ carbapenemase. However, this extensive drug resistance came at a fitness cost compromising virulence in both KP16 and KP30, which were only positive for *iut* and *kfu* and for *mrkABCDFHIJ,* respectively. This phenomenon is frequently associated with *bla*$_{\text{NDM-1}}$ carriage (Göttig, Riedel-Christ, Saleh, Kempf, & Hamprecht, 2016).

Moreover, this is the first description of *K. quasipneumoniae* in the region. The lack of reports is mainly due to misidentification of the species *K. quasipneumoniae* as *K. pneumoniae* because of the minor phenotypic, genetic, and biochemical differences between them that are usually overlooked by clinicians (Brisse, Passet, & Grimont, 2014; Long et al., 2017). Few previous reports of hypervirulent and carbapenem-resistant *K. quasipneumoniae* exist and thus proper identification is of high importance (Arena et al., 2015; Long et al., 2017; Shankar et al., 2017). Both KQ1 and KQ2 were carbapenemase producers harboring the *bla*$_{\text{OXA-48}}$ on IncL/M plasmids. Additionally, both isolates harbored the *bla*$_{\text{SHV-1}}$ gene carried on a second multi-replicon plasmid having IncFIIK and IncFIB replicons. This finding was in harmony with previous reports, where *bla*$_{\text{SHV-1}}$ was found to be linked to IncFIIK plasmids (Al-Marzooq et al.,

2015; Gaëlle Cuzon et al., 2010). The newly described 42-22-26-96-86-20-51 MLST profile was detected in both of these isolates. Upon curation, the new ST2720 corresponding to the new allelic profile was assigned and deposited on the Institute Pasteur ST database. Moreover, KQ1 and KQ2 were closely clustered with other isolates by PFGE, differing by only few bands from the remaining *K. pneumoniae* isolates. Yet, wgSNPs analysis clustered the two isolates with less than 30% similarity with the remaining clades and 85% similarity with a reference genome of *K. quasipneumoniae* KP-Z4175 (accession # GCA_001611185.1). Such a finding illustrates the importance and the high resolution of wgSNPs typing as compared to PFGE.

To our knowledge, this is the first comprehensive whole-genome based characterization of CRKP in Lebanon and the first detection and description of *K. quasipneumoniae* in the region. The multi-replicon nature of the detected plasmids, the observed diversity, and the coexistence of specific VFs with a very high number of ARGs all indicate the possible emergence of a population of untreatable invasive *K. pneumoniae.* Of importance were resistance determinants associated with: clones of high epidemicity (ST11, ST14, ST15, and ST147), broad host range plasmids (IncX3 or IncL), and multi-replicon plasmids (IncFIIK, IncFIB, and IncR). Thus, this is a problem that is too important to be ignored. As such building a deeper understanding by maximizing the potential benefits of genomic approaches, is needed to limit the cross-transmission of such pathogens in hospital settings, and to prevent outbreaks and global dissemination. Furthermore, the detected heteroresistance in multiple isolates and mechanisms of colistin resistance may be of interest for future studies.

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