Characterization of plasmids mediating carbapenem resistance in *Klebsiella pneumoniae* in Pretoria, South Africa

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by

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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained herein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree. I further declare that all sources cited are acknowledged by means of a list of references.

Signed _____ this _____ day of _____ 2020

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"With man this is impossible, but with God all things are possible"

Matthew 19:26

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List of abbreviations

AmpC	AmpC β-Lactamase
ARGs	Antimicrobial resistance genes
bla	β-lactamase gene
BLAST	Basic Local Alignment Search Tool
BHI	Brain heart Infusion
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-resistant Enterobacteriaceae
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
CTX-M	Cefotaximase-Munich
CPKP	Carbapenem-producing *Klebsiella pneumoniae*
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ESBLs	Extended-spectrum β-lactamases
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
GES	Guiana extended spectrum
GI	Gastrointestinal
HGT	Horizontal Gene Transfer
ICU	Intensive Care Unit
IMP	Imipenemase metallo-β-lactamase
Inc	Incompatibility
KPC	*Klebsiella pneumoniae* Carbapenemase
LB	Luria Bertani
MBLs	Metallo-β-lactamases
MDR	Multidrug resistant
MLST	Multi-locus sequence typing
M-PCR	Multiplex Polymerase chain reaction
NDM	New Dehli metallo-β-lactamase
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
OXA	Oxacillinase
PacBio	Pacific Biosciences
PBRT	PCR-Based Replicon Typing
PCR	Polymerase Chain Reaction
pMLST	plasmid Multi-locus Sequence Typing
REP-PCR	Repetitive Extragenic Palindromic-PCR
RND	Resistance Nodulation Division

ST	Sequence Type
UTI	Urinary Tract Infection
VIM	Verona Integron metallo-β-lactamase
WGS	Whole-genome Sequencing
WHO	World Health Organisation
WPS	Whole-plasmid Sequencing

CONFERENCE PRESENTATIONS

1. Kopotsa K, Osei Sekyere J, Mbelle NM (2019).
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SUMMARY

Klebsiella pneumoniae is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. This pathogen is implicated in community- and hospital-acquired infections, particularly in neonates, the elderly, and immunocompromised patients. Risk factors such as extended hospital stay, being immunocompromised, and excessive antibiotic use lead to disease severity and potential acquisition of resistance to antibiotics.

Carbapenems are usually the treatment of choice in multidrug-resistant *K. pneumoniae* infections. Since the last decade, carbapenems has become less effective as *K. pneumoniae* stains develop mechanisms of resistance against them. Among the various mechanisms of carbapenem resistance, enzyme (carbapenemases, ESBLs, AmpCs) production is the most dominant. Carbapenemases are classified into three classes viz., class A, B and D, and have the ability to hydrolyse β-lactams antibiotics, including "last resort" carbapenems. The most commonly reported carbapenemases worldwide in *K. pneumoniae* strains include the bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{OXA} genes. In South Africa, $bla_{OXA-48/181}$ and bla_{NDM-1} have been reported in *K. pneumoniae* in almost all provinces, with outbreaks being reported in other provinces within the past six years.

Plasmids are shuttles that mediate the acquisition and dissemination of carbapenemases. This is because plasmids are mobile and may be transferred from one specie to another via horizontal gene transfer. Conjugative plasmids such as IncF, A/C, IncL/M, IncN and IncX plasmids have been associated with commonly reported carbapenemases worldwide. In South Africa, little is known about plasmid types associated with carbapenemase genes and their molecular

characteristics. This study aimed to identify and characterise plasmids mediating carbapenem resistance in Pretoria, South Africa.

Sixty *K. pneumoniae* clinical isolates were collected from the National Health Laboratory service (NHLS, Pretoria) in 2018. Carbapenem resistance and carbapenemase production was determined using MicroScan automated system and PCR assays, respectively. The isolates' plasmids were characterized to determine their size, number and replicon types using gel electrophoresis, and PCR-based replicon typing techniques, respectively. Molecular characteristics of the isolates' carbapenemases and plasmids were analysed using both PCR assays and whole-genome sequencing (WGS). All *K. pneumoniae* isolates were multidrug resistant. Carbapenemase production was identified in 65% ($bla_{\text{OXA-48}}$-like) and 29% ($bla_{\text{NDM-1}}$) of the isolates. Multi-locus typing revealed five sequence types: ST307, ST607, ST17, ST39, and ST3559. Both PCR and WGS revealed multiple plasmid replicons associated with the carbapenem-resistant *K. pneumoniae* (CRKP) isolates viz., IncF, A/C, IncL/M and IncX3 plasmids. WGS proved to be more useful in characterising plasmids over the PCR-based replicon typing (PBRT). The PBRT could not identify the IncX3 replicons, which were detected by WGS. Identified plasmids could be transferred from donor CRKP strains to recipient *E. coli* strains. Phylogenomic analysis showed that strains in this study were closely related to stains from the United States, China, Thailand, and South Korea more than other countries. These strains shared similar antimicrobial resistance mechanisms, however, they belonged to different sequence types including ST14, ST11, ST147, and ST152.

This study shows an ongoing plasmid-mediated dissemination of carbapenemase genes in 2018 in Pretoria, with $bla_{\text{OXA-48}}$-like and $bla_{\text{NDM-1}}$ genes being the major resistance determinants. This study has also shown the important role conjugative or mobile plasmids play in the acquisition and dissemination of carbapenemase genes from one specie to another via conjugation.

CHAPTER 1

INTRODUCTION

Antibiotics have played a major role in treating clinical infections, which were difficult to treat before the antibiotic era[1]. Antibiotics such as β-lactams, have a broad spectrum of activity against both Gram-negative and Gram-positive bacteria[2]. The development of β-lactamases in the 1960s, threatened the use of β-lactams[3]. To overcome this, β-lactamase inhibitors such as clavulanic acid was introduced clinically to treat in combination with β-lactams[3]. However, this was not active to complex microbial infections, which led to the introduction of carbapenems. Carbapenems are unique in structure with a β-lactam ring conferring defence to most β-lactamases. The broad spectrum of antibacterial activity possessed by carbapenems; led to the classification of these agents as "last resort"[2].

Since the 1990s, an increase in the use of carbapenems to treat multidrug-resistant *K. pneumoniae* resulted in the development of carbapenem resistance in these strains[2]. In the past decade, carbapenem-resistant *K. pneumoniae* (CRKP) isolates have been listed as high priority pathogen due to their high attributable morbidities and mortalities[4]. *Klebsiella pneumoniae* is a non-motile, Gram-negative and facultative anaerobic bacteria belonging to the family *Enterobacteriaceae* [5]. This species was first isolated from the lungs of a patient who died of pneumonia and it was described by Carl Friedländer as *Friedländer's bacillus* in 1882, but subsequently speciated specifically[6]. This pathogen has been implicated in many hospital-acquired infections and outbreaks due to its ability to survive and multiply on wet surfaces as well as colonize human sites such as the bladder, upper respiratory tract and the skin[7]. Coupled with its virulent nature, the acquisition of drug resistance in this clinically ubiquitous pathogen makes the situation worrying.

Carbapenem resistance in *K. pneumoniae* is usually associated with carbapenemase-mediated hydrolysis of carbapenems, among other mechanisms[8]. Carbapenemases are categorized according to their amino acid sequences and three classes have been described based on the Ambler system: classes A, B, and D[9]. The Ambler class A and D are referred to as the serine β-lactamases and the class B as the metallo-β-lactamases (MBLs)[10]. The most commonly detected carbapenemases in *K. pneumoniae* include: the *Klebsiella pneumoniae* carbapenemase (KPC), New Dehli metallo-β-lactamase (NDM), Imipenemase metallo-β-lactamase (IMP), Verona integron metallo-β-

lactamase (VIM), and the oxacillinases (OXA-48-like and variants)[10]. The class A carbapenemases have the ability to hydrolyse penicillins, monobactams, 3rd generation cephalosporins, and carbapenems and can be inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam[11]. Similar to class A carbapenemases, MBLs hydrolyze most β-lactams including carbapenems and they are inhibited by metal-chelating agents such as ethylene diamine tetraacetic acid (EDTA)[11]. Furthermore, class D β-lactamases have the ability to hydrolyze β-lactams and cannot be inhibited by clavulanic acid or EDTA[11,12].

Carbapenemase genes are commonly found on mobile genetic elements such as integrons, plasmids, and/or transposons that disseminate them by horizontal gene transfer (HGT)[13]. The plasmid-mediated KPC-1, which was first detected in a *K. pneumoniae* outbreak, was referred to as the first important carbapenemase gene[14,15]. To date, other important plasmid-mediated carbapenemases such as NDM-1, IMP-1 or IMP-4, and OXA-48-like groups have been reported worldwide[14]. In South Africa, these plasmid-mediated carbapenemases have also been reported[16,17]. However, NDM and OXA-48 are recently the most reported in *Enterobacteriaceae*, particularly in *K. pneumoniae*[16]. Plasmids may carry one of these genes or a combination of these antimicrobial genes[18].

These plasmids are known to play an essential role in HGT of resistance genes through conjugation[18]. Bacterial conjugation results in a self-transfer of a larger plasmid and/or mobilization of smaller plasmids[19].

Various plasmids have been identified in *K. pneumoniae* that carry the bla_{KPC} gene[20]. These plasmids include the IncF, IncL2, IncX, A/C, LncR, and ColE1 groups[18,20,21]. However, Pitout reported the most predominant plasmid groups associated with bla_{KPC} as the IncF, with the FIIk replicons[18]. The bla_{NDM} gene is also found on plasmids such as A/C, IncF, IncR, IncN, IncL/M, and the IncX groups[22–26]. The bla_{NDM-1} gene has been predominantly associated with the A/C and IncX3 plasmid replicons[18,27]. Furthermore, the spread of bla_{OXA-48} is mainly mediated by the self-conjugative IncL/M groups of plasmids[28,29]. Other plasmids such as IncA/C groups and IncH groups have also been reported to carry the bla_{OXA-48} gene, while the bla_{OXA181} has been reported to be associated with IncX3 in South africa[30,31].

Detection and confirmation of carbapenemase production is done by finding the carbapenemase gene using molecular methods[32]. These molecular tests include PCR, microarrays, and whole-

genome sequencing (WGS)[33]. Molecular tests are sensitive and solve the limitations of phenotypic tests[32]. The disadvantage of molecular tests is cost, and most of the tests, including PCR and microarrays, can only target genes that are already known[32,33]. WGS is however a comprehensive molecular test that can be used to query the entire genome of bacteria and can reveal all resistance genes, both known and unknown[33].

Plasmids have been previously detected and classified according to their incompatibility (Inc) group identification through a method based on DNA hybridization, with probes recognizing basic replicons [34]. This method was labour intensive, particularly when used on a larger number of strains, so a PCR-based method was developed for detection of these plasmid groups[35]. Even though this method was less labour intensive, it was only limited to a few families of plasmids associated with antimicrobial resistance[35]. A better PCR-based replicon typing method was developed to encompass the major plasmid groups associated with antimicrobial resistance in *Enterobacteriaceae*[36]. This method consists of five multiplex PCRs and three simplex PCRs targeting the plasmid replicons[36,37].

More recently, more discriminatory and advanced methods have been adopted in the typing of plasmids including plasmid multi-locus sequence typing (pMLST), whole-plasmid sequencing (WPS), and whole-genome sequencing (WGS)[38]. pMLST is used for the subtyping of multiple plasmids such as IncF, IncN, IncH, and IncI plasmid groups[20]. Whole-plasmid sequencing and WGS can be used to track plasmids that are previously not identified and are not included in the other typing[39]. All these typing techniques play a major role in plasmid evolution and their epidemiology in different countries.

1.1 Background

The increasing acquisition of antimicrobial resistance genes and intrinsic resistance to antimicrobial agents limit treatment options for *K. pneumoniae* infections, leading to treatment failure[40,41] and high mortality. In particular, multiple resistance genes are borne by multidrug-resistant (MDR) *K. pneumoniae*[4], which the World Health Organization (WHO) has identified as an urgent threat to human health and described it as one of the ESKAPE pathogens [4]. MDR *K. pneumoniae* strains have resistance to extended-spectrum β-lactamases (ESBLs), aminoglycosides, fluoroquinolones, and "last resort" carbapenems[42] using different mechanisms including alteration of metabolic pathways, antimicrobial-inactivating enzymes, change in

membrane permeability, and overexpression of efflux pump systems[41,43]. The most common mechanism of resistance in *K. pneumoniae* is the production of enzymes such as ESBLs, AmpCs and carbapenemases[44]. Carbapenemases were believed to be chromosomally located until a plasmid-mediated carbapenemase was reported. Plasmid-mediated carbapenemases are usually located on mobile genetic elements such as plasmids, which mediate their inter- and intra-species spread[13].

In *K. pneumoniae,* multiple plasmids carrying different carbapenemases have been reported worldwide[27]. In South Africa, carbapenem resistance in carbapenemase-producing *K. pneumoniae* (CPKP) is mainly determined by plasmid-mediated *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$-like genes[16]. Recently, South Africa became the 3rd African country to document the emergence of carbapenemases especially by *bla*$_{\text{NDM-1}}$-producing *K. pneumoniae* strains. These strains have been associated with hospital outbreaks, especially in neonates with low birthweight in multiple South African provinces.

In South Africa the increase in antimicrobial resistance has been associated with the misuse of antibiotics, poor infection control and overcrowding in most of the public hospital sectors. Misusing antibiotics may result in bacteria developing resistant to a certain antibiotic due to natural selection pressure. This bacterium can be transferred between patients and/or mobile genetic elements such as plasmids in susceptible bacteria can acquire resistance genes and further disseminate to other bacterial species. IncF and IncX3 plasmids have been associated with spread of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-181}}$ in private hospital sectors in South Africa[31,45].

There is limited data about the epidemiology and evolution of plasmids harbouring carbapenemase genes in South Africa. Studies available addresses one characterized plasmid responsible for a single dissemination of a single gene in private hospital sector. More studies characterizing plasmids associated with the dissemination of carbapenemases in South Africa are needed to see the relationship between the private and public hospital sectors. Knowledge regarding plasmid mediated CRE expansion will facilitate easy comparison with findings from other countries and continents. The information obtained in this study will also be used as a baseline for data collection around the country. The results obtained in this will form part of awareness to the policy makers in South Africa to re-visit antimicrobial stewardship and the usage of antibiotics in the Pretoria region.

This study focuses on multiple plasmids that might be circulating among carbapenem-resistant *K. pneumoniae* strains in public academic hospitals in the Pretoria region. The study also characterizes these plasmids using both PCR-based replicon typing and whole-genome sequencing approaches. Characterization of these plasmids allows better understanding of the epidemiology and evolution of these plasmids compared to reported plasmids in other countries.

1.2 Aim

The aim of this study was to characterize plasmids mediating carbapenem resistance in *Klebsiella pneumoniae* isolates in Pretoria, South Africa.

1.3 Objectives

- To detect carbapenemase genes in *K. pneumoniae* isolates using Multiplex PCR (M-PCR) assays
- To determine the transferability of plasmids using plasmid conjugation/transformation assays
- To analyse the whole plasmid of representative isolates using PacBio sequencing
- To undertake plasmid typing and phylogenetics (evolutionary biology) using the PBRT, REP-PCR and whole-genome sequencing

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CHAPTER 2

Plasmid Evolution in Carbapenemase-Producing *Enterobacteriaceae*: A Review

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Running head: Plasmid-mediated evolution of carbapenemases

Abstract

Carbapenem-resistant *Enterobacteriaceae* (CRE) have been listed by the WHO as high-priority pathogens owing to their high association with mortalities and morbidities. Resistance to multiple β-lactams complicates effective clinical management of CRE infections. Using plasmid typing methods, a wide distribution of plasmid replicon groups has been reported in CREs around the world, including IncF, N, X, A/C, L/M, R, P, H, I, and W. We performed a literature search for English research papers, published between 2013 and 2018, reporting on plasmid-mediated carbapenem resistance. A rise in both carbapenemase types and associated plasmid replicon groups was seen, with China, Canada, and the United States recording a higher increase than other countries. *bla*$_{KPC}$ was the most prevalent, except in Angola and the Czech Republic, where OXA-181 (*n* = 50, 88%) and OXA-48–like (*n* = 24, 44%) carbapenemases were most prevalent, respectively; *bla*$_{KPC-2/3}$ accounted for 70% (*n* = 956) of all reported carbapenemases. IncF plasmids were found to be responsible for disseminating different antibiotic resistance genes worldwide, accounting for almost 40% (*n* = 254) of plasmid-borne carbapenemases. The *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1/9}$, *qnr*, and *aac-(6_)-lb* were mostly detected concurrently with carbapenemases. Most reported plasmids were conjugative but not present in multiple countries or species, suggesting limited interspecies and interboundary transmission of a common plasmid. A major limitation to effective characterization of plasmid evolution was the use of PCR-based instead of whole-plasmid sequencing–based plasmid typing.

Keywords: CRE; carbapenem resistance; plasmid typing; replicon types; incompatibility groups

2.1 Introduction

Prescription of carbapenems are increasing extensively worldwide owing to their relative safety and efficacy in resolving most fatal multidrug-resistant (MDR) bacterial infections. Subsequently, this is triggering and selecting resistance to carbapenems among an increasing number of Gram-Negative bacterial pathogens, including *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [1]. The increasing worldwide incidence and prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, with

their very high attributable mortalities ranging from 6.6% to 20%, are considered global threats to human and animal health [2–5]. Subsequently, they have been enlisted as priority 1 critical pathogens by the World Health Organization (WHO, 2017). Owing to the importance of carbapenems in the clinical management of MDR infections, the emergence and rapid dissemination of CREs that are also resistant to fluoroquinolones, aminoglycosides, colistin reduce therapeutic options [6,7]. Although CREs have been mostly isolated from healthcare-associated infections, *Enterobacteriaceae* also cause community-acquired infections, which helps explain the spread of CREs in the community [8].

Carbapenem resistance is mainly driven by carbapenemases found on mobile genetic elements (MGEs), such as integrons, insertion sequences, transposons, and mobile plasmids that can shuttle carbapenemase-expressing genes within and across bacterial cells of the same or different species [9–11]. The ability of plasmids to carry multiple antibiotic resistance genes (ARGs) and be mobilized across same and different species via conjugation make them very important in the molecular epidemiology of CREs [11,12]. This is further complicated by the ability of multiple plasmids, depending on their incompatibility (Inc), to be harbored in a single CRE cell. Coupled with their extrachromosomal and self-replicative characteristics, plasmids are crucial in bacterial adaptations and survival in unsuitable environments [12,13].

The centrality of plasmids in the epidemiology of antibiotic resistance necessitates an in-depth study into their structural and genetic characteristics. Plasmid replicon typing is the main technique used in identifying and classifying plasmids carrying virulence and/or ARGs [14]. These typing schemes, which includes the PCR-based replicon typing (PBRT) and plasmid mobility (MOB) typing, can determine whether antibiotic resistance is driven by a dominant or diverse plasmid type(s)[15].

2.1.1. Literature search strategy

PubMed was searched for all English research papers using the following search words: "carbapenems," "carbapenemase," "*Enterobacteriaceae,*" and "plasmids." This search yielded a total of 2098 articles after duplications were removed. A search period of 6 years, from January 1, 2013 to August 30, 2019, was applied, which decreased the number of papers to a total of 862. The title and abstracts of these papers were screened for eligibility according to our hypothesis and research questions, which resulted in 102 research papers being included in the analysis. The inclusion criteria included all papers retrieved using the keywords "carbapenem," "carbapenemase," *"Enterobacteriaceae*," and "plasmid," and reporting plasmid replicon groups associated with carbapenemases in *Enterobacteriaceae* species. We excluded all papers that reported plasmid replicon groups in non carbapenemase-producing *Enterobacteriaceae* (Fig. 2.1).

2.1.2. Statistical analysis

All pie and bar charts in this review were constructed after analysis and calculation of the results using Microsoft excel 365®. All charts were also designed using Microsoft excel 365®.

2.1.3. Evidence before this review

To our knowledge, at least two articles have been published on this topic. A mini review published by Carattoli[17] focused on plasmid families in *Enterobacteriaceae*; a second review, published by Mathers *et al.,*[18] focused on high risk clones in the spread of MDR *Enterobacteriaceae* and associated resistance plasmids. In contrast to the former minireview, our review provides an update on plasmid families associated with carbapenemases; in contrast to the latter review, which did not report the frequency of carbapenemase genes and their associated plasmid groups, our review aims to provide such data in addition to looking at all *Enterobacteriaceae* species instead of specific clones.

2.1.4. Purpose of this review

Our systemic review aims to provide insights on plasmids mediating the dissemination of carbapenem resistance in *Enterobacteriaceae*. It focusses on the following aspects: classification of carbapenemases, methods used in plasmid classification, plasmid biology and incompatibility plasmid groups, plasmid epidemiology and MGEs associated with Inc groups. Thus, our review aims to highlight the frequency and evolution of plasmids carrying carbapenemase genes over the last 6 years. Information that we provide also shows the evolution of the genetic structures in different incompatibility groups, which helps to explain the spread of carbapenemases and plasmids worldwide.

2.2. Carbapenems used as last resort antibiotics

Carbapenems are β-lactam antibiotics that differ from other β-lactams by the presence of a carbon instead of a sulfone at the fourth position of the lactam ring[19]. Carbapenems have broad-spectrum activity against both Gram-Negative and Gram-Positive bacteria and are usually reserved for serious infections caused by Gram-Negative bacteria (GNB)[20]. However, each carbapenem differs in stability, ability to inhibit or induce β-lactamases, and resistance to β-lactamases[21]. These characteristics have been used to classify carbapenems into three groups. Group 1 carbapenems such as ertapenem and panipenem, have limited activity against nonfermentative GNB and are suitable for community-acquired infections. Group 2 carbapenems include biapenem, doripenem, imipenem, and meropenem and are active against nonfermentative GNB and suitable for hospital-acquired infections. Group 3 carbapenems such as PZ-601 (not licensed) comprise of the cationic and dithiocarbamate carbapenems and have extended spectrum of activity; they are also active against methicillin-resistant *Staphylococcus aureus*[22,23]. Carbapenems are usually saved for β-lactamase-producers that are resistant to almost all classes of β-lactams except carbapenems.

However, some *Enterobacteriaceae* and other nonfermenters may produce carbapenem-hydrolyzing enzymes that enable them to resist carbapenem activity [23]. Carbapenemase production is thus the major mechanism of carbapenem resistance in *Enterobacteriaceae*.

2.3. Classification of Carbapenemases

Carbapenemases hydrolyze carbapenems and all other β-lactams[24] by breaking the β-lactam ring structure of β-lactam antibiotics, thus disrupting their function. β-lactamases are classified into different classes according to either their amino acid sequence or their functionality, that is, substrate specificity. In the 1980s, Ambler grouped β-lactamases into four classes, that is, class A–D, based on their amino acid sequence homology[25]. These classes function by different mechanisms based on the molecules at their active sites. Classes A, C, and D have serine at their active sites and use serine ester hydrolysis mechanism, while class B members have a zinc ion(s) at their active sites, which facilitate substrate catalysis[26,27]. Among these four classes, carbapenemases are placed in only three: classes A, B, and D.

The functionality classification scheme consists of three major groups: groups 1 – 3. Group 1 consists of cephalosporinases; group 2 are the penicillinases, cephalosporinases, and broad-spectrum β-lactamases inhibitors; and group 3 is composed of the metallo-β-lactamases (MBLs) [28,29]. In this scheme, carbapenemases are placed in Group 2 (class A and D) and Group 3 (class B), with the former being serine carbapenemases (SBLs) [10,28,29].

2.3.1. Class A carbapenemases

The first class A carbapenemase to be described was chromosomally located and reported in both clinical and environmental GNB[30]. It was only in the 1990s that plasmid-mediated class A carbapenemases were commonly described in clinical GNB including, *Enterobacteriaceae, P.*

aeruginosa, and *Acinetobacter* species[31]. Both chromosomally and plasmid-mediated carbapenemases are capable of hydrolyzing almost all β-lactams including carbapenems, while SBLs are inhibited by commercially available β-lactamase inhibitors such as clavulanic acid and tazobactam[30,32]. The most commonly described plasmid-mediated class A carbapenemases are the *Klebsiella pneumoniae* carbapenemase (KPC) and Guiana extended-spectrum β-lactamase (GES).

The GES family has more than 20 variants, with GES-1 showing activity towards other β-lactams but not carbapenems[33,34]. Most GES variants have activity towards broad-spectrum cephalosporins, but amino acid substitution in other variants extends their activity towards carbapenems[34]. Such variants with carbapenemase activity include GES-2, GES-4, GES-5, GES-6, GES-14, GES-16 and GES-18[33,35–39]. GES-2 is commonly detected in *Pseudomonas* spp., and it was first identified in a clonal outbreak of *P. aeruginosa* in South Africa[37]. Additionally, GES-5 is also described in *Pseudomonas* spp. and *Enterobacteriaceae*, and have been widely reported in South America, with few reports in Canada, the Czech Republic, Turkey, Portugal, South Africa, and South Korea[12,34,41–44]. Other GES variants are also reported, although rarely[45,46].

Klebsiella pneumoniae carbapenemases (KPCs) have broad-spectrum activity against almost all β-lactams, including carbapenems, and they are mostly reported in *K. pneumoniae* clinical isolates[47,48]. However, in the last decade, KPC has also been reported in other species of *Enterobacteriaceae*, including *Escherichia coli*, *Enterobacter* spp., *Klebsiella oxytoca*, *Proteus mirabilis*, *Serratia marcescens*, *Morganella morganii*, and *Citrobacter freundii*[48-51]. The KPC carbapenemases are widely distributed worldwide, but they are mostly reported in the United States, where they cause majority of the reported cases of infection[52,53]. In the United States, KPC-producers are usually associated with hospital outbreaks caused by patient-to-patient transmission of clonally related resistant organisms[54]. More than 20 KPC variants have been described, but

KPC-2 and KPC-3 are mostly reported and widely distributed[48,55]. KPCs have been reported in several *K. pneumoniae* sequence types (ST), although ST258 and ST11 are the major players associated with pandemic spread[12,54,56-58].

2.3.2. Class B Metallo-β-lactamases (MBLs)

Class B carbapenemases or MBLs are broad-spectrum β-lactamases capable of hydrolyzing all clinically available β-lactams except monobactams, and are not inhibited by commercially available β-lactamase inhibitors: clavulanic acid, tazobactam, or sulbactam [34,59]. However, MBLs are inhibited by metal ion chelators such as ethylene diamine tetra-acetic acid (EDTA) and dipicolinic acid (DA) [59–62] as their hydrolytic activity is dependent on the interaction between the active site zinc ion (Zn^{2+}) and the β-lactam[61]. The most common MBLs reported in *Enterobacteriaceae* include Verona-integron metallo-β-lactamase (VIM), Imipenemase (IMP) and New Delhi metallo-β-lactamase (NDM)[56,60,61,63,64].

IMP-types were among the acquired MBLs first identified in *Enterobacteriaceae*, the most common variant being IMP-1[61]. In 1991, IMP-1 was isolated for the first time in *Serratia marcescens* in Japan, and was located on a class 1 integron [34]. Since then, more than 40 variants have been reported in Japan, Taiwan and around the whole world [30].

The first occurrence of VIM-type (VIM-1) β-lactamase was in 1997 in Verona, Italy in a *Pseudomonas aeruginosa* isolate; and VIM-2 was reported in France [60,65–67]. So far, more than 40 variants of VIM have been described, albeit VIM-2 is the most common worldwide [34,68] VIM-2 is usually common in *Pseudomonas* spp. while VIM-1 is common in *Enterobacteriaceae* [17,69]. VIM-types carbapenemases have been reported in more than 17 countries but they are mostly prevalent in Africa and Europe[70]. *K. pneumoniae* species are mostly associated with VIM-variants, followed

by *Enterobacter cloacae*, *Citrobacter* spp., and *E. coli* in Greece, Spain, and, rarely in Germany and Czech Republic[70–72]. Since 2014, Sporadic reports of VIM-4-producing *K. pneumoniae* and *E. cloacae* were identified in Mediterranean countries[72].

First emergence of NDM was described in *K. pneumoniae* and *E. coli* clinical isolates in 2009 from a Swedish patient in New Delhi, India[73]. Since August 2010, NDM has spread worldwide to Canada, China, Europe, Japan, South Asia, Africa, Australia and the United States [74–76]. Epidemiologically analysis of the NDM-1 gene shows that it originated from the Indian subcontinent. NDM-1 is the most common variant described worldwide, but NDM-1 to NDM-9 has been published and 12 variants have been assigned[77,78]. NDM-4, NDM-5, NDM-7 were reported to have increased carbapenemase activity than NDM-1[61,79–81].

2.3.3 Class D carbapenemases

Class D β-lactamases are called the oxacillin-hydrolyzing enzymes and comprise of more than 200 enzymes, although a few have carbapenemase activity[61]. The most prevalent variants are OXA-48 and OXA-181, which weakly hydrolyze carbapenems[82]. Most OXA variants are commonly reported in *Acinetobacter baumannii* and rarely in *Enterobacteriaceae*[66,83]. This class of β-lactamases is not inhibited by commercially available β-lactamase inhibitors and/or EDTA[82].

Since the emergence of OXA-48, it has been increasingly reported in *Enterobacteriaceae* species including *E. coli*, *Enterobacter* spp., *Citrobacter freundii*, *Klebsiella oxytoca*, *Providencia rettgeri*, and *Salmonella marcescens*[40,84]. Although OXA-48s hydrolyze carbapenems to a lesser extent, their co-occurrence with other resistance mechanisms such as membrane impermeability may result in high-level resistance [40]. OXA-48 is widespread in *Enterobacteriaceae* worldwide and has been reported in countries in the Middle East (Saudi Arabia, Israel), Africa (Libya, Egypt,

Algeria, Morocco, South Africa), Asia (Russia, India, China, Taiwan), and South America (Argentina, Brazil, Colombia) [76,85–90]. The geographical distribution frequency of carbapenemase genes reported per country in the articles included in this review are shown in figure 2.2.

2.4 Methods used in plasmid classification

The identification and classification of plasmids form the foundation of research looking at different plasmid groups in bacteria. Scientists gave much attention to this topic after discovering the role of plasmids in the acquisition and dissemination of virulence and resistance genes by horizontal gene transfer[14]. Classification of plasmids is very important in studying the biology, adaptation, and evolution of microbial populations. Size and number of plasmids in a bacterial cell are usually determined using gel electrophoresis and/or pulsed field gel electrophoresis[91]. Plasmids are classified according to their incompatibility (Inc) or replicon group, which is based on the replication factors expressed by the plasmid in the bacteria. Incompatibility was determined by introducing a plasmid of unknown replicon group in a recipient with a plasmid of known replicon group. The two plasmids are assigned to the same replicon group if the resident plasmid is eliminated. If this plasmid is not eliminated, the two plasmids are assigned to different incompatibility groups[92]. This method was used for several years to trace the dissemination of antibiotic resistance plasmids and the evolution of new plasmids. Couturier *et al*. proposed a new method based on hybridization of the major plasmid replicon groups in *Enterobacteriaceae*[93]. This method was labor intensive and almost impossible on large sample sizes. To overcome these limitations, new typing schemes were introduced to facilitate the characterization and epidemiological analysis of resistance plasmids[94].

Numerous plasmid classification schemes, including replicon and degenerate primer MOB typing (DPMT), which, respectively, targets loci encoding replicons and mobility functions, are widely used in research[15,95,96]. Carattoli *et al*. developed a PCR-based replicon typing (PBRT) method, which uses five multiplex-PCRs and three simplex PCRs with 18 sets of primers that target the major plasmid replicon groups in *Enterobacteriaceae*[96]. Subsequently, this method was updated to incorporate emerging plasmid replicon groups such as IncR and IncU. Until recently, this method has been useful in the identification and classification of major antibiotic resistance plasmids circulating among *Enterobacteriaceae*. The PBRT scheme increased our knowledge of plasmid diversity and revealed that conjugative plasmids belonging to a few widespread replicon groups carry clinically relevant ARGs[98]. Real-time PCR has also been used with the same principle as the PBRT method, which speeds up the detection and classification of plasmids and reduces human error and contamination[99].

In 2011, a commercially available PCR-based typing kit was introduced, which includes all the modifications that have been incorporated since 2005 to 2010[100]. This kit contains all reagents and primers needed to perform the PCR, but still uses the same principle as the original PBRT method. The PBRT kit detects 28 replicons and is composed of eight multiplex-PCRs and positive control plasmids for all the PCRs[100]. Even though this method is still labor intensive and time consuming, it may detect more plasmid replicons than the 2005 PBRT scheme[100].

A technique based on plasmid mobility, called DPMT, was introduced by Francia and colleagues in 2004[101,102]. This technique uses degenerate primers to target relaxase sequences for separating plasmids into MOB types identified by *in silico* MOB typing[95,103]. The MOB typing overcomes replicon typing limitations in that it targets relaxases, of which only one can be encoded in a

plasmid. Unlike the PBRT, which detects plasmids at higher resolution, the MOB typing uses lower resolution to classify plasmids[94,98]. However, PBRT and DPMT have been combined to successfully classify plasmids in clinically relevant pathogens[104].

However, these typing schemes have a relatively lower discriminatory power than recent techniques such as plasmid multi-locus sequence typing (pMLST), whole-plasmid sequencing (WPS) and whole-genome sequencing (WGS)[94]. The PBRT methods have a few setbacks such as: i) the presence of multiple replicons in a single plasmid, which complicates plasmid classification; ii) rapid evolution of plasmid replicons; iii) and the presence of hybrid replication regions that make plasmid classification complicated[94,98]. Nevertheless, PCR-based typing methods may be used preliminarily for screening plasmids prior to using higher resolution techniques. All the typing techniques discussed above have played a major role in plasmid evolution and epidemiology research in different countries worldwide.

pMLST is a tool used to further subtype already known plasmid Inc groups that occur very frequently in bacterial cells [105]. This technique has been used to successfully subtype IncF, IncHI1, IncHI2, IncI1, and IncN plasmids (www.pubmlst.org/plasmid/). A/C subtyping was also developed to increase the discriminatory power for plasmid epidemiology studies. Hancock *et al*., recommended the use of pMLST and other PCR methods to further subtype A/C plasmids[106]. Garcia-Fernandez and colleagues (2011) suggested that pMLST can be used as a second-line plasmid typing technique after using PCR-based methods to identify plasmids[107]. pMLST has been used for epidemiological description of virulence and resistance plasmids in both human and animal reservoirs; moreover, more plasmid groups can be classified by pMLST[107]. In cases where pMLST is not available and plasmid subtyping is needed, a conventional technique, called

Restriction Fragment Length Polymorphism (RFLP), can be used. However, the results produced by this method can be difficult to interpret and may be very subjective[94].

WGS overcomes the defined limitations of typing methods and many plasmids can be typed in a reasonable timescale[108]. According to Carloni and colleagues, plasmid sequencing was able to detect novel plasmids previously not identified over the years by the PBRT scheme[100]. One major advantage of WGS is its ability to provide researchers with sequences of new/unknown plasmids[100]. Short read sequencers such as Illumina and Ion Torrent as well as long read sequencers such as PacBio and Oxford Nanopore are used for WGS or WPS, albeit PacBio is preferred for complete plasmid sequencing and gapless assembly[100]. Long-read sequencers are able to sequence repetitive sequences and/or multiple copies of the same mobile elements, which are usually longer than the read length covered by short-read sequencers; assembly programs will collapse such reads, identifying them as a single contig[11]. Long-read sequencing therefore provides a comprehensive insight into the epidemiology and evolution of plasmids, although it is more expensive and error prone due to lower throughput or coverage[109]. Subsequently, hybrid (short and long-read) sequencing and assembly, has been proposed and proven to override the deficiencies of both long- and short-read sequencers[43,110]. For instance, Li and colleagues, used Illumina and PacBio to yield high quality sequence reads; PacBio's proovread pipeline was used to correct the long reads errors[51].

Plasmid prediction database servers such as Plasmidfinder, pMLST, PLACNET, and plasmidSPAdes enable easy identification and annotation of relevant plasmid sequences from large WGS datasets[43,96,111], as well as assemble plasmids from WGS data[43,96,112,113]. PlasmidFinder is a web-based tool that allows submission of raw or assembled reads, which are searched for through

a plasmid replicon database to identify replicons and assign the plasmid to an Inc group[96]. The plasmid constellation network (PLACNET) is a graph-based tool that reconstructs plasmids from short read WGS raw data and is applied in plasmid diversity and adaptation[114]. The PLACNET tool uses three types of data for reconstruction of plasmids: (1) scaffold links and coverage; (2) comparison to a reference plasmid; and (3) sequences such as replication initiator proteins[112]. Although, this tool assemble plasmid contigs automatically, it relies on manual trimming of the graph[113].

Furthermore, in 2017, PLACNETw (https://castillo.dicom.unican.es/) was developed based on the PLACNET database, automating all BLAST searches. PLACNETw only extracts the needed plasmid information and the graph-based presentation is automated[115]. In 2016, Antipov and colleagues developed a novel plasmid prediction database (PlasmidSPAdes) which also allows *de-novo* plasmid contigs assembly by manipulating differences in coverage in raw sequence reads[113]. PLACNET and PlasmidSPAdes are Linux-based applications that do not run on Windows and use raw sequence reads instead of assembled fasta files. A more recent Linux-based application for identifying known plasmid sequences from WGS data is PlasmidSeeker[116], which also use raw reads and *k-mers* abundance to identify plasmid sequences. PlasmidSeeker is unable to assemble plasmid sequences from raw reads *de novo.*

2.5 Plasmid biology and incompatibility groups

Plasmids are usually double-stranded (ds) extra-chromosomal material or DNA that can replicate independently from the chromosome. These dsDNA materials occur naturally in bacterial cells and are essential for bacterial adaptability and persistence[117]. Thus, bacterial fitness may also be gained under some ecological conditions via the accessory genes carried on these plasmids[118]. For

example, increased survival and competitive fitness is seen in bacteria carrying plasmids with heavy-metal resistance genes and ARGs[119]. Plasmids that occur naturally vary in size (1-100s kilobases) and in copy number (1- 100s in a cell).

Plasmids mediate the acquisition and dissemination of ARGs, including carbapenemases, through conjugation[120], which is only achievable by mobile/conjugative plasmids. The conjugative machinery share the same relaxase, a key protein that recognizes the origin of transfer (*ori*T) in conjugation [121]. Conjugative plasmids carry all the genes that are responsible for self-transfer, including the type IV coupling protein (T4CP) and all the components needed for mating channels that assemble a type IV protein secretion systems (T4SS)[121]. These systems are responsible for transporting proteins such as virulence factors and toxins extracellularly. The conjugative T4SS also exports DNA substrates[122].

Hedges and Datta defined plasmids based on their stability (Inc) and defined four Inc types including: i) the type F pili-producing plasmids, which are susceptible to phage Ff (IncF); ii) the type I pili-producing plasmids, susceptible to phage Ifl (IncI); iii) plasmids related to N3, susceptible to phage Ike (IncN); iv) and plasmids related to RP4, susceptible to phage PRR1 (IncP) [17]. Numerous plasmid incompatibility (Inc) replicon groups have been associated with carriage of ARGs, thereby facilitating intra- and inter-species transfer**.**

2.6 Plasmid types and incompatibility groups associated with carbapenemases

To date, 27 major plasmid incompatibility groups are associated with ARGs in *Enterobacteriaceae*[17,93,123]. A wide distribution of plasmid replicon groups has been reported in CREs, including IncF, N, X, A/C, L/M, R, P, H, I, and W. These replicon groups are associated with different carbapenemases, with IncF, A/C, and X being the most prevalent in carbapenemase

production compared with the other Inc groups. The most prevalent incompatibility types in *Enterobacteriaceae* are the IncF plasmids, which have been reported in different sources around the world[17,18].

Plasmid host range is usually a term used to describe the range of hosts in which a plasmid can replicate. This host range varies amongst plasmids, and the terms narrow-host range and broad-host range are used for the plasmid host range differentiation[124,125]. Narrow-host range self-transmissible plasmids are mainly of IncF, IncH and IncI types, while IncL/M, IncN, IncP, and IncW can replicate in broad-host ranges [124,126]. Table 2.1 and Figures 2.2-2.4 show the different ARGs reported in each country and their associated plasmid replicon groups mediating the spread of these genes.

2.6.1 IncF plasmids

IncF plasmids are narrow-host-range plasmids that rely on both host-encoded and self-encoded factors for replication[123]. They are usually large in size (>100 kb), but with low copy number and often carry an additional replicon type to initiate replication[127]. This a strategy used by narrow-host range plasmids to obtain broad-host range replication. An example of this was seen in plasmid pKPX-1 from NDM-producing *K. pneumoniae* clinical isolates, which contains a narrow-host range (IncFIB) and a broad-host range (IncR) replicons, assisting with broad-host range replication[128]. This is an important characteristic of IncF plasmids, but these plasmids still encode regions essential for conjugative transfer, replication, and segregational stability[117]. Moreover, the plasmid's multi-replicon state can allow for acquisition of a plasmid carrying an incompatible replicon when replication is controlled by a compatible replicon, allowing the replicon not responsible for replication to undergo genetic alteration[123,129].

IncF plasmids are mostly associated with extended-spectrum beta-lactamases (ESBLs), particularly the *bla*$_{\text{CTX-M-15}}$ gene. A major IncF plasmid carrying the *bla*$_{\text{CTX-M-15}}$ gene was reported by Coque *et al*., to contain an MDR region containing *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$, and *aac(6')-Ib-Cr* genes, and other determinants of aminoglycoside and tetracycline resistance[130]. Moreover, these plasmids have been recently associated with carbapenemases in *Enterobacteriaceae*. Their great intracellular versatility and rapid evolution of their replicons' regulatory sequences allow them to succeed in their spread in *Enterobacteriaceae*[123]. This has been shown in most studies focusing on KPC- and NDM-producing *E. coli* and *K. pneumoniae* in different countries[128,131–134].

The first occurrence of an IncF plasmid (pKpQIL) in *K. pneumoniae* ST258 was reported by Villa and colleagues, which was a 113-kb plasmid belonging to the IncFII replicon group[123]. Since then, IncF plasmids have been reported in other countries where they mediate the spread of *bla*$_{\text{KPC}}$. Examples of IncF plasmids in *K. pneumoniae* carrying KPC include pBK30683 (140-kb) and pBK30661 (73,6-kb) plasmids, which were reported in US hospitals from patients with urinary tract infections[135]. pBK30661 was identified as an IncFIA plasmid harboring nine ARGs such as β-lactam resistance (*bla*$_{\text{KPC-3}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-9}}$), aminoglycoside resistance (*aacA4*, *aadA1*, *strA*, *strB*), sulfonamide resistance (*sul2*), and trimethoprim resistance (*dfrA14*) genes[135]. Other IncF types such as pKP1504-KPC and pGR-1780, have also been reported to spread *bla*$_{\text{KPC-2}}$ in *K. pneumoniae* clinical isolates, specifically ST258 and ST147[136].

These narrow-host range (IncF) plasmids are not only responsible for disseminating KPC, but also NDM in *E. coli* and *K. pneumoniae*[137–139]. Multiple plasmids have been reported, since 2012 and until recently, to carry NDM variants particularly on IncFIB and IncFII plasmid types in *K. pneumoniae* and *E. coli*, respectively (Table 2.1, Fig. 2.3)[128,131–133,140]. Bigger plasmids such as pPMK1-NDM (304,5-kb) and pNDM-EcoGN568 (166,7-kb), are examples of NDM-1-containing

plasmids, which contained other resistance determinants including β-lactamases, with pPMK1-NDM containing a large conjugative transfer module[132,141]. Other IncF plasmids including pEh1A, pNDM-Ec1GN574, pKOX-NDM-1, and pCRCB-101_1 are also responsible for the dissemination of *bla*$_{NDM-1}$ in other species such as *Citrobacter freundii*, *Enterobacter hormaechei*, and *Klebsiella michiganensis*[128,134,141,142]. pNDM-Ec1GN574 and pKOX-NDM1 plasmids were similar in size (110,8-kb), with the NDM region being flanked by 256-bp direct repeats, which are suggested to be responsible for the acquisition of the *bla*$_{NDM-1}$ gene[141]. An IncFII plasmid was also reported in an isolate in China carrying both NDM-5 and MCR-1 genes[143].

Although IncF plasmids are the most prevalent, other narrow-host range incompatibility types such as IncI, L/M, and IncX, are widely distributed and are associated with multiple carbapenemases, ESBLs and MBLs. Only in a few instances have they been associated with the class D carbapenemases, specifically OXA-181 gene (Table 2.1).

2.6.2 IncX plasmids

IncX plasmids were previously described as less predominant in *Enterobacteriaceae*, because of underestimations by PBRT. The first plasmids in this group, R6K, were discovered by Kontomichalou and colleagues in 1970, during the pre-antibiotic era in a *Salmonella* spp. isolate. This was a 39,8-kb self-transmissible low copy number (10-15 replicons) plasmid, containing ampicillin and streptomycin resistance determinants[144]. Comparison studies looking at plasmid R6K and modern plasmids revealed that this plasmid is different from other plasmids in the IncX group, suggesting that subdivisions are required in the IncX group[145]. Firstly, only two subgroups (IncX1 and IncX2) were characterized based on restriction analysis[145]. The expansion of this plasmid family to IncX3 and IncX4 was proposed by Johnson *et al*., based on a phylogeny deduced from polymorphisms of all conserved regions of sequenced IncX plasmids[146]. Another subgroup,

IncX5, was further added shortly after this expansion in a KPC-5-producing *K. pneumoniae* isolate[147]. Since these expansions, IncX plasmids have been found to play a major role in the dissemination of β-lactamases, including carbapenemases.

IncX1 was previously described as more predominant than IncX2 in environmental isolates[145]. However, Dobiasova and Dolejska reported a high prevalence of IncX1 and IncX4 in environmental isolates and none in human isolates in Africa[148].

IncX plasmids are usually associated with carbapenemase genes in *Enterobacteriaceae*, particularly *bla*$_{KPC}$, *bla*$_{OXA-181}$ and *bla*$_{NDM}$ (Table 2.1). According to recent studies, IncX3 is the predominant subgroup reported to harbour both *bla*$_{KPC}$ and *bla*$_{NDM}$ genes[149–152]. These studies reported this subgroup as predominantly associated with *bla*$_{NDM}$ variants than with *bla*$_{KPC}$ variants. Further, *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ were frequently associated with IncX3 than any other *bla*$_{NDM}$ variant. Only in a few instances have IncX4 and IncX5 plasmids been associated with carbapenemase genes (Table 1)[150,153]. In addition, an IncX5 plasmid encoding *bla*$_{IMP-4}$ was reported in Australia from an *E. coli* of animal origin[154]. These suggest that an essential role is played by IncX3 in the acquisition, emergence and dissemination of *bla*$_{NDM}$. IncX3 plasmids that have been associated with the spread of *bla*$_{NDM}$ are the following: pEc2A (74,8-kb), pM213_X3 (43,5-kb), pNDM-NJ-IncX3 (39,5-kb), and pKW53T-NDM (46,1-kb)[140,142,153]. Other IncX3 plasmids recovered from Czech hospitals in Europe have been reported in *E. cloacae* isolates that express *bla*$_{NDM-4}$[155]. An IncX plasmid was reported in China in an *E. coli* isolate co-expressing both *bla*$_{NDM-5}$ and *mcr-1*[143]. Occurrence of IncX6 was reported in 2016 in *E. cloacae*. Moreover, the dissemination of this plasmid type has been shown in at least six *Enterobacteriaceae* species in China[51,156]. IncX6 was reported to carry both *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ in China (Table 2.1)[51,156]. In *bla*$_{KPC}$-producing *Enterobacter* spp., another subgroup, IncX7, has also been reported in the United

States[53]. These findings suggest the wide dissemination of IncX subgroups in *Enterobacteriaceae* in China and the United states.

2.6.3 L/M plasmids

L/M plasmids have been considered an emerging threat due to their increasing prevalence in MDR clinical and environmental isolates[157]. L/M plasmids are broad host-range plasmids with an average size of 50 to 80-kb and a low copy number[98]. Foster *et al*., reported that the pEL60 plasmid in *Erwinia amylovora* has a basic L/M plasmid backbone but it lacks genetic elements and resistance determinants[158]. Moreover, genomic analysis of L/M plasmids have shown backbone genes such as replication and stability modules, conjugative transfer system and a *mucAB*-like mutagenic DNA repair system[158].

Separation of this group into IncL and IncM was suggested by Carattoli *et al*., because of differences in the proteins expressed, namely, ExcA, TraY and TraX. This separation was accepted, and the PBRT scheme has been updated to incorporate these separate plasmids. This incompatibility group has been associated with multiple ESBLs, AmpCs, and carbapenemases, specifically class B and D genes[157,159]. Several IncL plasmids in $bla_{\text{NDM-1}}$- and $bla_{\text{OXA-48}}$-expressing clinical isolates have been widely reported, some of which include: pNDM-OM (87,1-kb), pNDM-HK (88,8-kb), E71T (63,5-kb), and pOXA-48-4963 (63,5-kb)[157,160,161]. Although IncL/M plasmids usually harbour bla_{NDM} and $bla_{\text{OXA-48}}$ genes, they are also reportedly associated with bla_{IMP} in *Enterobacteriaceae* (Table 2.1, Fig. 2.3). A few studies have identified these plasmids in $bla_{\text{IMP-4}}$-producing isolates[162]. From isolates of animal origin, Dolejska and colleagues reported the presence of $bla_{\text{IMP-4}}$ in *E. aerogenes* on an IncM plasmid (pEa1631, 85-kb)[154]. Bryant *et al*., have also reported L/M plasmids (pNE1280, 66,5-kb) in bla_{KPC}-expressing isolates from a female with

a medical history of mitral and aortic valve stenosis, pulmonary hypertension, restrictive lung disease and diabetes[163].

2.6.4 A/C plasmids

Another important broad-host range incompatibility type is the A/C plasmids, which harbour various carbapenemase genes. These plasmid types are different from other plasmid types in that they contain an integron with the theta replicon, three integrative hotspots, putative transcriptional regulators, and hypothetical genes[164,165]. These plasmids are large with low copy numbers. The IncA/C plasmid types are usually associated with cephalosporinases eg. *bla*$_{CMY}$ and MBLs eg. *bla*$_{NDM}$ (Table 1)[166,167]. However, these plasmids have also been associated with the dissemination of carbapenemases such as *bla*$_{NDM}$, *bla*$_{VIM}$ and *bla*$_{KPC}$[155,168,169]. Two A/C groups have been identified and are named A/C$_1$ and A/C$_2$, with A/C$_2$ being the most predominant[170]. However, all A/C plasmid types share most of the conserved regions such as the genes responsible for conjugative transfer (*tra* genes) and replication (*repA*), as well as other genes with unknown functions[171]. Only a few plasmids belong to the first A/C plasmid types, including plasmids pRA1 and pIncAC-KP4898[172,173]; pIncAC-KP4898, encoding *bla*$_{VIM-1}$, is a recently isolated 156,2-kb plasmid[173].

bla$_{NDM-1}$ has been associated with A/C$_2$ plasmid types in different *Enterobacteriaceae* species; recently, *bla*$_{NDM-4}$ was detected on A/C$_2$ plasmids[140]. A/C$_2$ plasmids reported to carry *bla*$_{NDM-1}$ include pM214_AC2 (176-kb), pNDM-EcoGN568 (166,7-kb), pNDM-KN (162,7-kb), and pNDM-PstGN576 (147,8-kb)[140,141,167]. pNDM-EcoGN568 is a multi-replicon (IncF and A/C) circular plasmid, which was reported to be identical to pNDM10-0505, an A/C plasmid with the same size as the pNDM-EcoGN576 plasmid[141]. All these three plasmids shared similar conserved

sequences and genes, suggesting a lateral transfer between different species, albeit their independent acquisition of genes cannot be ruled out[141].

2.6.5 IncN plasmids

IncN plasmid types are also of broad-host range, with high transmission efficiency. They are also important in the dissemination of carbapenemase genes including *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$ (Table 2.1)[74,147,174,175]. Within this group of plasmids, three subgroups with similar plasmid scaffolds and less similarity in backbone sequences have been described: IncN1 (R46), IncN2 (p271A), and IncN3 (pN-Cit)[85,176]. These characteristics might be the reason for their stability and success in disseminating multiple carbapenemases. IncN plasmids are usually medium-sized conjugative plasmids documented to be associated with *bla*$_{VIM}$-expressing *Enterobacteriaceae* and *bla*$_{KPC}$-expressing *K. pneumoniae* isolates[177]. Plasmids including p9 (70,6-kb), p12 (75,6-kb), pKPC-629 (80,1-kb), pBK31551 (83,7-kb), pKO6 (65,5-kb), and pKp58-N (69,8-kb) have been documented as carriers of *bla*$_{KPC}$[52,135,178]. Most of these plasmids have been deposited into GenBank without a corresponding published article.

The pKOX105 (54,6-kb) plasmid carried regions encoding genes conferring resistance to carbapenems (*bla*$_{VIM-1}$), cephalosporins (*bla*$_{SHV-12}$), aminoglycosides (*aacA4*), trimethoprim (*dfrA14*) and quinolones (*qnrS1*)[179]. This plasmid was compared with other previously reported IncN plasmid types i.e., plasmids 9 (70,6-kb) and 12 (75,6-kb) that carried a *bla*$_{KPC}$ gene[178]. The scaffolds between these IncN plasmids were found to be the same, but the MDR regions were different in all the plasmids[179]. The major differences that are usually reported among IncN plasmids are related to their acquired genes[179].

2.6.7 Other plasmid groups

Other incompatibility groups such IncI, ColE, IncB/O, IncH and IncP have also been reported to be associated with carbapenemases in *Enterobacteriaceae*, albeit they are reported in few species and are limited to a few carbapenemase genes (Table 2.1).

2.7 Molecular epidemiology of plasmids in *Enterobacteriaceae*

2.7.1 *K. pneumoniae*

In the United States, KPC is the major carbapenemase associated with antibiotic resistance (Fig. 2-4)[24,49,135,180]. KPC variants, such as KPC-2, KPC-3, and KPC-4, were reported in several studies in the US and were associated with multiple plasmid replicon groups, facilitating their spread. KPC-2 was commonly associated with multiple STs, but ST258 was the most prevalent in the US. The IncF groups dominate in the spread of KPC-2 and KPC-3 in the US and other countries including Australia, Canada, China, Italy, Romania, and Spain[42,50,52,56,152,153,156,174] Only one study has reported on IncF groups in KPC-3 in Portugal and Romania[56,181]. Few occurrences were also reported in other countries including USA, Mexico and Spain. This plasmid replicon group is commonly reported in KPC-producing *K. pneumoniae* species[182].

Other plasmid replicon groups such as IncN, IncP, IncX, IncU, IncI, A/C, IncR, and L/M are also occasionally reported in KPC-producing *K. pneumoniae*[48,52,53,57,175,180,183,184]. Moreover, these plasmid replicon groups were also associated with *K. pneumoniae* strains producing other carbapenemases. VIM was only reported by two studies in Italy and Kuwait to be hosted by IncN and A/C[174,177].

OXA variants in *K. pneumoniae* are usually spread by IncF and L/M replicon groups. L/M has been reported to spread OXA-48 in different countries including the US, Czech Republic,

Romania, and Australia (Table 2.1)[56,161,162,180]. OXA-181 was reported in Angola and Australia on IncF, A/C, and IncX plasmid replicon groups (Fig. 2.4)[138,162].

IncX is commonly associated with the spread of NDM variants and has been mostly described in China[185,186]. Other plasmid replicon groups including IncF, IncR, IncCol, L/M, and A/C have also been described in NDM-producing *K. pneumoniae* in Australia, China, Mexico, and Vietnam (Fig. 3)[139,143,162,184,185,187]. Furthermore, other *Klebsiella spp*. such as *K. oxytoca,* do not have a wide distribution of replicon groups as only the IncN and IncF groups have been described in them in few countries (Table 1). IncN plasmids have been reported in *K. oxytoca* strains producing VIM-1 and IMP-6 β-lactamases[175,177]. These plasmid types were occasionally reported in VIM-1-producing *K. oxytoca* isolated from river samples[177]. Moreover, IncN plasmids have been found with bla_{IMP} genes in Japan[175].

2.7.2 *E. coli*

Similar to *K. pneumoniae* species, *E. coli* strains have a wide distribution of plasmid replicon groups that have been reported worldwide. Most carbapenemase-producing *E. coli* usually harbor IncF plasmids, which is also dominant in *K. pneumoniae* species. IMP variants in *E. coli* are spread by multiple plasmid replicon groups such as IncHI, IncN, IncQ, IncX, IncI, and IncW[162,175,188]. Most NDM variants in *E. coli* were detected in China, except NDM-4, which is mostly reported in Australia[162]. NDM-1 has been disseminated worldwide through various plasmid replicon groups including IncF, IncI, IncX, and IncA/C; however, IncX is the most prevalent replicon facilitating the spread of bla_{NDM} genes[138,141,142,189,190]. Among these plasmid types, IncX-3 was mostly associated with the dissemination of $bla_{\text{NDM-1}}$ in China, a finding different from other countries[190–192].

2.7.3 *Enterobacter spp.*

In the United States, *Enterobacter spp.* are ranked 8th among all other pathogens causing health-care-associated infections[49]. MDR *E. cloacae* isolates have been associated with bloodstream infections, resulting in bacteremia and mortality as high as 40%[162]. *bla*$_{NDM}$ and *bla*$_{IMP}$ are the most predominant carbapenemases isolated from *Enterobacter* spp. in the United States, Australia, China and Vietnam[162,189,193]. In Vietnam, *bla*$_{NDM-1}$ was disseminated by IncF (IncFII and IncFIB) plasmids and, in a few cases, by the A/C plasmids (Table 2.1). Similar results were reported in other countries including the United Kingdom, Canada and the United States (Table 2.1). A/C plasmids have been identified in unrelated *E. cloacae* clinical isolates in China. As well, IncHI2 and IncN have been also implicated in the dissemination of *bla*$_{NDM-1}$[194]. IncHI2, L/M and IncP are usually associated with *bla*$_{IMP}$ gene in countries such as Australia, China and the United States in *Enterobacter spp*.[162,188,194]. However, IncP plasmids have so far been identified with IMP-27-producing *Enterobacter spp*. in the United States[188]. These plasmids are also present in Spain and carry the *bla*$_{KPC-2}$ gene in sewage[195]. Chavda *et al*., reported a wide distribution of plasmid Inc groups in KPC-producing *Enterobacter spp*. in New York City, which included IncN and IncX7 (*bla*$_{KPC-2}$), IncF and L/M (*bla*$_{KPC-3}$), and A/C (*bla*$_{KPC-4}$)[53]. The complexity, diversity and wide geographical distribution of these Inc groups disseminating major groups of classes A and B carbapenemases pose a major challenge to the control of MDR *Enterobacter spp*.

2.7.4 *Providencia, Proteus, Citrobactor* and *Salmonella* spp.

Other *Enterobacteriaceae* species including *Proteus* spp., *Providencia* spp., *Citrobacter* spp. and *Salmonella* spp. have been only reported in a relatively few cases, with few carbapenemases being identified in them (Table 2.1). Two major carbapenemases, *bla*$_{IMP}$ and *bla*$_{NDM,}$ are predominantly detected in these species, with IncHI2, A/C, IncP and IncX3 being the plasmid types responsible

for their dissemination between species[138,141,162,185,188,196,197]. The isolates in these reports were recovered from both clinical and environmental samples, including river water and domestic cats in Angola, Australia, Canada, China, India, Spain and the United States (Table 2.1)[138,139,141,162,188,196,197]. *Citrobacter* spp. also harbor IncX, IncR, IncHI2, IncP and IncN plasmids mediating bla_{NDM}, bla_{KPC}, and bla_{IMP} carbapenemases[97,198,199]. A hospital sewage isolate in China was found to contain *C. freundii* carrying an IncX3 plasmid harboring the bla_{NDM-1} gene[199]. Another study in China reported IncX3 plasmids in NDM-1-producing isolates collected from ready-to-eat vegetables[200]. Other countries, such as Australia, Canada and Italy, reported other plasmid types such IncFII, IncR, IncP and L/M in *Citrobacter* spp.[41,50,97,162]. Recent studies are reporting on the increased isolation of A/C plasmids in *Enterobacteriaceae* species, including *E. coli*, *K. pneumoniae* and *Salmonella spp.*[201]. Most isolates reported in our review here were clinical isolates from human, and only few studies evaluated here addressed carbapenemases and plasmid replicon groups in animal and Environmental isolates (Fig. 2.5).

2.8 MGEs associated with plasmid incompatibility types

Most MGEs are commonly found on plasmids and play an important role in disseminating antimicrobial resistance determinants. MGEs such as integrons, transposons and insertions sequences, may be associated with specific incompatibility groups and carbapenemases.

In most A/C plasmids, the antimicrobial resistance island is usually embedded in or upstream of the *rhs*1 gene, and also contain an integron, multiple transposons, a Tn*21*-tnp module, and a Tn*21*-mer module, which is interrupted by an insertion sequence IS*4321*[202]. Integrons, particularly class 1 integrons, are usually associated with A/C plasmids and gene cassettes carrying ARGs, specifically bla_{NDM}[141]. This was shown in multiple A/C plasmids, and one IncF (pNDM-

EcoGN568) plasmid, which was identical to A/C plasmids; the IncF plasmid only differed from the A/C ones by the number of ARG cassettes on the class 1 integron[141]. Other NDM-carrying plasmids, such as pM109-FII and pGUE-NDM, carry a 12-kb ARG region that surrounds the *bla*$_{NDM}$ gene[140]. An additional gene cassette bracketed by two IS*26* elements and carrying *bla*$_{TEM-1}$ was found downstream the *rmtB* gene (an aminoglycoside resistance determinant)[140].

The *bla*$_{NDM}$ variants such as NDM-4/5/6 have been reported on IncX3 plasmids. The genetic structure of IncX3 plasmids is usually highly similar in almost all plasmids. A study performed in Myanmar (Burma) found NDM-4 and NDM-7 on IncX3 plasmids that were highly similar to previously reported IncX3 plasmids; suggesting a common ancestor[140]. NDM-4 was carried on a Tn*3* transposon unit and flanked by insertion sequences, with no other resistance gene being reported on this plasmid. The *bla*$_{KPC}$ region of IncX6 plasmids are highly similar with Tn*6296* derivatives and an IS*Kpn19* element, However one plasmid reported by Li *et al* (2018) contained a Tn*6296* derivative and an IS*Kpn19*-containing Tn*6292* derivative[51,156].

The MGEs in A/C plasmids carrying other carbapenemases, such as *bla*$_{KPC,}$ are usually different from those carrying *bla*$_{NDM}$. Transposons are mostly associated with the acquisition of *bla*$_{KPC}$ genes. The Tn*4401* transposon, which is approximately 10-kb in size and delimited by two 39-bp inverted repeat (IR) sequences, are associated with a 5-bp target-site duplications (TSDs) on both site adjacent to the IR sequences[203]. The 5-bp TSDs adjacent to the IR sequences are the target-site sequences for the Tn*4401* transposons. This is an important characteristic identified in plasmid p9 and p12, which contained a functional conjugative apparatus with a ~10-kb region carrying the Tn*4401b* element with *bla*$_{KPC}$ and other ARGs[178]. The Tn*4401b* element in plasmid p9 was inserted

in an inverted orientation downstream the EcoRII restriction/antirestriction system and the *uvp1* gene[178].

Similar characteristics are seen in IncF plasmids carrying *bla*$_{KPC}$ with additional elements. pBK30661, an IncF plasmid whose backbone genes are separated by multiple insertion sequence elements (IS*3*, IS*26*, IS*1294* and IS*66*), had a Tn*4401d* variant with a 68-bp deletion upstream of the *bla*$_{KPC}$ gene[135]. The region upstream the Tn*1331* was truncated by an 8-kb nickel resistance operon (*nic* operon), which resulted in a deletion of the corresponding 5-bp sequence and leaving a unique 5-bp sequence adjacent to the upstream IR sequence[135]. pNE1280, an IncL/M plasmid carrying the *bla*$_{KPC}$ gene, contained a major insertion of a 13-kb Tn*3* family transposon, the Tn*4401f*, with the *bla*$_{KPC-4}$ flanked by IS*kpn6* on the left and IS*kpn7* on the right[163].

The genetic structure of the *bla*$_{OXA-48}$ gene in L/M plasmids is different from that of other carbapenemases. This gene is usually part of the Tn*1999* transposon (Tn*1999* – Tn*1991.4*), with Tn*1991.2* being the most prevalent[157,204]. In 2016, Cuzon and colleagues reported an L/M plasmid carrying GES-5 and GES-6 on the same plasmid (Table 2.1). This plasmid harboured additional ARGs, including *aadA1* and *sul*1[205]. *bla*$_{GES-5}$ and *bla*$_{GES-6}$ genes were located on a class 1 integron, and both sides were flanked by IS*26* and IS*6100*. This pEB-1 plasmid was compared to other L/M plasmids, pEL60 and pNDM-OM, and similar characteristics were observed, except that the integration site of the ARGs array was different[205]. In South Africa, a *bla*$_{GES-5}$ gene ;was reported on an IncQ plasmid, but it was still harboured on a class 1 integron, with an additional *aadA4* on an integron mobilization unit[12].

2.9 Conclusion

Our review showed a high frequency of *bla*$_{KPC}$ (n=956, 73%) genes in almost all the countries reported, with China, Canada, Greece and United States having the highest percentages. These genes are associated with multiple plasmid groups including IncF (n=254, 48%), IncN (n=125, 24%), IncX (n=38, 7%), A/C (n=39, 7%), and L/M (n=14, 3%) in different *Enterobacteriaceae* species. Furthermore, specific plasmid type, such as IncF, L/M and IncX3, have been reported to be associated with the dissemination of *bla*$_{KPC}$, *bla*$_{OXA-48}$, and *bla*$_{NDM}$, respectively. We also showed the frequency of carbapenemases and plasmid replicon groups in the articles used for this review. Other countries, such the United States and China had high frequencies due to more research being performed in these countries. Our review has shown the important role played by MGEs, such as plasmids, transposon, and insertion sequences, in acquisition and dissemination of ARGs among *Enterobacteriaceae* species, increasing the need for new antibiotics and antibiotic stewardship strategies. We also found that a major limitation to effective characterization of plasmid evolution was the use of PCR-based instead of WPS-based plasmid typing. WGS has proven to give enough data for plasmid characterization, albeit PBRT still forms the basis of most plasmid characterization studies, particularly in low-income countries. Obviously, long-read WPS and WGS hold the key to an efficient characterization of plasmid types, epidemiology and evolution, and towards an efficient description of antibiotic resistance dissemination and expansion among *Enterobacteriaceae*. By overriding the deficiencies of PBRT, WPS and WGS will likely increase the effective identification and control of resistant bacteria, reducing mortalities, morbidities, and healthcare-associated expenses involved in long-term hospitalization of infected patients.

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Table 2.1: Major plasmids mediating carbapenem resistance in *Enterobacteriaceae*

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Australia	2018	*K. pneumoniae*	ST258	FIB, FII	_	_	*bla*$_{KPC-2}$	_	Tn*4401*	[169]
		K. oxytoca	_	FII	_	_	*bla*$_{KPC-2}$	_	_	
		C. farmeri	_	FII, R	_	_	*bla*$_{KPC-2}$	_	Tn*4401*	
		C. freundii	_	R	_	_	*bla*$_{KPC-2}$	_	Tn*4401*	
	2016	*S. enterica*	ST19	HI2	339 kb	conjugative	*bla*$_{IMP-4}$	TEM-1, *sul1*, OXA-1, *aacA4*, *qnrB2*	class 1 integron	[186]
	2015	*E. cloacae*	ST127	FII	_	conjugative	*bla*$_{NDM-1}$	_	_	[131]
			ST265	X3	_	conjugative	*bla*$_{NDM-1}$	_	_	
			ST45	L/M	_	conjugative	*bla*$_{IMP-4}$	TEM-1, SHV, CTX-M, *qnr*, *aac (6')-Ib*	class 1 integron	
			ST1	HI2	_	non-conjugative	*bla*$_{IMP-4}$	TEM-4, *qnrB2*, *aaCA4*	class 1 integron	
		E. hermannii	ST1	HI2	_	non-conjugative	*bla*$_{IMP-4}$	*qnrB*, TEM-1, SHV, *aac(6')-Ib*	class 1 integron	
		E. aerogenes	ST45	L/M	_	conjugative	*bla*$_{IMP-4}$	*qnrB*, TEM-1, *aac(6')-Ib*	*class 1 integron*	
		E. asburiae	ST1	HI2	_	non-conjugative	*bla*$_{IMP-4}$	TEM-1, *aac(6')-Ib*	class 1 integron	
		E. coli	_	HI2	_	non-conjugative	*bla*$_{IMP-4}$	*qnrB*, TEM-1, *aac(6')-Ib*	class 1 integron	
		K. pneumoniae	_	HI2, L/M	_	conjugative	*bla*$_{IMP-4}$	*qnr*, TEM-1, SHV, *aac(6')-Ib*	class 1 integron	
		C. freundii	_	HI2	_	non-conjugative	*bla*$_{IMP-4}$	TEM-1, SHV, CTX-M, *qnr*, *aac (6')-Ib*	class 1 integron	
		C. koseri	_	HI2	_	non-conjugative	*bla*$_{IMP-4}$	*qnrB*, TEM-1, *aac(6')-Ib*	class 1 integron	
		P. mirabilis	_	HI2	_	non-conjugative	*bla*$_{IMP-4}$	*qnrB*, TEM-1, SHV, *aac(6')-Ib*	class 1 integron	
China	2018	*K. pneumoniae*	ST11	FII	_	_	*bla*$_{KPC-2}$	CTX-M-65, SHV-12, TEM-1	Tn*1721*-Tn*3*-IS*26*	[146]
			ST11	FII, I1	_	_	*bla*$_{KPC-2}$	CTX-M-55, SHV-12, DHA-1	Tn*1721*-Tn*3*-IS*26*	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
China	2018	*K. pneumoniae*	ST11	FII, N	_	_	*bla*$_{KPC-2}$	CTX-M-65, SHV-12, TEM-1	Tn*1721*-Tn*3*-IS*26*	[146]
			ST571	A/C	_	_	*bla*$_{NDM-1}$	CMY-2, TEM-1	_	
			ST1723	P, FII	_	_	*bla*$_{IMP-4}$	CTX-M, SHV-12, TEM-1	_	
		K. aerogenes	_	X6		conjugative	*bla*$_{KPC-2}$	TEM-1	Tn6296 & ISkpn19	[104]
		P. mirabilis	_	X6		conjugative	*bla*$_{KPC-2}$	TEM-1	Tn6296 & ISkpn19	
		S. marcescens	_	X6		conjugative	*bla*$_{KPC-2}$	TEM-1, *qnr*S1	Tn6296 & ISkpn19 (Tn6292)	
		M. morganii	_	X6		conjugative	*bla*$_{KPC-2}$	_	Tn6296 & ISkpn19	
		E. hormaechei	ST177	FII	109-kb	conjugative	*bla*$_{NDM-1}$	-	-	[193]
		E. coli	ST167	FII/FIA	144-kb	conjugative	*bla*$_{NDM-5}$	*aadA2, aadA5*, TEM-1, *Sul*1, *drfA12*, *drfA15*	IS*26*	[194]
			ST167	X3	80-kb	-	*bla*$_{NDM-1}$	-	IS*Aba125*	[195]
			ST1114	X3	46-kb	conjugative	*bla*$_{NDM-20}$	-	IS*Aba125*	[196]
			ST405	FII	-	-	*bla*$_{NDM-1}$	-	-	[197]
			-	X3	46-kb	-	*bla*$_{NDM-1}$	-	IS*Aba125*	
		C. freundii	-	X3	80-kb	-	*bla*$_{NDM-1}$	CTX-M-15	IS*Aba125*	
	2017	*K. pneumoniae*	_	_	_	_	*bla*$_{KPC-2}$	*rmtB*, CTX-M-65, TEM-1, SHV-11, *catA2*, *fosA*, *oqxA*	_	[185]
			ST14	X3	46 161-bp	conjugative	*bla*$_{NDM-5}$	CTX-M-15	_	[176]
		E. coli	ST48	X3	47-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-64, TEM-1b, *sul2*, *aadA5*, *rmtB*	IS	[144]
			ST10	X3	102 512-bp	conjugative	*bla*$_{NDM-5}$	mcr-1, *aadA2*, *sul1*, *dfrA12*, *aac(3)-IId*	IS*3000*	[137]
			ST4981	FII	92-kb	conjugative	*bla*$_{NDM-5}$	mcr-1, TEM-1B, erm	IS*30*	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
China	2016	*K. pneumoniae*	ST105	FI	50-kb	conjugative	*bla*$_{NDM-1}$, *bla*$_{IMP-4}$	*qnrS1*, *qnrB4*, *aacA4*, CTX-M-15, SHV-1	IS*3000*	[198]
			ST2250	FII	30-kb	conjugative	*bla*$_{NDM-5}$	-	IS*3000*	[175]
			ST3835	X3	54-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, SHV-12, CMY-42, OXA-1		[52]
		C. sakazakii	_	B/O	80-kb	conjugative	*bla*$_{NDM-9}$	mcr-1, *fosA3*, CTX-M-55, *qnrS*	IS26	[199]
		P. mirabilis	_	X3	40-kb	conjugative	*bla*$_{NDM-5}$	_	IS*3000*	[175]
		E. cloacae	ST231	A/C	130 573-bp	non-conjugative	*bla*$_{NDM-1}$	MBL, strA,strB, aadA2, armAmph2, mel, sul1 and sul2, dfrA12, qacE1	class 1 integron	[148]
				X6	10 756-bp	conjugative	*bla*$_{KPC-3}$	TEM-1	Tn3-Tn1722	
	2015	*E. cloacae*	ST120	HI2	340-kb	conjugative	*bla*$_{NDM-1}$	*armA*, *fosA3*	IS*Aba*125	[184]
			ST93	A/C	55-kb	conjugative	*bla*$_{NDM-1}$	*armA*	IS*Aba*125	
			ST88	N	65-kb	conjugative	*bla*$_{NDM-1}$	TEM-1, CTX-M-3	IS*Aba*125	
			_	X3	54 035-bp	conjugative	*bla*$_{NDM-1}$		IS*5*	[180]
		K. pneumoniae	ST11	FII-FIB	110 786-bp	conjugative	*bla*$_{NDM-1}$	*sul1*, *rmtC*	IS*CR3*, IS*Ehe3*	[200]
			Clone B, A	X3	7.8-kb	conjugative	*bla*$_{NDM-1}$	_	_	[180]
		C. sakazakii	_	B/O	80-kb	conjugative	*bla*$_{NDM-9}$	MCR-1, CTX-M-9, CTX-M-1,	IS	[199]
		R. planticola	_	X3	53 134-bp	conjugative	*bla*$_{NDM-1}$	SHV-12	IS*26*, IS*Aba125*, IS*5*	[200]
	2014	*K. pneumoniae*	ST889/966	A/C	245-kb	conjugative	*bla*$_{NDM-1}$	TEM-1, CTX-M-15	_	[177]
			ST113	N	55-kb	conjugative	*bla*$_{NDM-1}$	_	_	
		E. cloacae	ST40	FIB	310-kb	conjugative	*bla*$_{NDM-1}$	TEM-1, CMY-30, FosA3	-	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
China	2014	*E. cloacae*	ST410	I1	60-kb	conjugative	*bla*$_{NDM-1}$	TEM-1, CTX-M-15, CMY-30	_	[177]
		C. freundii	_	A/C	170-kb	conjugative	*bla*$_{NDM-1}$	FosA3, CMY-73	_	
United States	2018	*E. cloacae*	ST171	HI2	315-kb	-	*bla*$_{KPC-4}$	-	Tn*4401b*	[201]
			ST171	FIA	141-kb	-	*bla*$_{KPC-3}$	-	Tn*4401d*	
	2017	*K. pneumoniae*	ST111	N	69 888-bp	conjugative	*bla*$_{KPC-2}$	*aac(6')-Ib*, *aadA1*,OXA-9, TEM-1, *strB*, *strA*, *sull2*	Tn*4401b*	[49]
		K. michiganensis	_	N	68 763-bp	conjugative	*bla*$_{KPC-2}$	aac(6')-Ib, aadA1,OXA-9, TEM-1, strB, strA, sull2	Tn*4401b*	
		E. coli	ST218	Q1	10-kb	non-conjugative	*bla*$_{IMP-27}$	CMY-2	_	[178]
		P. mirabilis	_	Q1	10-kb	non-conjugative	*bla*$_{IMP-27}$	_	_	
		P. vulgaris	_	Q1	10-kb	non-conjugative	*bla*$_{IMP-27}$	_	_	
		E. cloacae	_	Q1	10-kb	non-conjugative	*bla*$_{IMP-27}$	_	_	
		C. farmeri	_	Q1	10-kb	non-conjugative	*bla*$_{IMP-27}$	_	_	
	2016	*E. coli*	ST617	N	108-kb	conjugative	*bla*$_{KPC-3}$	TEM-1	Tn*4401b*	[84]
			ST131	FII	116-kb	conjugative	*bla*$_{KPC-2}$	TEM-1	Tn*4401a*	
			ST2289	FIA, A/C	99-kb	conjugative	*bla*$_{KPC-2}$	TEM-1, OXA-9, FOX-5, PSE-1	Tn*4401d*	
			ST405	X3	39 520-bp	conjugative	*bla*$_{NDM-5}$	*strA*, *strB*, *aac(6')-Ibcr*, OXA-1, *sul1*	_	[147]
			ST595	_	44-kb		*bla*$_{KPC-3}$	OXA-9, TEM-1A, *aac(6')-Ib*, *aadA1*, *qnrB19*	Tn*4401b*	[50]
		E. xiangfangensis	ST114	F	_		*bla*$_{KPC-3}$	*qnrS1*, TEM-1A	Tn*4401b*	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
United States	2016	*E. hormaechei*	ST594	Col	_		*bla*$_{KPC-2}$	TEM-1B, SHV-12, *strB*, *strA*, *aadA2*, *aac(6')-Iic*, *qnrB2*, *sul1*, *sul2*, *dfrA18*	Tn*4401*	50
			ST269	_	44-kb		*bla*$_{KPC-2}$	TEM-1B, *qnrB2*, *sul1*, *dfrB3*	Tn*4401*	
			ST113	A/C	66-kb		*bla*$_{KPC-4}$	TEM-1A, OXA-1, *aadA1*, *aac(3)-via*, *aph*, *mph* (A), *catB3*, *arr-3*, *qnrS1*, *sul1*, *dfrA14*, TEM-1B, *sul2*, *strA*, *strB*	Tn*4401*	
	2015	*E. cloacae*	ST171	FIA	63 481-bp	_	*bla*$_{KPC-3}$	TEM-1, OXA-9	Tn*1331*/Tn*4401*	202
			ST171	HI2	_	_	*bla*$_{KPC-4}$	TEM-1, OXA-1	Tn*4401b*	168
			ST78	N	_	_	*bla*$_{KPC-4}$	_	Tn*4401b*	
		K. pneumoniae	ST101	L/M			*bla*$_{OXA-48}$	_	Tn*1991*	
			ST258	R			*bla*$_{KPC-2}$	_	Tn*4401a*	
			ST113	N			*bla*$_{KPC-4}$	_	Tn*4401b*	
			ST258	I2			*bla*$_{KPC-3}$	_	Tn*4401b*	
			ST258	A/C2			*bla*$_{KPC-2}$	_	Tn*4401e*	
			ST16	X3			*bla*$_{KPC-3}$	_	Tn*4401b*	
		E. coli	ST131	X3	116 803-bp	_	*bla*$_{KPC-3}$	TEM-1, OXA-9, *sul2, strAB*	Tn*4401*	202
	2014	*K. pneumoniae*	ST258	FIA	73 635-bp	non-conjugative	*bla*$_{KPC-3}$	TEM-1, OXA-9, *aacA4*, *aadA1*, *strB*, *sul1*, *dfrA14*	Tn*4401*/Tn*1331*	129
			ST963	FII	139 941-bp	conjugative	*bla*$_{KPC-3}$	_	Tn*1331*/Tn*4401d*	
		E. cloacae	ST93/253/171	N	90-kb		*bla*$_{KPC-3}$	SHV-5	_	48
				N	90-kb	_	*bla*$_{KPC-2}$	CTX-M-15, SXT	_	
				FIB	30-kb		*bla*$_{KPC-2}$	_	_	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Japan	2018	*K. pneumoniae*	ST1471	L	-	-	*bla*$_{IMP-1}$	-	-	[203]
	2015	*K. pneumoniae*	ST5	N	47 236-bp	conjugative	*bla*$_{IMP-6}$	CTX-M-2, *aacA4',aadA2*, *tetR-tetA*	Class 1 integron (In*722*)	[164]
		K. oxytoca	ST37	N	_	conjugative	*bla*$_{IMP-6}$	CTX-M-2	Class 1 integron (In*722*)	
		E. coli	ST37	N	_	conjugative	*bla*$_{IMP-6}$	CTX-M-2	Class 1 integron (In*722*)	
Mexico	2017	*K. pneumoniae*	ST392	IIIk	130-kb	conjugation	*bla*$_{NDM-1}$	_	_	[183]
			ST309	FII	130-kb	conjugation	*bla*$_{NDM-1}$	_	_	
		E. cloacae	ST182	FII	150-kb	conjugation	*bla*$_{NDM-1}$	_	_	
		E. coli	ST10	FII	130-kb	conjugation	*bla*$_{NDM-1}$	_	_	
	2015	*K. pneumoniae*	ST22	FII	_	conjugation	*bla*$_{NDM-1}$	CTX-M-15	_	[179]
		E. coli	ST617	FII	_	conjugation	*bla*$_{NDM-1}$	CTX-M-15	_	
		E. cloacae	ST182	FII	_	conjugation	*bla*$_{NDM-1}$	CTX-M-15	_	
Spain	2017	*E. coli*	ST1434	N	70-kb	conjugation	*bla*$_{KPC-2}$	OXA-1, *aac(6')-Ib-cr*, *qnrB6*	_	[174]
			ST5001	R	48-kb	_	*bla*$_{KPC-2}$	_	_	
			ST216	R	48-kb	non-conjugative	*bla*$_{KPC-2}$	*aac(6')-Ib*	_	
			ST131	L/M	61 395-bp	conjugative	*bla*$_{OXA-48}$	_	Tn*1991.2*	
		E. cloacae	ST822	FIB	170-kb	_	*bla*$_{IMI-2}$	_	_	
			ST823	N	70-kb	conjugative	*bla*$_{KPC-2}$	*aac(6')-Ib*, *qnrB6*	_	
		K. oxytoca	_	N	60-kb	_	*bla*$_{KPC-2}$, *bla*$_{VIM-1}$	OXA-1, *aac(6')-Ib*	_	[174]
		R. ornithinolytica	_	R	70-kb	_	*bla*$_{VIM-1}$	OXA-1, *aac(6')-Ib*, *qnrB5*	_	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Spain	2017	*R. ornithinolytica*	_	P6	_	_	*bla*$_{KPC-2}$	TEM-1	IS*Kpn6*-IS*pn27*	185
		C. freundii	_	P6	40-kb	conjugative	*bla*$_{KPC-2}$	TEM-1	IS*Kpn6*-IS*pn27*	
		E. cloacae	_	P6	_	_	*bla*$_{KPC-2}$	TEM-1	IS*Kpn6*-IS*pn27*	
		K. pneumoniae	_	N	_	_	*bla*$_{KPC-2}$	TEM-1	IS*Kpn6*-IS*pn27*	
		Kluyveraa sp.	_	U	_	_	*bla*$_{KPC-2}$	TEM-1	IS*Kpn6*-IS*pn27*	
Poland	2016	*K. pneumoniae*	ST11	R	90-kb	non-conjugative	*bla*$_{NDM-1}$	CTX-M-15, TEM-1, OXA-1	Tn*125*	54
			ST11	FII	100-kb	conjugative	*bla*$_{NDM-1}$	TEM-1	Tn*125*	
			ST11	R+FII	80-kb	non-conjugative	*bla*$_{NDM-1}$	TEM-1, OXA-1	Tn*125*	
Italy	2015	*K. pneumoniae*	ST101	FII	_	conjugative	*bla*$_{KPC-2}$	CTX-M-1	_	204
			ST1789	FII	_	conjugative	*bla*$_{KPC-2}$	CTX-M-1	_	
			ST512	FII	_	conjugative	*bla*$_{KPC-3}$	_	_	
			ST405	FII	_	conjugative	*bla*$_{KPC-3}$	_	_	
		E. coli	ST131	N	_	conjugative	*bla*$_{VIM-1}$	_	_	
			ST5	X3, FIB, colE			*bla*$_{KPC-3}$	SHV-11	Tn*4401a*	91
		C. freundii	ST91	X3	_		*bla*$_{KPC-3}$	SHV-11	Tn*4401a*	
			ST96	X3			*bla*$_{KPC-3}$, *bla*$_{VIM-2}$	SHV-11, TEM-1, CTX-M-9	Tn*4401a*	
				X3, N, HI1			*bla*$_{KPC-3}$, *bla*$_{VIM-2}$	SHV-11, TEM-1, CTX-M-9	Tn*4401a*	
Canada	2016	*K. pneumoniae*	ST258	FIA	_	_	*bla*$_{KPC-3}$	SHV, TEM	_	42
			ST512	FIA, FII	_	_	*bla*$_{KPC-3}$	SHV, TEM	_	
			ST15	N	-	-	*bla*$_{KPC-3}$	SHV, TEM, CTX-M, OXA-1, CMY-2	-	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance genes	Genetic elements	References
Canada	2016	*K. pneumoniae*	ST15	N	_	_	*bla*$_{KPC-3}$	SHV, TEM, CTX-M, OXA-1, CMY-2	_	[42]
			ST437	R	_	_	*bla*$_{NDM-1}$	SHV, CTX-M	_	
			ST11	A/C	_	_	*bla*$_{NDM-1}$	SHV, OXA-1	_	
			ST147	R	_	_	*bla*$_{NDM-1}$	SHV, CTX-M, OXA-1	_	
			ST15	R	_	_	*bla*$_{NDM-1}$	SHV, TEM, CTX-M	_	
			ST16	A/C	_	_	*bla*$_{NDM-1}$	SHV-1, CTX-15, OXA-1, CMY-6	_	
			ST101	N	_	_	*bla*$_{OXA-48}$	SHV, OXA-1	_	
		E. coli	cluster II	FIIA			*bla*$_{KPC-3}$	_	_	
			cluster VI	N			*bla*$_{KPC-3}$	_	_	
		E. cloacae	cluster IV	P, L/M	_	_	*bla*$_{KPC-3}$	_	_	
			_	L/M			*bla*$_{KPC-3}$	_	_	
			_	FIIA	_	_	*bla*$_{KPC-3}$	_	_	
			cluster VI	N			*bla*$_{KPC-3}$	_	_	
			_	Y	_	_	*bla*$_{VIM-1}$	_	_	
			_	R	_	_	*bla*$_{VIM-1}$	_	_	
		E. aerogenes	cluster VI	N			*bla*$_{KPC-3}$	_	_	
		C. freundii	cluster IV	P, L/M	_	_	*bla*$_{KPC-3}$	_	_	
		C. koseri	cluster IV	P, L/M	_	_	*bla*$_{KPC-3}$	_	_	
		C. youngae	cluster IV	P, L/M			*bla*$_{KPC-3}$	_	_	
		R. planticola	_	N	_	_	*bla*$_{KPC-3}$	_	_	
			cluster VI	N	_	_	*bla*$_{KPC-3}$	_	_	
			cluster IV	P, L/M	_	_	*bla*$_{KPC-3}$	_	_	

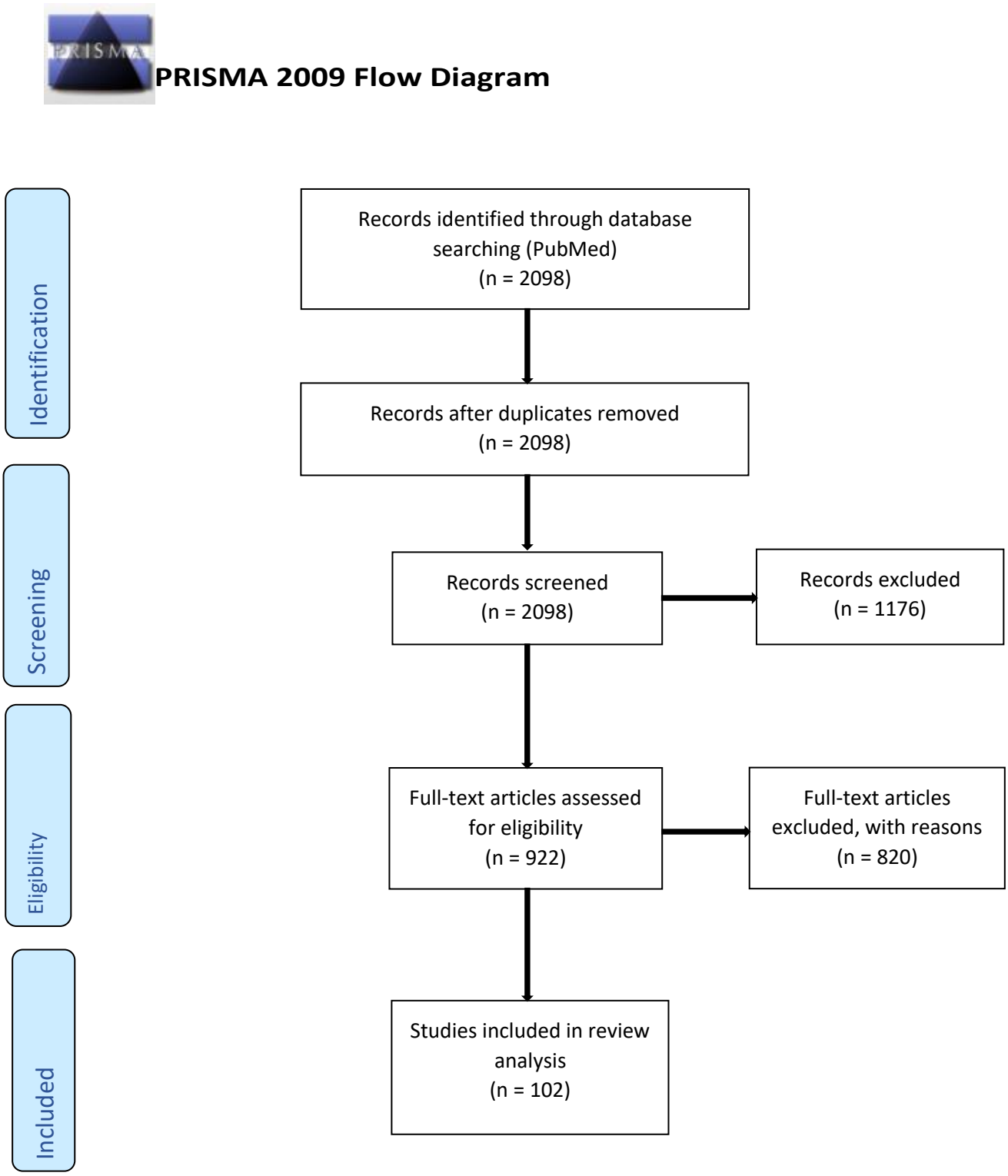
Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Canada	2014	*K. pneumoniae*	ST258	F, I2	120-kb, 80-kb	_	*bla*$_{KPC-3}$	TEM-1, SHV-11	Tn*4401b*	[47]
			ST258	I2	70-kb	_	*bla*$_{KPC-3}$	TEM-1, SHV-11	Tn*4401b*	
			ST258	A/C, FII	100-kb	_	*bla*$_{KPC-2}$	TEM-1, SHV-11	Tn*4401a*	
			ST258	FII, I2	80-kb	_	*bla*$_{KPC-2}$	TEM-1, SHV-11	Tn*4401a*	
			ST258	N, FII	50-kb	_	*bla*$_{KPC-2}$	OXA-1, SHV-11	Tn*4401b*	
		E. cloacae	-	HI2	120-kb	_	*bla*$_{KPC-3}$	TEM-1	Tn*4401b*	
		C. freundii	_	A/C	180-kb	_	*bla*$_{KPC-2}$	TEM-1	Tn*4401b*	
		R. ornithinolytica	_	F	70-kb	_	*bla*$_{KPC-2}$	_	Tn*4401b*	
Myanmar	2019	*E. coli*	ST167/101/410	FII	-	-	*bla*$_{NDM-5}$	CTX-M-15	IS*Sba14*	[205]
			ST410	X3	50-kb	-	*bla*$_{NDM-4/7}$	-	IS*sba14*	
	2017	*E. coli*	-	A/C		conjugative	*bla*$_{NDM-1}$	CTY-4	Tn*125*, Tn*1548*	[134]
			_	X3	47-kb	conjugative	*bla*$_{NDM-4}$	_	Tn*3*	
			_	X3		conjugative	*bla*$_{NDM-7}$	_	Tn*3*	
			_	X3		conjugative	*bla*$_{NDM-5}$	_	Tn*3*	
			_	FII		conjugative	*bla*$_{NDM-4}$	_	IS*26*	
			_	FII		conjugative	*bla*$_{NDM-5}$	_	IS*26*	
Germany	2018	*E. coli*	ST131	HI2	300-kb	-	*bla*$_{VIM-1}$	*aac(6')-Ib-cr*, *aacA4*, *aadA1*, ACC-1, CMY-2, *catA1*, *strA/B*, *Sul*1	Tn21	[206]
		S. Infantis	ST32	HI2	300-kb	-	*bla*$_{VIM-1}$	*aac(6')-Ib-cr*, *aacA4*, *aadA1*, ACC-1, CMY-2, *catA1*, *strA/B*, *Sul*1	Tn21	
Portugal	2018	*E. coli*	ST131	Q2	13-kb	non-conjugative	*bla*$_{KPC-21}$	-	IS*kp6*	[207]
Denmark	2018	*E. coli*	ST410	F	-	-	*bla*$_{OXA-181}$	CTX-M-15, TEM-30	-	[208]

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Denmark	2018	*E. coli*	-	X3	-	-	*bla*$_{NDM-5}$	CMY-2	-	208
		K. pneumoniae	ST35	HI2	314-kb	conjugative	*bla*$_{OXA-436}$	-	IS*91*/IS*CR 1*	209
		C. freundii	ST22/65	HI2	314-kb	conjugative	*bla*$_{OXA-436}$	-	IS*91*/IS*CR 1*	
		E. asburiae	-	HI2	314-kb	conjugative	*bla*$_{OXA-436}$	-	IS*91*/IS*CR 1*	
Romania	2015	*K. pneumoniae*	ST258	FII		conjugative	*bla*$_{KPC-2}$	CTX-M-15, TEM-1, OXA-1, OXA-9, AAC-6'-1b	Tn*4401*	53
			ST101	L/M		conjugative	*bla*$_{OXA-48}$	CTX-M-15, TEM-1, OXA-9, AAC-6'-1b-cr	Tn*1999.2*	
		E. cloacae	ST93	FII		conjugative	*bla*$_{VIM-4}$	CTX-M-15, TEM-1, OXA-1, AAC-6'-1b	class 1 integron	
Kuwait	2017	*K. pneumoniae*	ST1399	A/C	165-kb	conjugative	*bla*$_{VIM-4}$	TEM-1, SHV-12, CTX-M-15, CMY-4, aac(6')-Ib-cr	In416	210
		E. aerogenes	_	FII		conjugative	*bla*$_{KPC-3}$	TEM-1, OXA-30, CTX-M-15	Tn*4401b*	
		E. coli	ST58	FII		_	*bla*$_{KPC-3}$	TEM-1	Tn*4401b*	
		K. pneumoniae	ST11	FII		conjugative	*bla*$_{KPC-3}$	TEM-1, SHV-11, OXA-30, CTX-M-15	Tn*4401b*	
			ST147	FII		conjugative	*bla*$_{KPC-3}$	TEM-1, SHV-11	Tn*4401b*	
			ST1138	FII		conjugative	*bla*$_{KPC-3}$	TEM-1, SHV-36	Tn*4401b*	
Lebanon	2018	*E. coli*	ST354	L/M	-	-	*bla*$_{OXA-48}$	CTX-M-15, CMY-42, TEM-1b, OXA-1	IS*1999*	211
			ST410	X3	-	-	*bla*$_{OXA-181}$	CMY-2/4, CTX-M-15, TEM-1B, OXA-1	-	
Thailand	2018	*K. pneumoniae*	-	H1B	297-kb	-	*bla*$_{NDM-1}$	*aadA2*, *armA*, *aph(3')*-Vla, *Sul*1, CTX-M-15, *qnrB1*	-	212
Pakistan	2018	*K. pneumoniae*	ST101	L/M	-	-	*bla*$_{OXA-48}$	CTX-M-15, SHV-28, TEM-1, OXA-10	-	213
Czech Republic	2017	*E. coli*	ST4956/ ST216	L	64-Kb	_	*bla*$_{OXA-48}$	_	-	153
		E. cloacae	ST109	L	64-kb	_	*bla*$_{OXA-48}$	CTX-M-15, OXA-1, TEM-1	Tn*1999.2*	
		K. pneumoniae	ST101	L	64-kb	_	*bla*$_{OXA-48}$	CTX-M-15, TEM-1	Tn*1999.2*	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Czech Republic	2017	*K. pneumoniae*	ST18	X3	51-kb	_	*bla*$_{OXA-181}$	CTX-M-15, OXA-1, TEM-1	IS*26*	153
			ST15	colE2	13-kb	_	*bla*$_{OXA-232}$, *bla*$_{NDM-1}$	CTX-M-15, OXA-1	Tn*1000*	
			ST11	L	65-kb	_	*bla*$_{OXA-48}$	CTX-M-15	Tn*1999.5*	
		P. stuartii	_	A/C	100-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15	_	
			ST1301	X3	>150-kb	non-conjugative	*bla*$_{OXA-181}$	CTX-M-15, *qnrS*	IS*Esp1*-*IS3000*-IS*Kpn19*	
			ST15	X3	120-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15	_	
Taiwan	2018	*K. pneumoniae*	ST11	-	86-kb	-	*bla*$_{KPC-2}$	CTX-M, SHV, TEM	IS*1*	214
Egypt	2016	*K. pneumoniae*	ST147	colE, R	60-97-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, SHV-11, *aac(3)-IIa*, *aph(30)-Ia*, *aac(60)-Ib-cr*, *rmtF*, *qnrB*	IS*Aba125*	215
			ST11	colE, R, F	55-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, SHV-11, *aac(3)-IIa*, *aph(30)-Ia*, *aac(6')-Ib-cr*, *rmtF*, *qnrB*	IS*Aba125*	
Gabon	2017	*K. pneumoniae*	ST307	X3	_	conjugative	*bla*$_{NDM-7}$	CTX-M-15, SHV-28, OXA-9, *aac(6')-Ib*, *sul*, *fosA*	transposon	216
		E. cloacae	_	X3	_	conjugative	*bla*$_{NDM-7}$	OXA-9, ampR, SHV-12, TEM-104	transposon	
India	2019	*K. pneumoniae*	ST347	FII	153-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, *qnr*S1, *qnr*B1, *oqxAB*, *aac(6')-Ib-cr*	IS*Aba125*	217
			ST29	FII	115-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, *qnr*S1, *oqxAB*	IS*Ec33*	
			ST1224	FII	270-kb	conjugative	*bla*$_{NDM-1}$	CTX-M-15, *qnr*S1, *qnr*B1, *oqxAB*, *aac(6')-Ib-cr*	IS*Aba125*	
			ST2558	FII	173-kb	conjuagtive	*bla*$_{NDM-1}$	CTX-M-15, *qnr*S1, *aac(6')-Ib*	IS*Ec33*	
	2016	*S. enterica*	_	A/C	146-kb	conjugative	*bla*$_{NDM-1}$	CMY -4	IS*26*, IS*4321*	187
Vietnam	2015	*K. pneumoniae*		FII, A/C	_	_	*bla*$_{NDM-1}$	TEM, CTX-M	_	173
		E. cloacae		FII, A/C	_	_	*bla*$_{NDM-1}$	TEM, CTX-M, SHV	_	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
Vietnam	2015	*E. coli*		FII	_	_	*bla*$_{NDM-1}$	TEM, CTX-M, SHV	_	173
		C. freundii		FII, A/C	_	_	*bla*$_{NDM-1}$	TEM, CTX-M	_	
		K. oxytoca		FII	_	_	*bla*$_{NDM-1}$	TEM, CTX-M	_	
Brazil	2015	*E. hormaechei*		F	96 124-bp	conjugative	*bla*$_{NDM-1}$	_	Tn*3000*	136
Korea	2018	*K. pneumoniae*	ST340	X3	-	-	*bla*$_{NDM-1}$	-	IS	218
		E. coli	ST1642	X3	69 409-bp	conjugative	*bla*$_{KPC-2}$	SHV-11	Tn*4401*	145
France	2018	*K. pneumoniae*	ST395	L	62-kb	-	*bla*$_{OXA-48}$	CTX-M-15, *aac(6')-Ib-cr*, *qnr*S	-	219
			-	L/M	63-kb	conjugative	*bla*$_{OXA-48}$	CTX-M-1	IS*9999*	
			-	L/M	167-kb	conjugative	*bla*$_{OXA-48}$	-	IS*9999*	
Belgium	2016	*E. cloacae*	ST346	L/M	78 907-bp	conjugative	*bla*$_{GES6/7}$	_	class 1 integron	192
Ireland	2014	*K. pneumoniae*	_	L/M	63 578-bp	conjugative	*bla*$_{OXA-48}$	_	Tn*1999*	152
			_	FIB	63-kb	conjugative	*bla*$_{OXA-48}$	_	Tn*1999*	
			_	FII	63-bp	conjugative	*bla*$_{OXA-48}$	_	Tn*1999*	
			_	Y	_	_	*bla*$_{OXA-48}$	_	_	
Tunisia	2018	*P. mirabilis*	-	P & A/C	-	-	*bla*$_{NDM-1}$	CMY-4, *qnr*A6, *aph*3 VIa, *aph*3 Ia	-	220
Saudi Arabia	2018	*K. pneumoniae*	ST152	F, N	-	conjugative	*bla*$_{NDM-1}$	-	IS*Aba125*	221
			ST37/974	L/M	-	conjugative	*bla*$_{OXA-48}$	CTX-M-15, TEM-1, SHV-11	-	
South Africa	2018	*E. coli*	ST167	X3	46 253-bp	_	*bla*$_{NDM-5}$	_	_	12
		K. pneumoniae	ST101	Col	6 141-bp	_	*bla*$_{OXA-232}$	_	_	

Country	Year	Species	Clone	Plasmid type (Inc)	Size	Plasmid conjugation/ mobility	Carbapene-mase gene	Other resistance	Genetic elements	References
South Africa	2018	*K. pneumoniae*	ST101	FIB	223 434-bp	conjugative	*bla*$_{NDM-1}$	qac/sul1, DHA-1	Tn*1548*-like	[12]
			ST2017	R, FIB, FII	212 326-bp	conjugative	*bla*$_{NDM-1}$	qac/sul1, DHA-1	Tn*1548*-like	
			ST101	Q	8 201-bp	_	*bla*$_{GES-5}$	aacA4	Class 1 integron	
Sao Tome and Principe	2018	*E. coli*	ST1163	X3	66-kb	conjugative	*bla*$_{OXA-181}$	TEM-1	IS*kpn19*	[222]
			ST410	X3	60-kb	conjugative	*bla*$_{OXA-181}$	CTX-M-15, TEM-1	IS*kpn19*	
		K. pneumoniae	-	X3	64-kb	conjugative	*bla*$_{OXA-181}$	TEM-1	IS*kpn19*	
Croatia	2018	*K. pneumoniae*	-	L/M	70-kb	conjugative	*bla*$_{OXA-48}$	CTX-M-15, TEM-1, OXA-1, *qnr*A/B	IS*1999*/IS*1R*	[223]
		E. coli	-	L/M	70-kb	conjugative	*bla*$_{OXA-48}$	TEM-1	IS*1999*	
		E. cloacae	-	L/M	70-kb	conjugative	*bla*$_{OXA-48}$	CTX-M, TEM-1	IS*1999*	



Eligibility: only articles from 2013 to 2019 were assessed. All articles not reporting plasmid replicon groups in carbapenemase-producing *Enterobacteriaceae* were excluded.

Figure 2.1: PRISMA-adapted flow diagram of included and excluded studies. Adapted from the PRISMA website (http://prisma-statement.org/PRISMAStatement/CitingAndUsingPRISMA.aspx).

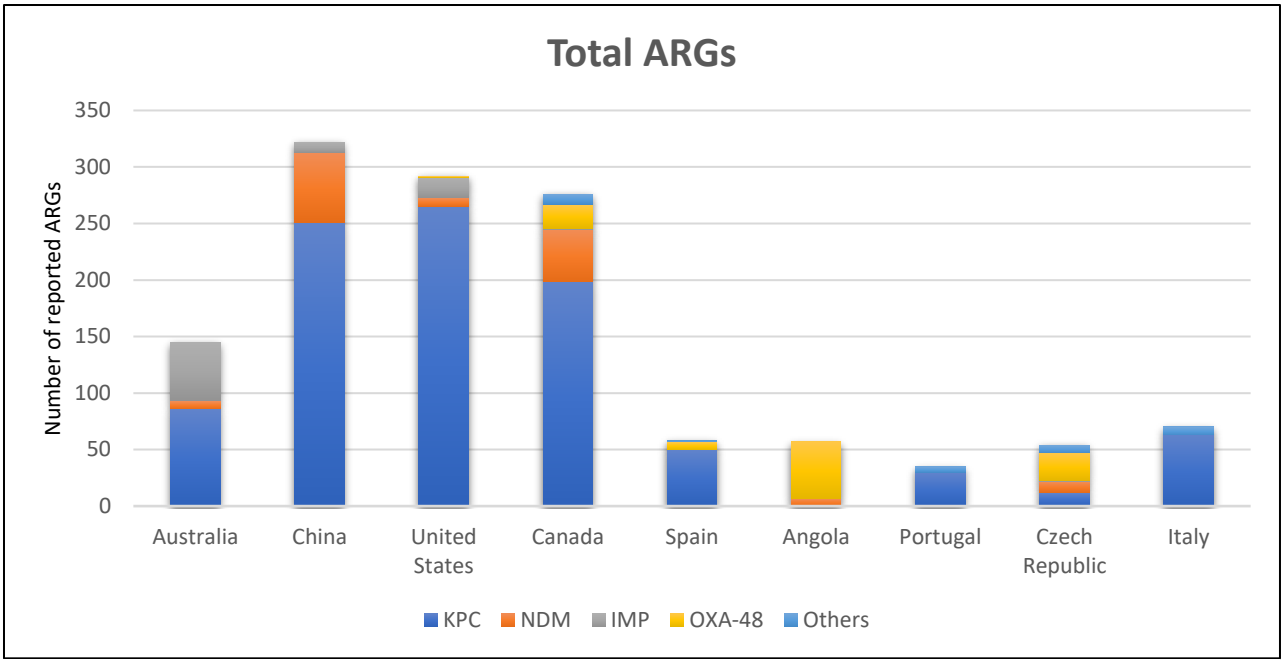


Figure 2.2: Frequency distribution of carbapenemase genes reported in countries represented by the included articles. KPC (blue bars) were the commonest per country except in Angola and Czech Republic. China, the USA and Canada had more included studies and hence, higher carbapenemase incidence. ARGs, antibiotic resistant genes.

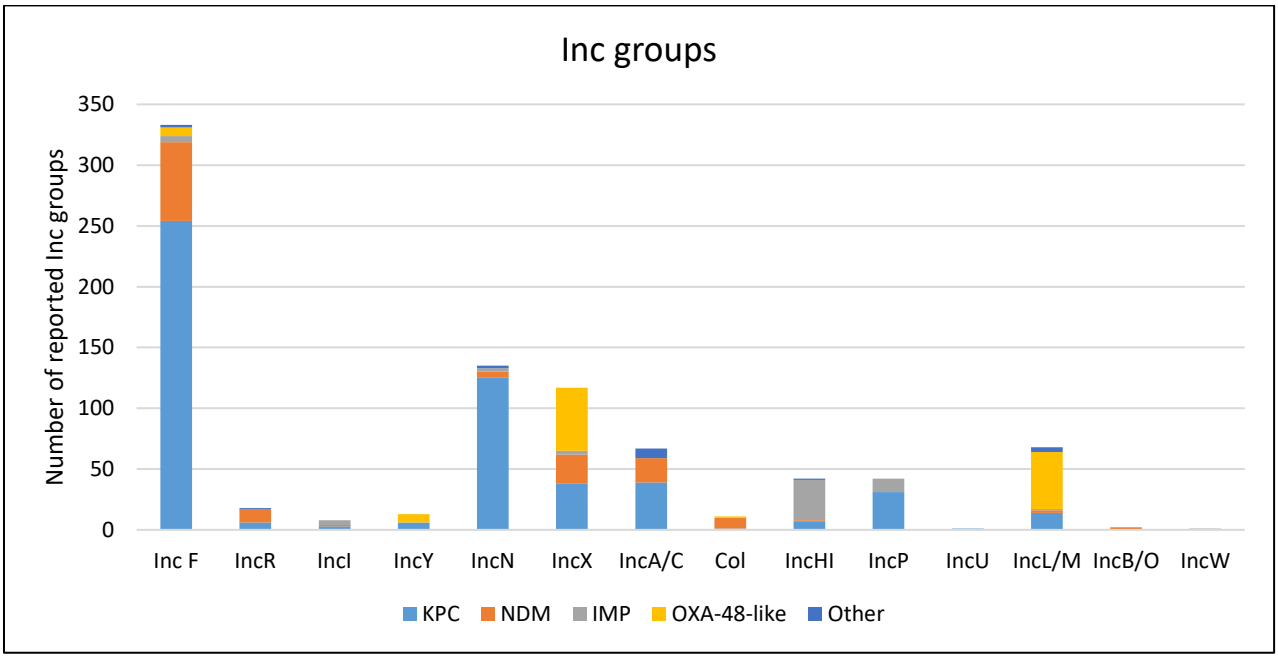


Figure 2.3: Frequency of plasmid incompatibility groups associated with different carbapenemase genes reported in *Enterobacteriaceae* in 23 countries. The commonest of these is the IncF types, followed by IncN, IncX, IncL/M, IncA/C, IncHI and IncP. which are mostly associated with KPC, NDM and OXA-48-like carbapenemases

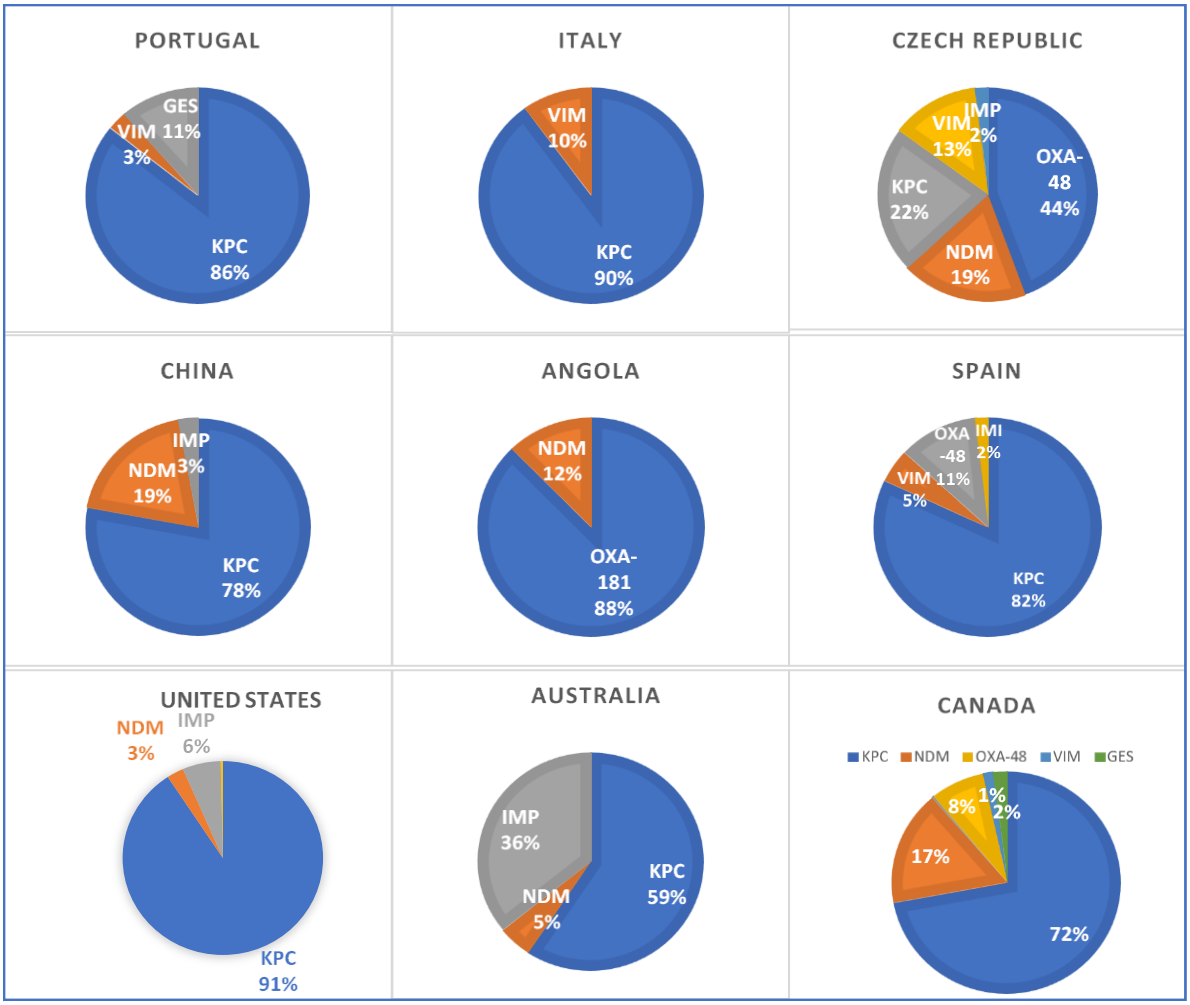


Figure 2.4. Charts showing the frequency of carbapenemase genes per country reported in the set of papers included in this review. KPC has been reported as the most prevalent in almost all shown countries, except the Czech Republic and Angola, where OXA-48-like were the most prevalent. KPC, Klebsiella pneumoniae carbapenemase; VIM, Verona-Integron metallo-β-lactamase; NDM, New-Delhi metallo-β-lactamase; GES, Guiana extended-spectrum β-lactamase; IMP, Imipenemase; OXA-48, Oxacillinase-48.

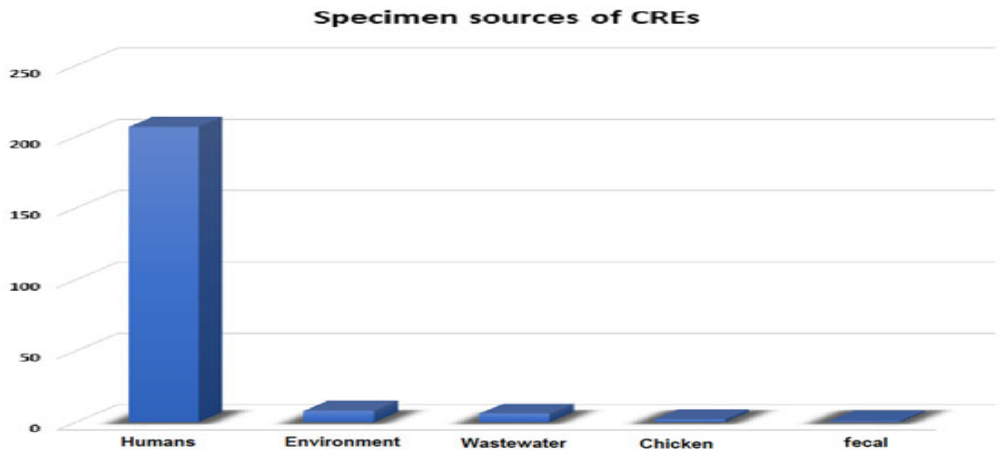


Figure 2.5. Specimen sources of CREs identified in the included articles reported in this review. Most of the specimens were obtained from humans with a small number being obtained from environmental and animal sources. Frequencies were manually calculated and graphically represented using Microsoft Excel.

2.10 References

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CHAPTER 3

Plasmids Associated with Carbapenemase-producing *Klebsiella pneumoniae* in the Pretoria Region, South Africa

The editorial style of the Journal of Antimicrobial Chemotherapy was followed in this chapter

Abstract

Mobile genetic elements such as plasmids play a major role in the acquisition and dissemination of antimicrobial resistance determinants in carbapenem-resistant *Klebsiella pneumoniae* (CRKP). This study aims to characterize plasmids mediating carbapenem resistance in *K. pneumoniae* isolates in Gauteng, South Africa.

A total of 60 *K. pneumoniae* isolates previously routinely identified by the national referral laboratory in Pretoria using the VITEK 2® (Biomerieux, Marcy-l'Étoile, France) automated system were collected. Antimicrobial susceptibility testing was performed using the MicroScan Gram-negative MIC 44 panel (Beckman Coulter, California, United States). All *K. pneumoniae* resistant to one or more carbapenem(s) were screened for carbapenemase-encoding genes (*bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{IMP}}$) using multiplex-PCR. These isolates were genotyped by Repetitive Element Palindromic-Polymerase Chain Reaction (REP-PCR). Plasmid extraction was performed on all isolates and electrophoresis was used to determine their number and size. The PCR-based replicon typing (PBRT) scheme was used to determine the incompatibility/replicon groups of all the extracted plasmids. Whole-genome sequencing was performed to characterize resistance genes and plasmids responsible for their spread.

The isolates showed reduced susceptibility to almost all tested antibiotics including ertapenem (98.2%), imipenem (66.1%), doripenem (50%), meropenem (44.3%) and colistin (41.1%). Multiplex-PCR analysis showed that 55 isolates harboured at least one of the detected carbapenemase genes, with 41 (73.2%) harbouring *bla*$_{\text{OXA-48}}$ and 18 (32%) harbouring *bla*$_{\text{NDM-1}}$. Co-expression of two carbapenemase genes were observed in four isolates with reduced susceptibility to all carbepenems, two of which were also colistin-resistant. The isolates were resolved into four major strains/genotypes by the REP-PCR. Electrophoresis revealed that the isolates carried between one and five plasmids, with the majority carrying 2 or 3 plasmids; the plasmid sizes ranged between 1.6-kb to >48.5-kb. IncF (FII, FIB, FIC), IncL, IncM, and A/C plasmid replicons were detected. The IncA/C, IncL and IncFIB were associated with both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$-producers while the IncM was mostly associated with only *bla*$_{\text{OXA-48}}$-producers. Almost 90% of the isolates showed multi-replicon carriage. WGS revealed IncX3

plasmid in *bla*$_{\text{OXA-181}}$-producing ST307 *K. pneumoniae* strain which could not be detected with PBRT technique.

This study shows an ongoing plasmid-mediated endemicity/dissemination of CRKP in the Gauteng province, with *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$-producing CRKP being predominant in 2018. Of concern, strains in this study were closely related to strains from different countries with different sequence types but similar resistance determinants. Indicating the importance of HGT, also shown in this study to play an important role in acquisition and dissemination of multiple resistance genes between different species Rigorous infection prevention and control, including contact precautions, should be adopted in all Gauteng hospitals to curtail further escalation of CRKP.

Keywords: Carbapenems, Carbapenemase, Plasmid and REP-PCR

3.1 Introduction

Klebsiella pneumoniae is an encapsulated, non-motile, Gram-negative bacterium first isolated from the lung of a demised patient who was suffering from pneumonia in 1882 [1]. These bacteria are known to colonize the human gastrointestinal (GI) tract and oropharynx mucosal surfaces[2]. To date, *K. pneumoniae* causes most nosocomial infections, accounting for 3% to 8% of all reported nosocomial infections[3]. These infections are specifically a problem in elderly, immunocompromised patients and neonates, but less frequently, *K. pneumoniae* infections such as sepsis and pneumonia are community-acquired[4].

According to the World Health Organisation (WHO), antimicrobial resistance in bacteria such as *K. pneumoniae* has become a major public concern worldwide. Antimicrobial resistance in the clinical settings is usually due to misuse and overuse of antibiotics, leading to increase and evolution of antimicrobial resistance genes and antimicrobial-resistant bacteria[5]. Resistance may be intrinsic i.e., acquired through mutations, and/or transferred horizontally from one bacterium to another through mobile genetic elements[6].

Among the various resistance mechanisms in *K. pneumoniae*, acquired resistance through mobile genetic elements has been shown to be of increasing importance[7]. Acquisition of antimicrobial-inactivating enzymes and efflux pump systems are important in the development of multi-drug resistant (MDR) *K. pneumoniae*[8]. The resistance-nodulation-division (RND) family of efflux pumps are responsible of ejecting charged and amphiphilic antimicrobials such as aminoglycosides, β-lactams and fluoroquinolones[9]. The use of β-lactams over the years has

resulted in β-lactamases and/or carbapenemase-producing *K. pneumoniae*[10], resulting in increased treatment failure, morbidity, and mortality[11].

Carbapenemases are categorised into three classes, class A (e.g. KPC, SME, IMI, and GES), class B (e.g. NDM, VIM, and IMP), and class D (OXA-48-like)[12]. These are defined as enzymes that are capable of slightly and/or completely hydrolysing β-lactams, including "last resort" carbapenems[13]. Class B carbapenemases, particularly *bla*$_{NDM}$ genes have been reported to be more potent than the other groups and cannot be inhibited by commercially available β-lactamase inhibitors such as clavulanic acid, tazobactam, or sulbactam[14].

Mobile genetic elements such as plasmids, transposons, phages and integrons play a major role in the acquisition and dissemination of antimicrobial resistance genes (ARGs) in carbapenem-resistant strains. Among these are large conjugative plasmids that have been associated with horizontal gene transfer (HGT) of carbapenemases between and within Gram-negative bacteria[7]. These plasmids have been reported in *K. pneumoniae* strains and are associated with multiple replicon groups such as IncF, A/C, L/M, N, and X[15,16,17,18]. IncF replicon plasmids are the most predominant and are mainly reported to carry the *bla*$_{KPC}$ and *bla*$_{NDM}$ genes in the United States, Canada, Greece, South Africa and Taiwan[15,19–21]. The L/M plasmids in *K. pneumoniae* are more frequently reported in the Czech Republic and Ireland, carrying the *bla*$_{OXA-48}$ gene and more rarely, in Oman carrying the *bla*$_{NDM}$ gene[17,22,23]. IncX plasmids are the major vehicles for the *bla*$_{NDM}$ gene in China and India while IncN plasmids, which are reported rarely, are associated with *bla*$_{NDM}$ and *bla*$_{KPC}$ genes in *K. pneumoniae* strains[16,18,24]. Only few reports of replicon groups in South Africa are available, increasing the need for studies focusing on replicon groups associated with carbapenemases in South Africa.

3.2 Materials and Methods

3.2.1 Bacterial strains and antimicrobial susceptibility testing

A total of 60 non-repetitive *K. pneumoniae* isolates were collected from a referral laboratory (National Health Laboratory Service/NHLS) in Pretoria. These *K. pneumoniae* clinical isolates were identified using the VITEK 2® automated system (BioMerieux-Vitek, Marcy-l'Étoile, France) and only those resistant to at least one carbapenem (Ertapenem, meropenem, imipenem, doripenem) by the VITEK 2 ® automated system (BioMerieux-Vitek, Marcy-l'Étoile, France) were collected. The *K. pneumoniae* isolates were received on blood agar plates (NHLS, SA) and incubated (Scientific Incubater Vacutec, Roodepoort, SA) at 37°C for 24 hours. Following incubation, confirmation of the Minimal Inhibitory Concentrations (MIC) of the isolates were

determined using the MicroScan Neg MIC 44 panels on the MicroScan automated system (Beckman Coulter, California, United States). Procedures were performed according to the manufacturer's instructions.

3.2.2 DNA extraction of carbapenem-resistant *K. pneumoniae* isolates

The total genomic DNA was extracted from all isolates that were confirmed to be resistant or showed reduced susceptibility to at least one carbapenem according to the MicroScan automated system (Beckman Coulter, California, United States). The DNA was extracted from an overnight Brain Heart Infusion (BHI) broth using the boiling method. The cells were heated at 95°C using a digital dry bath (Labnet International, New York, United States) for 15 minutes and transferred to an ultrasonic bath (Lasec Ltd, Midrand, South Africa) for another 15 minutes. The resulting supernatant was stored at -20°C freezer (Defy Ltd, Midrand, South Africa) until needed for further analysis and it was used as a template for the PCR assays.

3.2.3 Detection of carbapenemase genes using PCR assays

PCR was used to screen for the presence of six carbapenemase genes viz., *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, and *bla*$_{GES}$. Specifically, multiplex PCR was used for determining the presence of *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{NDM}$ while simplex PCR was used for *bla*$_{IMP}$, *bla*$_{KPC}$, and *bla*$_{GES}$ screening. The oligonucleotide primers were synthesized by Inqaba Biotechnical Industries (Pretoria, SA) and their sequences are shown in the Table 3.1. For the PCR reaction, 1 µl of template DNA was added to 12.5 µl of MyTaq^{TM} HS mix (Bioline, London, United Kingdom) while 0.4 µM of each primer and nuclease-free water (Qiagen, Hilden, Germany) were added to make up the volume to 25 µl in each PCR tube. The multiplex PCR conditions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 57°C for 45 sec, and 72°C for 30 sec, and a final extension step at 72°C for 7 min. The PCR amplicon were analysed using 1.5% Seakem agarose gel (Whitehead Scientific (Pty) Ltd, Cape Town, SA) with 5 µl ethidium bromide and visualised under Ultraviolet light using the Gel Doc™ EZ Gel (BioRad Laboratories, California, US) bioimaging system. A 100bp ready-to-use DNA ladder (Celtic Molecular Diagnostics, Cape Town, SA) was used to determine the size of the expected genes. All PCR amplicons were run alongside a positive and negative control.

3.2.4 Genotyping of CRKP isolates using Repetitive Extragenic Palindromic (REP) PCR assay

Total genomic DNA from all carbapenem-resistant *K. pneumoniae* isolates were used as template in the REP-PCR assay. The primer pair sequences REP 1 (5'-

IIIGCGCCGICATCAGGC-3') and REP 2 (5'-ACGTCTTATCAGGCCTAC-3') and PCR conditions described previously were used in this assay[25]. For the PCR reaction, 1 µl of template DNA was added to 12.5 µl of MyTaq™ HS mix (Bioline, London, United Kingdom) while 0.4 µM of each primer and nuclease free water (Qiagen, Hilden, Germany) was added to make up the volume to 25 µl in each PCR tube. The PCR conditions were as follows: an initial denaturation of 94°C for 3 min, followed by 30 cycles of 94°C for 45 sec, 45.8°C for 1 min, and 72°C for 8 min and a final extension step of 72°C for 16 min. The amplified DNA amplicons (10 µl) were separated by electrophoresis using 1.5% SeaKem agarose gel (Whitehead Scientific (Pty) Ltd, Cape Town, South Africa) with 5 µl ethidium bromide. The gels were run for 3 hour 20 minutes at 80 volts. The DNA amplicons bands were visualised under Ultraviolet light using the Gel Doc™ EZ Gel (BioRad Laboratories, California, United States) bioimaging system and banding patterns were compared to a 1 kb plus ready-to-use DNA ladder (Thermo Fisher Scientific, Massachusetts, United States). Analysis of REP-PCR fingerprints was performed using the GelCompare II software (Applied Maths, Belgium, Europe). Relatedness was determined by means of the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA). In this study a similarity coefficient of 75% was used to determine different strains of CRKP, i.e. isolates that showed a similarity of 75% were considered part of the same strain.

3.2.5 Plasmid characterisation using the PBRT scheme

Plasmid DNA extracted using the plasmid midi kit (Qiagen, Hilden, Germany) was used as template in characterising plasmids using the PCR-based inc/rep typing scheme. This method was carried out as previously described with few modifications[26,27]. Modifications were made in multiplex 5, where A/C and IncT were detected in a multiplex and IncFII plasmids were detected in a simplex PCR assay instead of multiplex. The $IncFII_k$ virulence plasmids in *K. pneumoniae* were also detected. The PCR assays were performed using a SimpliAmp Thermal cycler mini (Thermo Fisher Scientific, Massachusetts, US) and the PCR conditions used were described previously[26,27]. The PCR conditions were as follows: initial denaturation of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min, and a final extension of 72°C for 5 min. For IncF, IncFII, and $IncFII_k$ plasmids, same conditions were used except that an annealing temperature of 54°C for 30 sec was used instead. Table 3.2 shows all the primer sequences that were used for these assays.

3.2.6 Carbapenem-resistant plasmid transferability from *K. pneumoniae* isolates

Transferability of meropenem-resistant plasmids was determined using conjugation experiments. The experiments were performed on 26 isolates showing reduced susceptibility to meropenem using a broth mating method. The meropenem-resistant isolates were used as plasmid donors and the *E. coli* J53-A^r (sodium-azide resistant) strain served as a recipient strain. For broth mating, 3-hour growth cultures of donor and recipient strains grown in Luria Bertani (LB) broth (VWR international, Pennsylvania, US) were mixed with each other at a ratio of 1:4 (donor to recipient) and incubated at 37°C for 3 hours. Grown cells (200 µl) of the mixtures were spread onto Mueller-Hinton agar (Sigma-Aldrich (Pty) Ltd, Missouri, US) containing 0.5 µg/ml meropenem (Sigma-Aldrich (Pty) Ltd, Missouri, US) and 100 µg/ml sodium azide (VWR international, Pennsylvania, US) to select only for plasmid-encoded carbapenem resistance and then incubated at 37°C for 24 or 48 hours. PCR assay was used to confirm the carbapenemase gene ($bla_{\text{NDM-1}}$ and/or $bla_{\text{OXA-48}}$) carriage by transconjugants as their donors.

3.2.7 Whole-genome sequencing of *K. pneumoniae* isolates

Genomic DNA was extracted from the *K. pneumoniae* isolates Kp8, Kp10, Kp15, Kp29, Kp32, and Kp33 using a Zymo Research Fungal/Bacterial kit (Inqaba biotec, Pretoria, South Africa) according to the manufacturer's instructions. Genomic DNA was sent for sequencing at Inqaba Biotec (Pretoria, South Africa) on the PacBio RSII sequencer (Pacific Biosciences, Menlo Park, CA, United States).

3.2.8 Sequence annotation and genome comparison

Genomic sequences were aligned using WebACT (https://www.webact.org) software and the BLASTN (https://blast.ncbi.nlm.nih.gov) searches. Resistance genes, plasmids, and sequence types were annotated using online databases including ResFinder[28], Plasmidfinder[29], and MLST[30] at the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/) website. *K. pneumoniae* capsule polysaccharide-based serotyping (K-type) was performed using the Kaptive Web database[31]. Prophages in *K. pneumoniae* isolates were identified using the PHASTER algorithm.

3.2.9 Phylogenomic analysis of *K. pneumoniae* genomes

Whole genome sequences of carbapenem-resistant *Klebsiella pneumoniae* strains were downloaded from the PATRIC website (https://www.patricbrc.org/). These genomes and genomes obtained in this study (deposited in NCBI Bioproject database under accession no. PRJNA565241) were used for the whole-genome phylogeny analysis. The genomes were from Thailand (n = 189), South Africa (n = 80), China (n = 53), Italy (n = 38), United States and

Vietnam (n = 14, each), France (n = 10), Spain (n = 5), India and South Korea (n = 2, each), Austria, Australia, Brazil and Japan (n = 1, each). The phylogeny of these genomes was characterised using Parsnp (http://www.harvest.readthedocs.io/en/latest/content/parsnp.html) and the phylogeny trees were annotated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Genomes from the same country and/or with the same sequence types have the same colour labels and genomes of the same clade are highlighted with the same colour.

3.3 Results

The *K. pneumoniae* isolates were isolated from a variety of sources including aspirates (n = 4), blood cultures (n = 17), catheter tips (n = 7), swabs (n = 11), tissue (n = 3) and urine (n = 14). These specimens were submitted to the referral laboratory from six hospitals and centres including Kalafong hospital (n = 10), Mamelodi hospital (n = 1), Olievenhoutbosch clinic (n = 1), Steve Biko academic hospital (n = 36), Tembisa hospital (n = 5) and Tshwane rehabilitation centre (n = 3). The study population consisted of males (58.9%) more than females (39.3%) and results were not available for one participant. All the patient demographics obtained from the NHLS Laboratory Information System is summarised in Table 2.3.

Of the 60 *K. pneumoniae* with reduced susceptibility to carbapenems by VITEK®, reduced susceptibility could only be confirmed in 56 *K. pneumoniae* isolates by the MicroScan system. Susceptibility profiles of these isolates are shown in Figure 3.1. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and interpreted into resistant (R), intermediate (I), and susceptible (S). Almost all isolates showed reduced susceptibility to ertapenem (98.2%), followed by imipenem (66.1%), doripenem (50%) and meropenem (48.2%). Reduced susceptibility to colistin was also observed in 23 (41.1%) isolates. Among all the tested antibiotics, the isolates were susceptible to amikacin (82.1%), fosfomycin (82.1%), tigecycline (76.8%) and levofloxacin (60.7%).

Figure 3.2 shows the frequency of carbapenemase genes detected in this study using PCR assays. The most prevalent carbapenemase detected was $bla_{\text{OXA-48}}$ (65%), followed by $bla_{\text{NDM-1}}$ (29%). No bla_{GES}, bla_{KPC}, bla_{IMP} and/or bla_{VIM} were detected in any of the *K. pneumoniae* isolates.

Repetitive extragenic palindromic (REP) – PCR revealed four major genotypes, with strain 1 consisting of most of the *K. pneumoniae* isolates (n = 21), followed by strain 2 (n = 12), strain

3 (n = 8) and strain 4 (n = 5). The remaining isolates (n = 10) were not genotypically related to each other (Figure 3.4 & 3.5).

Plasmid characterization using gel electrophoresis revealed the number and size of plasmids in each *K. pneumoniae* strain. Most isolates (n = 17) carried 4 plasmids, followed by16 isolates with 2 plasmids, 15 isolates with 3 plasmids, 5 isolates with 5 plasmids, and 3 isolates with only 1 plasmid: plasmids sizes ranged from 1.4-kb to >48.5-kb, 48.5-kb and >48.5-kb, 1.6-kb to >48.5-kb, 1.5-kb to >48.5-kb, and 48.5-kb, respectively (Figure 3.5). These plasmids were typed into different replicon groups using PBRT assay targeting 19 replicon groups reported in *Enterobacteriaceae* and one replicon group targeting the virulence plasmid in *K. pneumoniae*. Among these, 11 replicon plasmid groups were identified in the tested *K. pneumoniae* isolates. All tested isolates (n = 56) were positive for $IncFII_k$ (virulent plasmid) replicon group. The majority of the isolates also tested positive for IncF (FII, FIB, FIC, FIB), IncL, A/C, and IncM plasmids, while only a few isolates were positive for IncHI1 and IncHI2 (Figure 4 and 5). Multi-replicons were reported in 75% (n = 42) of the tested isolates. Two isolates showed the highest multi-replicon combination: one $bla_{\text{OXA-48}}$-producer and one $bla_{\text{NDM-1}}$-producer had 6 and 7 replicon groups, respectively (Figure 3.3 & 3.5).

Of the 26 meropenem-resistant *K. pneumoniae* isolates that conjugation experiments were performed on; 20 donor strains were able to transfer their plasmids to the *E. coli* J53-A^r strain. Among the 20 transferred plasmids, 16 were positive for the $bla_{\text{NDM-1}}$ gene, followed by 3 $bla_{\text{OXA-48}}$ and 1isolate with both $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes (Figure 3.6).

A total of 6 representative isolates, based on their carbapenemase gene, REP pattern, plasmid number and type, were selected for WGS. These *K. pneumoniae* isolates were collected from three different hospitals in the Tshwane area in 2018. Four of these were from the same hospital collected from different wards and collection site including ward 4 and urine (Kp10); vascular surgery ward 4 and catheter tip (Kp15); neurology ward and swab (Kp29); and high-care multidiscipline ward and urine (Kp33) (Table 1, annexure B). The six *K. pneumoniae* isolates carried the $bla_{\text{OXA-181}}$ (n = 2), $bla_{\text{OXA-48}}$ (n = 1), $bla_{\text{NDM-1}}$ (n = 2), and $bla_{\text{NDM-7}}$ (n =1). These genes were associated with other resistance determinants causing resistance to aminoglycosides [*aac(3)-lla*, *aac(6')-lb-cr*, *aadA16*, *aph(3')-lb*, *aph(6)-ld*], quinolones [*aac(6')-lb-cr*, *oqxA*, *oqxB*, qnrB1, *qnrS1*], β-lactams ($bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, bla_{SHV}, $bla_{\text{TEM-1B}}$), tetracycline (*tetA*), sulphonamides (*sul1*, *sul2*), trimethoprim (*dfrA14*/*27*), phenicol (*catB3*/ *catA2*), and fosfomycin (*fosA*, *fosA7*) (Table 3.3). Five different sequence types (STs) were identified among the isolates

including ST39, ST307, ST607, ST17, and ST3559. Isolates Kp10 and Kp33 carrying the *bla*$_{\text{NDM-1}}$ gene both belonged to sequence type-39 (ST39).

The capsule polysaccharide-based serotyping or the K-loci results showed four different serotypes among the sequenced isolates, including serotypes KL2 (n = 2), KL25 (n = 2), KL27 and KL102 with one isolate each. The PHASTER algorithm was used to identify phage-associated sequences in carbapenem-resistant *K. pneumoniae* isolates and multiple intact phage regions were observed. These phages included the Klebsi_phiKO2, Cronob_ENT47670, Edward_GF_2, Pectob_ZF40, Phage_Gifsy, and different variant of Salmon (6), Entero (2), Escher (3) (Table 3.5 and Figure 3.7).

The phylogeny tree (Figure 3.8 & 3.9) shows that isolates from this study were more closely related to isolates from the United States and China than any other country. Figure 3.10 included other strains previously isolated in South Africa and none of these strains were related to the strains in this study. All the phylogeny trees, including figure 3.10 (South African strains only) showed that our strains were closely related to each other. Additionally, figure 3.10 also show that these strains were closely related to a few previously identified South African strains. Table 3.6 shows the comparison of closely related strains in different countries to strains in this study regarding the antimicrobial resistance genes, date of collection, sequence type and plasmid replicon groups. Strains from this study were closely related to multiple strains from different countries such as United States, China, Thailand, South Korea and South Africa. These strains belonged to different sequence types including ST14, ST11, ST147, ST392, and ST152 and these harboured plasmids of different replicons.

3.4 Discussion

This study showed *K. pneumoniae* isolates to have resistance profiles to most of the tested antibiotics, including colistin. Among all the tested antibiotics, only a few (amikacin, fosfomycin, and tigecycline) were still active against these isolates. This raises more concern about treatment options of CRKP, because colistin is one of the last resorts for infections caused by these pathogens. Due to this reason, and reports of poor outcome of colistin monotherapy[11], clinicians are left with limited or no treatment options.

In this study, we report on carbapenemase production among the CRKP isolates, with *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ facilitating carbapenem resistance in these isolates. These results are different to a South African report by the National institute for communicable diseases (NICD) in 2015. The NICD reported a high prevalence of *bla*$_{\text{NDM-1}}$-producing *K. pneumoniae* isolates in the

Gauteng and Kwazulu-Natal provinces and a few reports of *bla*$_{\text{OXA-48}}$-producing *K. pneumoniae* in Gauteng and Eastern Cape provinces. Similar results were also reported in 2016 by Perovic and colleagues, where a high prevalence of *bla*$_{\text{NDM-1}}$ was observed in the Gauteng province[32]. However, a paper published in 2019 reports an exponential increase in *bla*$_{\text{OXA-48}}$-like producing *K. pneumoniae* strains[33].This previous study and our current study suggest the change in carbapenem resistance determinants in *K. pneumoniae* strains in Gauteng.

REP-PCR revealed a major strain that was reported in majority of the *K. pneumoniae* isolates in this study, mainly carrying the *bla*$_{\text{OXA-48}}$ gene. One of the isolates in this group was sequenced and we were able to identify the *bla*$_{\text{OXA-181}}$ gene associated with the sequence type-307 (ST307). This sequence type has been associated with hospital outbreaks and harbors multiple antimicrobial resistance genes such as *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48}}$, and *mcr*-1 genes[34–38]. In South Africa, OXA-181-producing *K. pneumoniae* ST307 isolates were reported previously in the private sector hospitals in 6 provinces, including Gauteng province[33]. In this study, the *K. pneumoniae* ST307 isolates were collected from government sector hospitals in the Tshwane area. Another strain detected by REP-PCR was sequenced and we confirmed the carbapenemase gene as *bla*$_{\text{OXA-181}}$ and MLST revealed ST607 strain. Only a few studies have reported this ST607, including one Chinese isolate and 14 MDR isolates encoding ESBLs collected from a neonatal ICU in France[39]. The *bla*$_{\text{OXA-48}}$-producing *K. pneumoniae* strains in this study belonged to sequence type-3559 (ST3559). ST3559 has been recently reported as a novel sequence type in carbapenem-resistant *K. pneumoniae* isolates in South Africa[40]. Isolates in that study were collected from hospital wastewater, influent wastewater, river water, and riverbed sediments[40]. These *K. pneumoniae* isolates shared the same molecular characteristics with isolates from this present study (Table 3.3). This might mean the same strain is now circulating in the Tshwane area hospitals and causing infections. These *K. pneumoniae* strains are resistant to all carbapenems, leaving clinicians with little or no therapeutic options to treat patients with these strains.

Almost 79 capsule polysaccharide types based on the K-coli of *K. pneumoniae* have been described, and of these, only K1 and K2 serotypes are associated with hypervirulent strains while the others are associated with classical strains of *K. pneumoniae*[41,42]. Our results showed that two of the sequenced isolates (Kp10 and Kp33), which are highly resistant to carbapenems and harboured the *bla*$_{\text{NDM-1}}$ gene, were KL2 serotypes. This might indicate that these isolates are K2-hypervirulent *K. pneumoniae* (K2-hvKP) strains. K2-hvKP strains were not given attention until the report of a multidrug-resistant K2-hvKP strain harbouring the *bla*$_{\text{KPC-2}}$ and

bla$_{IMP-4}$ in China[43]. Following this report, multiple studies, including our current study have reported carbapenemase production associated with highly virulent stains of the K2 serotype[16,44]. Another study in China reported an ST11, *bla*$_{KPC-2}$-producing strain (CR-HvKP1) closely related to strains in this study; harboured a virulence plasmid (pLVPK-like) and showed a highly resistant profile[45].

WGS revealed the *bla*$_{NDM-7}$ gene in the ST17 *K. pneumoniae* strain. *bla*$_{NDM-7}$ has been reported previously in *K. pneumoniae* isolates in nosocomial cases in Canada, Gabon, Philippines, US and India[46–50]. These *bla*$_{NDM-7}$-producing *K. pneumoniae* were previously reported to belong to ST147 or ST273 in above mentioned countries. To our knowledge, this is the first- report of the bla$_{NDM-7}$ in *K. pneumoniae* ST17 in South Africa. A study in China reported a clinical *K. pneumoniae* isolate that belonged to ST17, which was clinically resistant to multiple antimicrobials including β-lactams, sulphonamides, and tetracycline; however, the strain remained susceptible to carbapenems[51].

Previous studies have reported on the presence of *bla*$_{NDM-1}$ on plasmids of different incompatibility/replicon groups in *K. pneumoniae* strains worldwide. *bla*$_{NDM-1}$ has been reported on IncF[52], IncL/M[53], IncN[54], A/C[55], and IncX[56] plasmid replicons. In this study, *bla*$_{NDM-1}$-producing *K. pneumoniae* were mostly associated with IncF (FII, F, FIB, FIC), followed by IncL and IncA/C plasmid replicons. Our results are comparable with reports in Nepal, Taiwan, Oman, Myanmar, Canada and South Africa[20,21,52,57]. However, in other countries such as China, Japan and India, reported NDM-variants in *K. pneumoniae* were on IncX plasmid replicons, particularly IncX3 plasmids[16,24]. In these countries bla$_{NDM-1}$ was the most predominant, while a study in Gabon reported *bla*$_{NDM-7}$ on IncX3 plasmids.

In this study, the *bla*$_{OXA-181}$-producing *K. pneumoniae* isolates were associated with ColKP3, IncX3, and IncF plasmids. This finding is in accordance with other previous studies in different countries including Czech Republic, Denmark, Sao Tome and Principle, and South Africa[17,33,58,59]. In South Africa, IncX3 has been previously associated with *K. pneumoniae* isolates collected during a hospital outbreak[33]. An earlier study reported the significant role that L/M plasmids play in the dissemination of *bla*$_{OXA-48}$ gene in *K. pneumoniae* strains worldwide[17,22,60–63]. Our present findings also prove that *bla*$_{OXA-48}$ gene is usually located on conjugative L/M plasmids.

Multiple strains were found to be closely related to strains in this study and these strains were reported in different countries. Strains were reported to be highly resistant to multiple antimicrobials, including carbapenems. *K. pneumoniae* strains KP33_1 and KP64, both ST14

and *bla*$_{NDM-1}$-producers harboured multiple resistance genes (Table 6) which were also detected in strains in this study. The IncHI1B plasmid replicon was responsible for the carriage of *bla*$_{NDM-1}$ in strain KP33_1[64]. This is different from *bla*$_{NDM-1}$-producers in this study, which were associated with IncFII and IncFIB replicons. A strain reported in the United States (CN1) which harboured an IncFII/FIB multi-replicon showed similar molecular characteristics with bla$_{NDM-1}$-producers in this study, but it belonged to a different sequence type (ST392). This explains that dissemination of resistance genes may occur in diverse strains with different sequence types and these genes can be accommodated in different plasmid replicon types.

Interestingly, strain G702R3B2 collected in 2016 in Kwazulu-Natal province, South Africa displayed similar resistance determinants as strains in this study; the difference was that strain belonged to a different sequence type (ST152) and the strain did not show resistance towards carbapenems[65]. This strain carried 8 replicon groups including IncFII, IncFII(k), IncN, IncQ1, ColpVC, and ColRNAI, among these 8, six were also reported in this study. Intact prophages such as Entero P88, Salmon 118970, Klebsi phiKO2 were also detected in this strain. These intact prophages were also detected in this study in two strains (KP8 and KP15).

The PCR-based plasmid typing scheme that was used in this study was unable to detect IncX3 plasmids, which were revealed with whole genome sequences and plasmidFinder. This is a limitation in areas where they only use this typing scheme. When using the typing scheme, it needs to be modified with new information of primers targeting all subtypes of the replicon groups.

3.5 Conclusion

This study has shown plasmid-mediated dissemination of carbapenemase genes in *K. pneumoniae* isolates in hospitals in Gauteng, 2018. The *bla*$_{OXA-48}$-like and *bla*$_{NDM}$ genes were the most predominant within this period and were carried on IncF, A/C, IncX3 and IncL/M plasmids, which were mostly transferable from *K. pneumoniae* to *E. coli* strains. All the *K. pneumoniae* strains were highly resistant to all the tested antimicrobials in this study. Of concern, strains in this study were closely related to strains from different countries with different sequence types but similar resistance determinants. This shows the importance of HGT, also shown in this study to play an important role in acquisition and dissemination of multiple resistance genes between different species. It is essential increases that rigorous infection prevention and control is adopted in Gauteng hospitals to avoid further escalation of this highly resistant *K. pneumoniae* strains.

3.6 References

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Table 3.1: **Primer sequences used for detecting carbapenemase genes in PCR assays of *K. pneumoniae* isolates**

Target	Gene	Primer (5' to 3')	Amplicon size (bp)	Reference
IMP	*bla*$_{IMP}$(F)	GGAATAGAGTGGCTTAAYTCTC	232	Poirel *et al*., 2011
	bla$_{IMP}$(R)	GGTTTAAYAAAACAACCACC		
KPC	*bla*$_{KPC}$(F)	TGTCACTGTATCGCCGTC CTCAGTGCTCTACAGAAAACC	900	Doyle *et al*., 2012
	bla$_{KPC}$(R)	TTGTCATCCTTGTTAGGCG		
VIM	*bla*$_{VIM}$(F)	GATGGTGTTTGGTCGCATA	390	Poirel *et al*., 2011
	bla$_{VIM}$(R)	CGAATGCGCAGCACCAG		
OXA	*bla*$_{OXA-48}$(F)	GCGTGGTTAAGGATGAACAC	438	
	bla$_{OXA-48}$(R)	CATCAAGTTCAACCCAACCG		
NDM	*bla*$_{NDM-1}$(F)	GGTTTGGCGATCTGGTTTTC	782	
	bla$_{NDM-1}$(R)	CGGAATGGCTCATCACGATC		
GES	*bla*$_{GES}$	AGTCGGCTAGACCGGAAAG	399	Dallenne *et al*., 2010
	bla$_{GES}$	TTTGTCCGTGCTCAGGAT		

F, sense primer, R, antisense primer

Table 3.2: **Primer sequences used for detecting replicons in CRKP isolates using the PBRT assay**

PCR type	Target	Primer (5' to 3')	Amplicon size	Reference
M1	I1-αγ(F)	cgaaagccggacggcagaa	139	Carattoli *et al*., 2005
	I1-αγ(R)	tcgtcgttccgccaagttcgt		
	HI1(F)	ggagcgatggattacttcagtac	471	
	HI1(R)	tgccgtttcacctcgtgagta		
	HI2(F)	tttctcctgagtcacctgttaacac	644	
	HI2(R)	ggctcactaccgttgtcatcct		
	X(R)	tgagagtcaatttttatctcatgttttagc		
M2	M(F)	ggatgaaaactatcagcatctgaag	738	Carattoli *et al*., 2015
	M(R)	gaactccggcgaaagaccttc		
	N(F)	gtctaacgagcttaccgaag	559	
	N(R)	gtttcaactctgccaagttc		
	L(F)	cggaaccgacatgtgcctact	854	
	L(R)	gaactccggcgaaagaccttc		
M3	W(F)	cctaagaacaacaaagcccccg	242	Carattoli *et al*., 2005; Villa *et al*., 2010
	W(R)	ggtgcgcggcatagaaccgt		
	FIA(F)	ccatgctggttctagagaaggtg	462	
	FIA(R)	gtatatccttactggcttccgcag		
	FIB(F)	ggagttctgacacacgattttctg	683	
	FIB(R)	tctgtttattcttttactgtccac		
M4	FIC(F)	gtgaactggcagatgaggaagg	262	Carattoli *et al*., 2005
	FIC(R)	ttctcctcgtcgccaaactagat		
	P(F)	ctatggccctgcaaacgcgccagaaa	534	
	P(R)	tcacgcgccagggcgcagcc		
	Y(F)	aattcaaacaacactgtgcagcctg	765	
	Y(R)	gcgagaatggacgattacaaaacttt		
M5	FIIA$_S$(F)	ctgtcgtaagctgatggc	270	Carattoli *et al*., 2005
	FIIA$_S$(R)	ctctgccacaaacttcagc		
	A/C(F)	gagaaccaaagacaaagacctgga	465	
	A/C(R)	acgacaaacctgaattgcctcctt		

F, sense primer, R, antisense primer

Table 3.2: **Primer sequences used for detecting replicons in CRKP isolates using the PBRT assay (continued)**

PCR type	Target	Primer (5' to 3')	Amplicon size	Reference
M5	T(F)	ttggcctgtttgtgcctaaaccat	750	Carattoli *et al*., 2005
	T(R)	cgttgattacacttagctttggac		
S1	B/O(F)	gcggtccggaaagccagaaaac	159	
	B/O(R)	tctgcgttccgccaagttcga		
S2	K(F)	gcggtccggaaagccagaaaac	160	
	K(R)	tctttcacgagcccgccaaa		
S3	F(F)	tgatcgtttaaggaattttg	270	
	F(R)	gaagatcagtcacaccatcc		
S4	FII(F)	ctgatcgtttaaggaatttt	258-262	Villa *et al*., 2010
	FII(R)	cacaccatcctgcactta		
S5	FII_k(F)	tcttcttcaatcttggcgga	142-148	
	FII_k(R)	gcttatgttgcacrgaagga		

F, sense primer, R, antisense primer

Table 3.3: Patient demographics from which *K. pneumoniae* isolates were collected

Variables	**Results**
Age, median (IQR)	43 (21,5)
Male, No. (%)	33 (58,9%)
Source of isolation, No (%)	
Urine	14 (25%)
Blood culture	17 (30,4%)
Rectal swab	7 (12,5%)
Tissue	3 (5,4%)
Aspirate/fluid	4 (7,1%)
Pus swab	4 (7,1%)
Catheter tip	7 (12,5%)
Hospital, No. (%)	
Steve Biko Academic	
Intensive care unit	12 (33,3%)
Surgery	5 (13,9%)
High care multidiscipline	4 (11,1%)
Others	15 (41,7%)
Kalafong hospital	
Ward 27	4 (40%)
Intensive care unit	1 (10%)
Others	5 (50%)
Tembisa hospital	5 (8,9%)
Tshwane rehabilitation Centre	3 (5,4%)
Mamelodi hospital	1 (1,8%)

Olievenhoutbosch clinic	1 (1,8%)

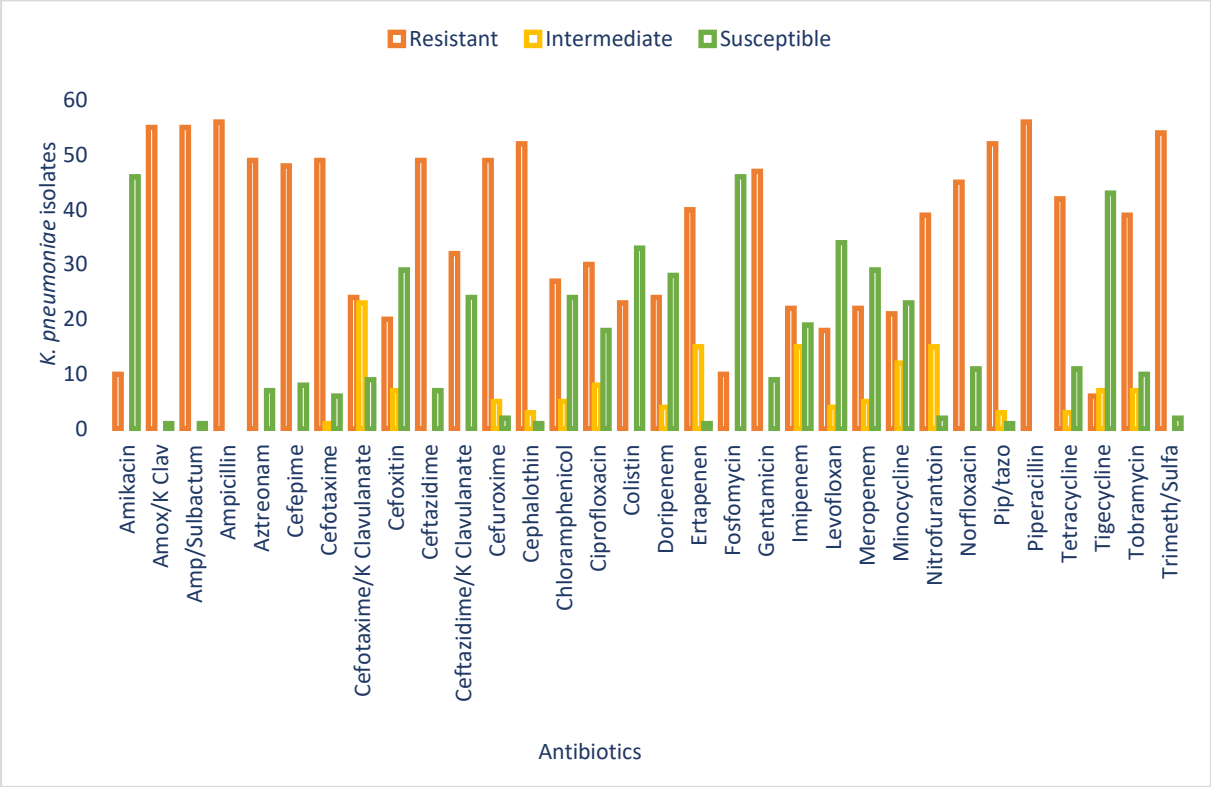


Figure 3.1: Antibiotic susceptibility patterns of 32 antibiotics in 56 *K. pneumoniae* isolates

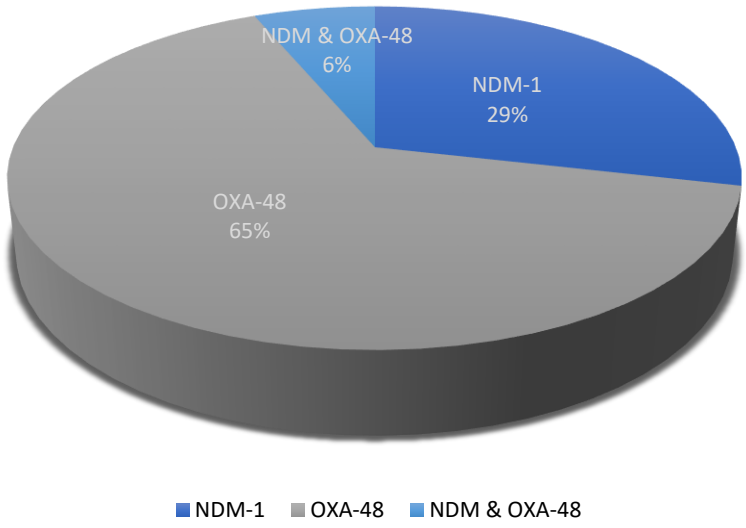


Figure 3.2: Frequency of carbapenemase genes detected in *K. pneumoniae* isolates using PCR assays

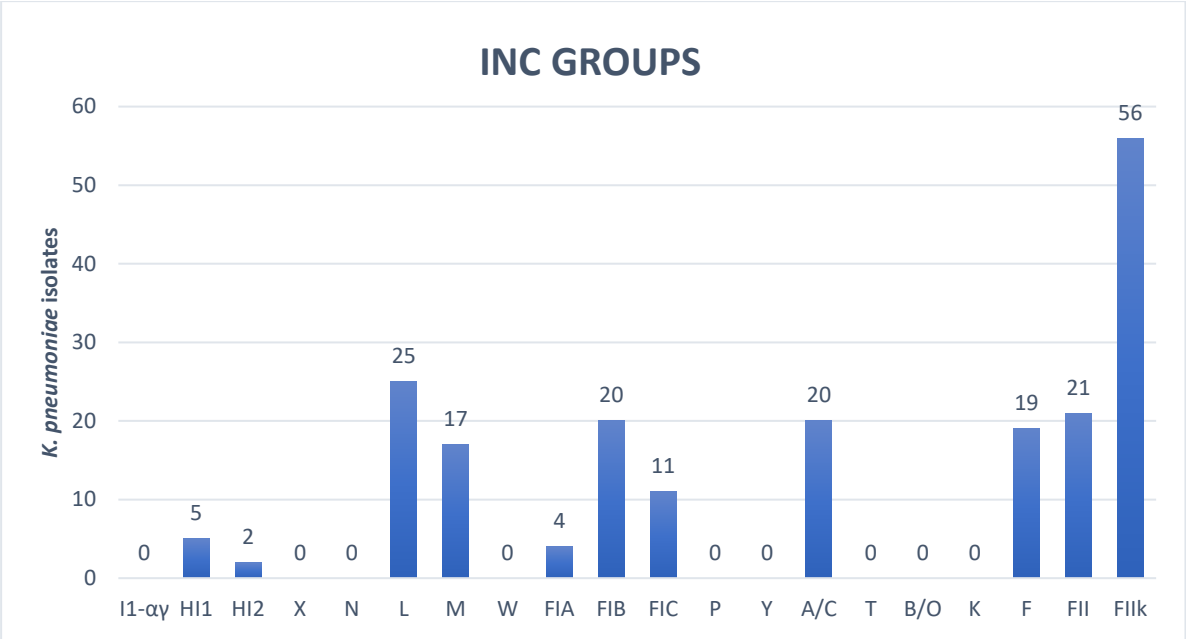


Figure 3.3: frequency of plasmid replicon groups detected in carbapenem-resistant *K. pneumoniae* isolates

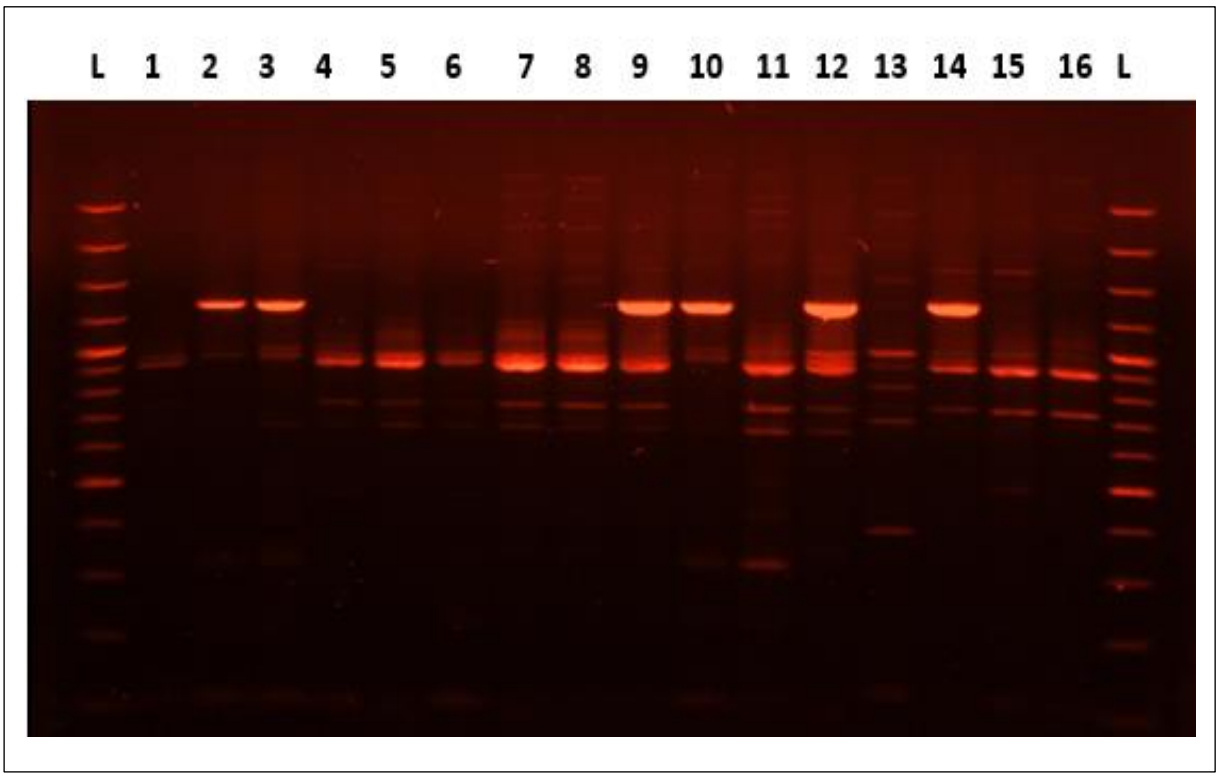
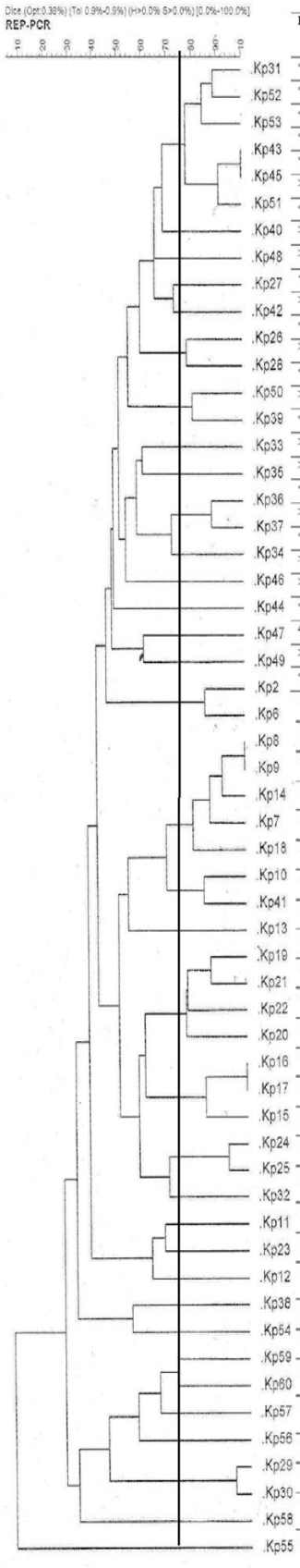


Figure 3.4: Gel image showing different REP patterns in carbapenem-resistant *K. pneumoniae* isolates



	Antimicrobial susceptibility testing						Molecular characteristics			
	Doripenem	Ertapenem	Imipenem	Meropenem	Colistin	Carbapenemases	Plasmid No	Size estimation (kb)	Plasmid Inc	Hospital
Kp31	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	>4	OXA-48	4	2.2, 48.5, >48.5	FIB, FIC, FIIk	A
Kp52	<=1 (S)	1 (I)	2 (I)	<=1 (S)	<=2	OXA-48	3	3, 48.5, >48.5	A/C, F, FII, FIIk	A
Kp53	<=1 (S)	1 (I)	2 (I)	<=1 (S)	4	OXA-48	3	3, 48.5, >48.5	F, FII, FIIk	A
Kp43	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	2	48.5, >48.5	L, A/C, F, FII, FIIk	A
Kp45	<=1 (S)	1 (I)	2 (I)	<=1 (S)	<=2	OXA-48	4	1.7, 3.3, 48.5, >48.5	L, A/C, F, FII, FIIk	C
Kp51	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	2	8, 48.5	L, FIB, A/C, F, FII, FIIk	A
Kp40	<=1 (S)	>1 (R)	2 (I)	<=1 (S)	4	OXA-48	3	4, 48.5, >48.5	L, FIB, F, FII, FIIk	B
Kp48	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	NDM-1	3	2.4, 48.5, >48.5	L, FIB, F, FII, FIIk	A
Kp27	>4 (R)	>1 (R)	>8 (R)	>8 (R)	4	NDM-1	2	2, 48.5-kb	FIIk	F
Kp42	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	4	OXA-48	4	2.4, 8, 48.5, >48.5	L, F, FII, FIIk	B
Kp26	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	2	2, 48.5-kb	FIIk	C
Kp28	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	>4	OXA-48	3	3.4, 48.5, >48.5	M, FIIk	B
Kp50	>4 (R)	>1 (R)	8 (I)	>8 (R)	<=2	NDM-1	4	1.4, 3.5, 8, 48.5	L, F, FII, FIIk	A
Kp39	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	<=2	OXA-48	5	2, 3, 3.2, 48.5, >48.5	L, A/C, FIIk	C
Kp33	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	NDM-1	4	2.4, 48.5, >48.5	L, M, FIB, A/C, FIIk	D
Kp35	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	2	48.5, >48.5	M, FIC, FIIk	A
Kp36	>4 (R)	>1 (R)	2 (I)	>8 (R)	>4	OXA-48	2	48.5, >48.5	L, M, FIB, FIC, FIIk	A
Kp37	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	NDM-1, OXA-48	4	2.4, 48.5, >48.5	L, M, FII, FIIk	A
Kp34	<=1 (S)	1 (I)	2 (I)	<=1 (S)	<=2	OXA-48	3	48.5, >48.5	M, FIB, FIC, FIIk	A
Kp46	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	4	2.4, 2.6, 48.5, >48.5	L, FIB, FII, FIIk	B
Kp44	<=1 (S)	>1 (R)	2 (I)	<=1 (S)	<=2	OXA-48	4	1.5, 2.5, 48.5, >48.5	FIB, F, FII, FIIk	A
Kp47	>4 (R)	>1 (R)	>8 (R)	>8 (R)	4	NDM-1	3	1.6, 2.4, 48.5	L, A/C, F, FII, FIIk	A
Kp49	4 (R)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	3	3, 4.5, 48.5	FIC, A/C, F, FII, FIIk	A
Kp2	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	<=2	OXA-48	3	8, 48.5, >48.5	HI2, M, FIIk	B
Kp6	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	OXA-48	2	48.5, >48.5	FIB, FIIk	E
Kp8	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	3	2, 48.5, >48.5	FIIk	A
Kp9	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	3	2, 48.5, >48.5	L, FIB, FIIk	A
Kp14	<=1 (S)	<=0.5 (S)	2 (I)	<=1 (S)	>4	OXA-48	2	48.5, >48.5	FIB, FIIk	A
Kp7	2 (I)	>1 (R)	2 (I)	2 (I)	<=2	NDM-1, OXA-48	4	2.7, 3.4, 48.5, >48.5	L, FIA, A/C, FIIk	A
Kp18	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1, OXA-48	4	2.4, 2.6, 48.5, >48.5	HI2, M, FIIk	A
Kp10	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	4	2.4, 2.6, 48.5, >48.5	FIB, F, FII, FIIk	B
Kp41	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	4	OXA-48	3	2.5, 48.5, >48.5	A/C, FIIk	C
Kp13	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	4	OXA-48	3	2, 48.5, >48.5	M, FIA, FIIk	A
Kp19	<=1 (S)	>1 (R)	2 (I)	<=1 (S)	<=2	OXA-48	3	3.4, 48.5, >48.5	M, FIA, FIIk	B
Kp21	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	<=2	OXA-48	2	48.5, >48.5	FIA, A/C, FIIk	D
Kp22	4 (R)	>1 (R)	4 (R)	2 (I)	<=2	OXA-48	5	1.7, 3, 3.8, 48.5, >48.5	FIB, A/C,FIIk	A
Kp20	<=1 (S)	1 (I)	<=1 (S)	<=1 (S)	<=2	OXA-48	5	1.7, 3, 3.8, 48.5, >48.5	L, A/C, FIIk	A
Kp16	4 (R)	>1 (R)	4 (R)	2 (I)	<=2	OXA-48	5	1.7, 3, 3.8, 48.5, >48.5	FIB, A/C,FIIk	A
Kp17	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	4	OXA-48	2	48.5, >48.5	M, FIIk	A
Kp15	2 (I)	>1 (R)	4 (R)	>8 (R)	>4	OXA-48	2	48.5, >48.5	M, A/C, FIIk	A
Kp24	2 (I)	>1 (R)	<=1 (S)	2 (I)	>4	OXA-48	2	48.5, >48.5	L, FIIk	A
Kp25	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	4	2.4, 2.6, 48.5, >48.5-kb	M, FIIk	B
Kp32	<=1 (S)	>1 (R)	2 (I)	<=1 (S)	<=2	OXA-48	2	48.5, >48.5	M, FIIk	B
Kp11	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	4	OXA-48	4	2.5, 48.5, >48.5	L, F, FII, FIIk	A
Kp23	<=1 (S)	>1 (R)	4 (I)	<=1 (S)	4	OXA-48	1	48.5	HI1, L, FIC, A/C, F, FII, FIIk	A
Kp12	<=1 (S)	>1 (R)	2 (I)	<=1 (S)	<=2	OXA-48	2	4.9, 48.5	HI1, L, FIC, F, FII, FIIk	A
Kp38	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	NDM-1	2	4, 48.5	L, FIB, FIC, A/C, FIIk	A
Kp54	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	3	3.9, 4.2, 48.5	HI1, L, FIB, FIC, A/C, F, FII, FIIk	B
Kp59	>4 (R)	>1 (R)	>8 (R)	>8 (R)	>4	NDM-1, OXA-48	1	48.5	HI1, L, M, FIB, FIC, F, FII, FIIk	D
Kp60	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	4	2.4, 48.5, >48.5	FIIk	A
Kp57	>4 (R)	>1 (R)	>8 (R)	>8 (R)	<=2	NDM-1	4	2.4, 48.5, >48.5	A/C, FIIk	A
Kp56	<=1 (S)	>1 (R)	<=1 (S)	<=1 (S)	<=2	OXA-48	4	3.2, 5, 5.5, 48.5	HI1, L, FIB, FIC, A/C, FIIk	C
Kp29	4 (R)	>1 (R)	>8 (R)	4 (R)	>4	OXA-48	1	48.5	L, F, FII, FIIk	A
Kp30										
Kp58										
Kp55										

Figure 3.5: The REP-PCR dendogram of the CRKP isolates using a similarity coefficient ratio of 75%.

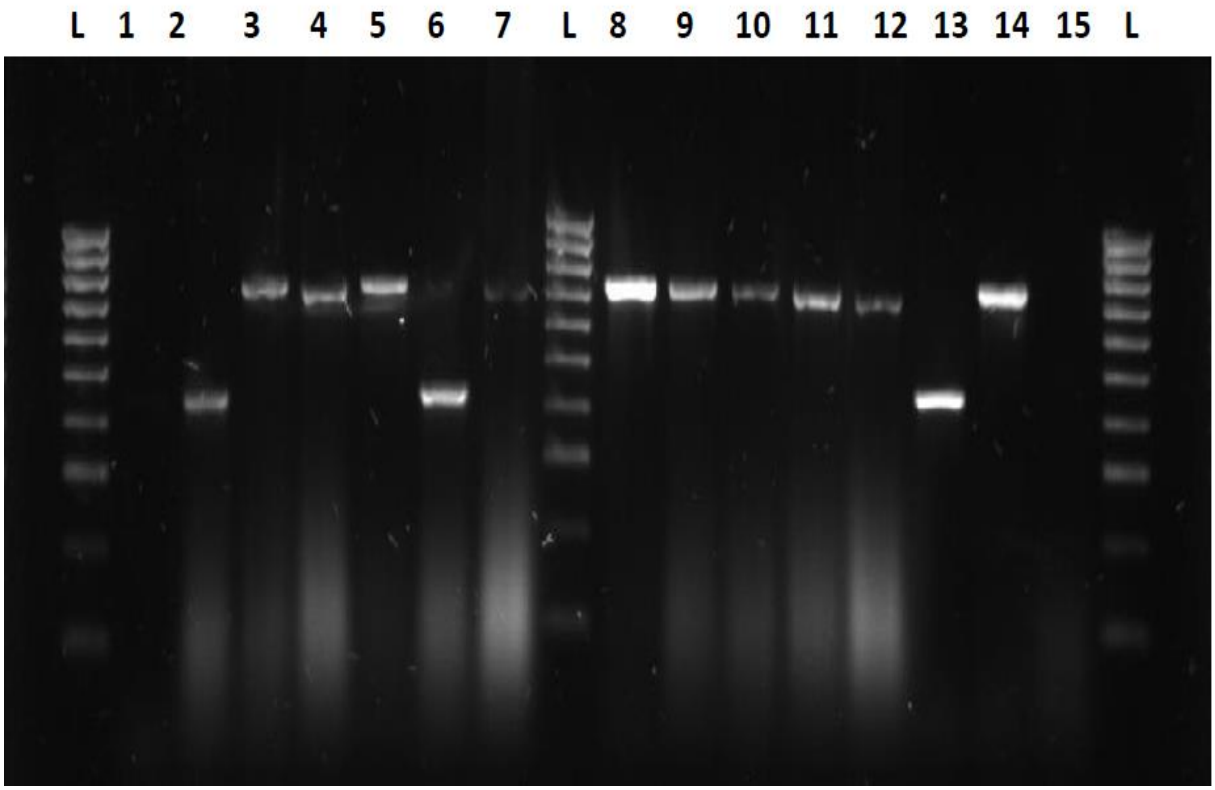


Figure 3.6: Colony PCR of carbapenemase genes in *E. coli* recipients. L: ladder, 1-12: isolates, 13: $bla_{\text{OXA-48}}$ positive control, 14: $bla_{\text{NDM-1}}$ positive control, 15: negative control

Table 3.4: **Resistance determinants of the carbapenem-resistant *K. pneumoniae* isolates**

Isolate	Resistance mechanisms								Sequence type	Plasmid (Inc) types
	B-lactams	**Tetracycline**	**Phenicol**	**Fosfomycin**	**Aminoglycosides**	**Quinolone**	**Sulphonamide**	**Trimethoprim**		
Kp8	**OXA-181**, OXA-1, CTX-M-15, SHV-28, SHV-106, TEM-1B	*tet(A)*	*catB3*	*fosA*	*aac(3)-lla*, *aac(6')-lb-cr*, *aph(3'')-lb*, *aph(6)-ld*	*aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB1*, *qnrS1*	*sul2*	*dfrA14*	307	ColKP3, IncX3, IncFIB (k), IncFII (k)
Kp10	**NDM-1**, OXA-1, CTX-M-15, SHV-40, SHV-56, SHV-79, SHV-85, SHV-89, TEM-1B	*tet(A)*	*catB3*	*fosA*	*aac(6')-lb-cr*, *aph(3'')-lb*, *aph(6)-ld*, *rmtC*	*aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB1*	*sul1*, *sul2*	*dfrA14*	39	IncFIB (k), IncFII (k), IncFII (yp)
Kp15	**OXA-181**, CTX-M-15, SHV-65, TEM-1B	*tet(A)*	*catA2*	*fosA*	*aac(3)-lla*, *aac(6')-lb-*cr, aac(6')-lb3, *aadA16*, *aph(3')-lb*, *aph(6)-ld*,	*aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB6*, *qnrS1*	*sul1*, *sul2*	*dfrA27*	607	ColKP3, IncA/C2, IncFIA (HI1), IncFIB (k), IncFII (k), IncR, IncU
Kp29	**NDM-7**, CTX-M-15, SCO-1, SHV-172, SHV-94, SHV-96, TEM-1B	-	*catA2*	*fosA*	*aac(3)-lla*, *aac(6')-lb-cr*, *aadA16*, *aph(3')-lb*, *aph(6)-ld*	*aac(6')-lb-cr*, *oqxA*, *oqxB*	*sul1*, *sul2*	*dfrA27*	17	IncFIA (HI1), IncFIB (k), IncFII (k), IncR, IncX3
Kp32	**OXA-48**, OXA-1, CTX-M-15, SHV-178, SHV-193, SHV-36, SHV-80, TEM-1B	*tet(A)*	*catB3*	*fosA*, *fosA7*	*aac(3)-lla*, *aac(6')-lb-cr*, *aph(3')-lb*, *aph(6)-ld*	*aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB1*	*sul2*	*dfrA14*	3559	IncFIB (k), IncFII (k), IncL/M

Table 3.4: **Resistance determinants of the carbapenem-resistant *K. pneumoniae* isolates (continued)**

Isolate	Resistance mechanisms								Sequence type	Plasmid (Inc) types
	B-lactams	**Tetracycline**	**Phenicol**	**Fosfomycin**	**Aminoglycosides**	**Quinolone**	**Sulphonamide**	**Trimethoprim**		
Kp33	**NDM-1**, OXA-1, CTX-M-15, SHV-40, SHV-56, SHV-79, SHV-85, SHV-89, TEM-1B	*tet(A)*	*catB3*	*fosA*	*aac(6')-lb-cr, aph(3')-lb, aph(6)-ld, rmtC*	aac(6')-lb-cr, oqxA, oqxB, qnrB1	*sul1*, *sul2*	*dfrA14*	39	IncFIB (k), IncFII (k), IncFII (yp)

Table 3.5: **Intact phages identified in carbapenem-resistant *K. pneumoniae* clinical isolates**

Phage identified	GC%	No CDS	Length (kb)	Isolates
PHAGE_Salmon_SEN5_NC_028701(21)	54.79	49	37.7	Kp8, Kp15, Kp29
PHAGE_Salmon_Fels_2_NC_010463(33)	51.82	42	41.5	Kp8, Kp32
PHAGE_Cronob_ENT47670_NC_019927(12)	53.15	71	49.1	Kp8
PHAGE_Salmon_SPN3UB_NC_019545(12)	52.02	68	55.6	Kp8
PHAGE_Salmon_118970_sal3_NC_031940(11)	50.44	44	39.8	Kp8, Kp15
PHAGE_Salmon_SJ46_NC_031129(4)	52.78	27	40.5	Kp8
PHAGE_Klebsi_phiKO2_NC_005857(43)	51.45	70	54.7	Kp8
PHAGE_Entero_mEp237_NC_019704(10)	52.26	67	56.4	Kp33, Kp10, Kp29
PHAGE_Edward_GF_2_NC_026611(21)	52.58	70	46.2	Kp33, Kp10
PHAGE_Entero_P1_NC_005856(3)	54.30	31	16.5	Kp33, Kp10
PHAGE_Escher_RCS47_NC_042128(3)	53.61	24	35.2	Kp15
PHAGE_Escher_RCS47_NC_042128(5)	53.99	34	27.1	Kp15, Kp29
PHAGE_Escher_phiV10_NC_007804(33)	50.84	59	59.9	Kp15
PHAGE_Salmon_RE_2010_NC_019488(32)	53.71	45	37.4	Kp29
PHAGE_Pectob_ZF40_NC_019522(14)	50.21	56	48.1	Kp29
PHAGE_Phage_Gifsy_1_NC_010392(11)	52.09	78	55.7	Kp32
PHAGE_Salmon_SEN34_NC_028699(25)	54.27	70	60.2	Kp32
PHAGE_Staphy_SPbeta_like_NC_029119(3)	54.40	29	25.5	Kp32

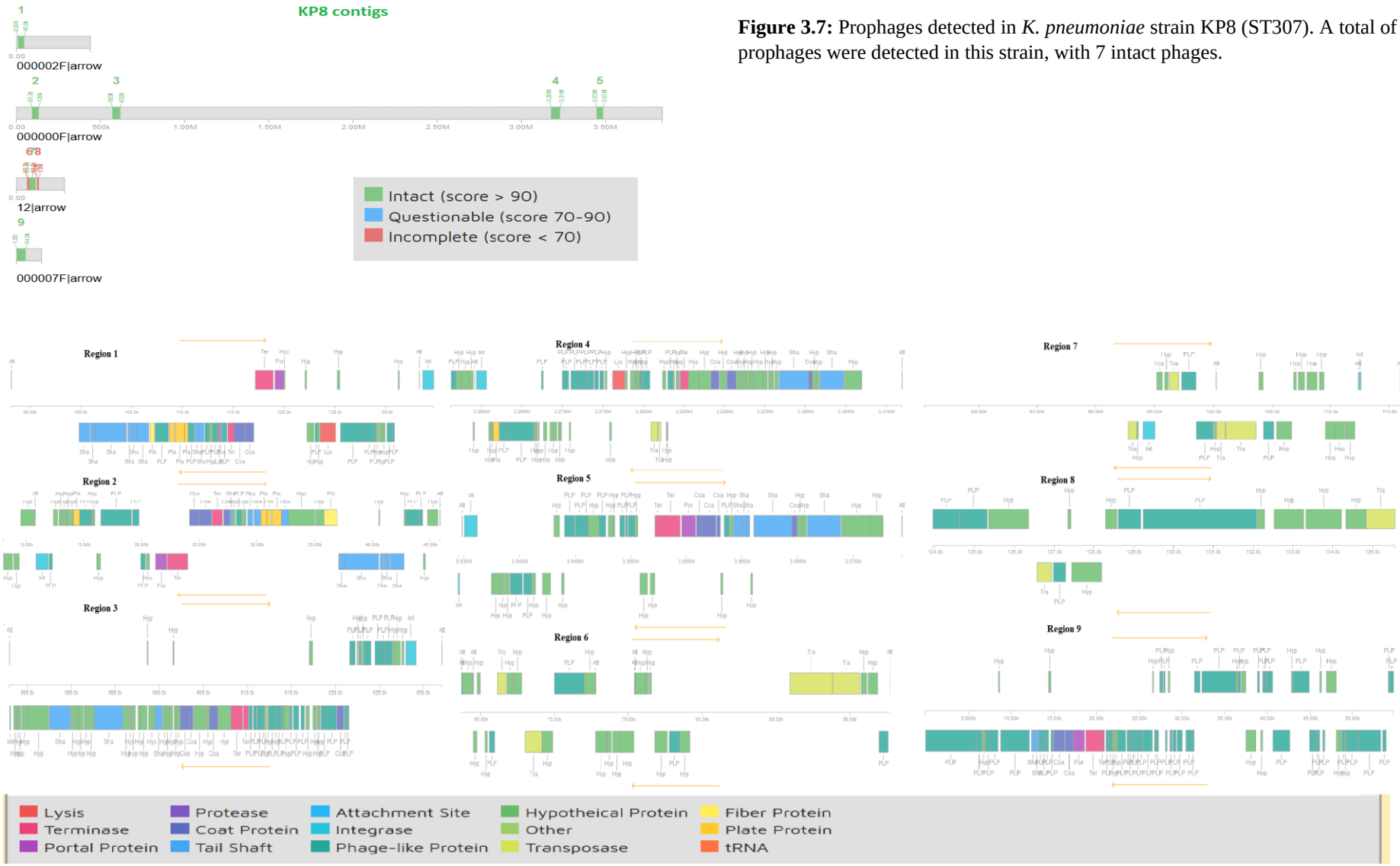


Figure 3.7: Prophages detected in *K. pneumoniae* strain KP8 (ST307). A total of 9 prophages were detected in this strain, with 7 intact phages.

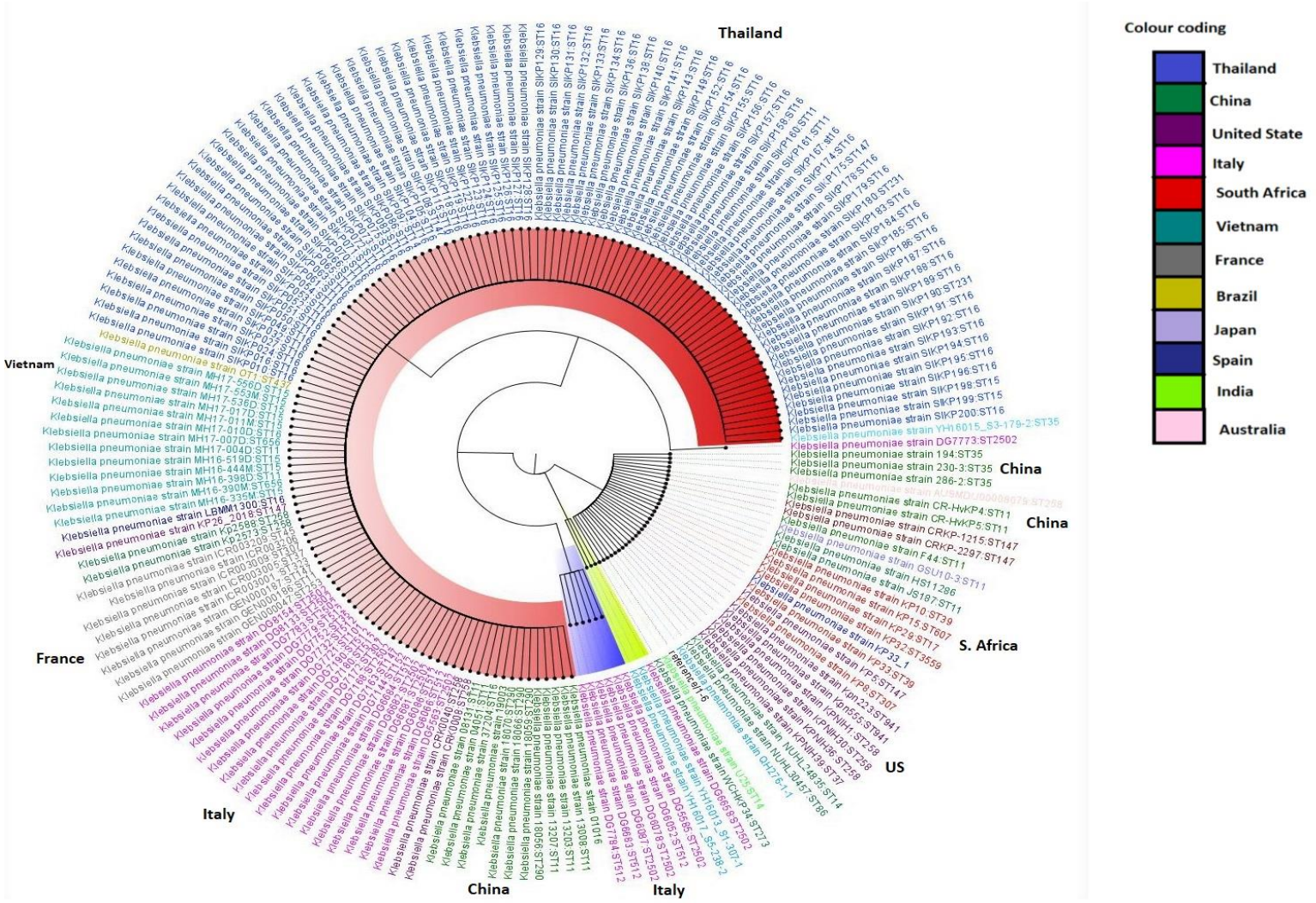


Figure 3.8: Phylogeny tree1 showing relatedness of carbapenem-resistant *K. pneumoniae* strains worldwide. Each strain is expressed in specie name, strain and the sequence type. Different countries are differentiated with different colour coding as shown in legend.

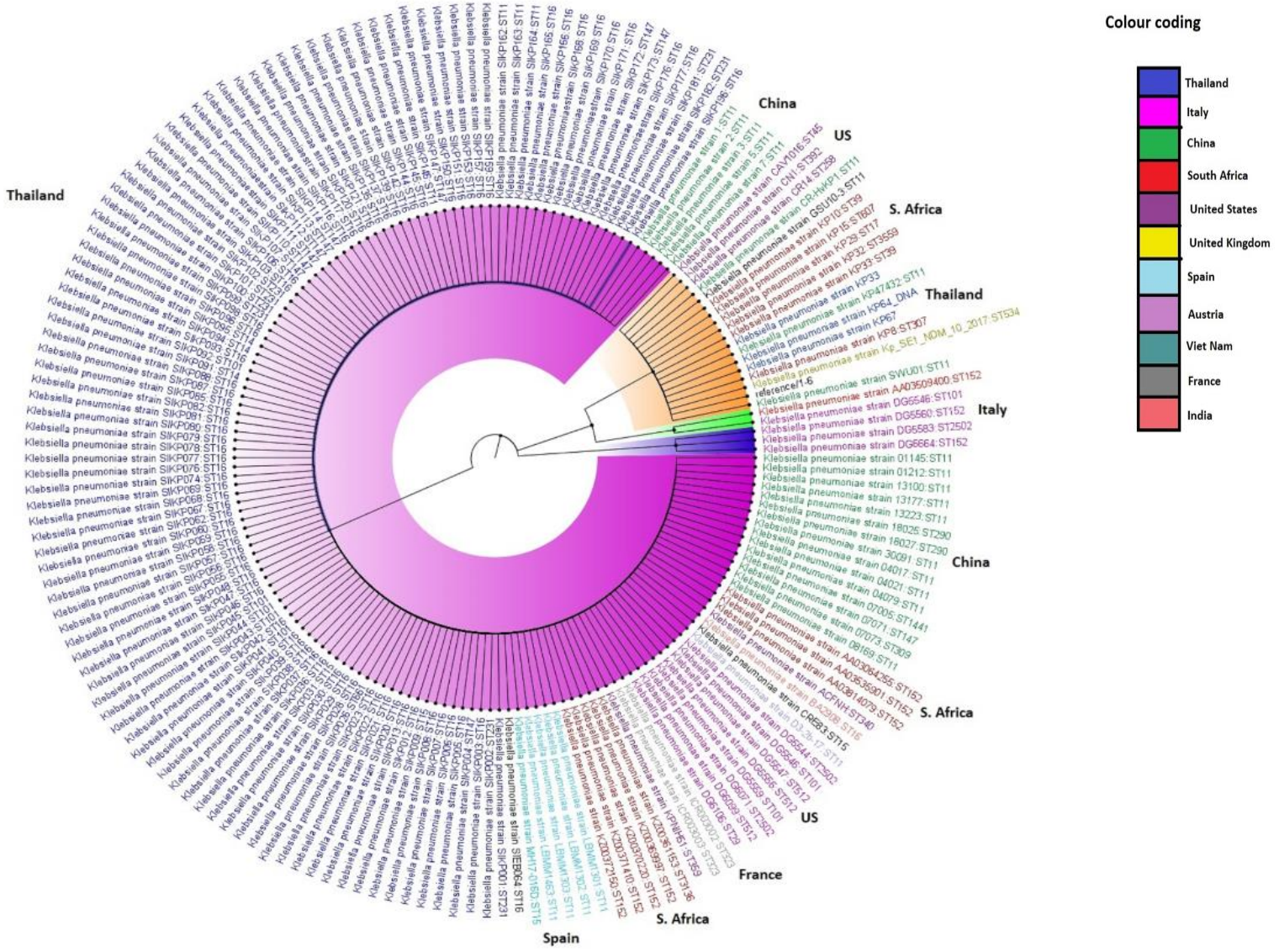


Figure 3.9: Phylogeny tree2 showing relatedness of carbapenem-resistant *K. pneumoniae* strains worldwide. Each strain is expressed in specie name, strain and the sequence type. Different countries are differentiated with different colour coding as shown in legend.

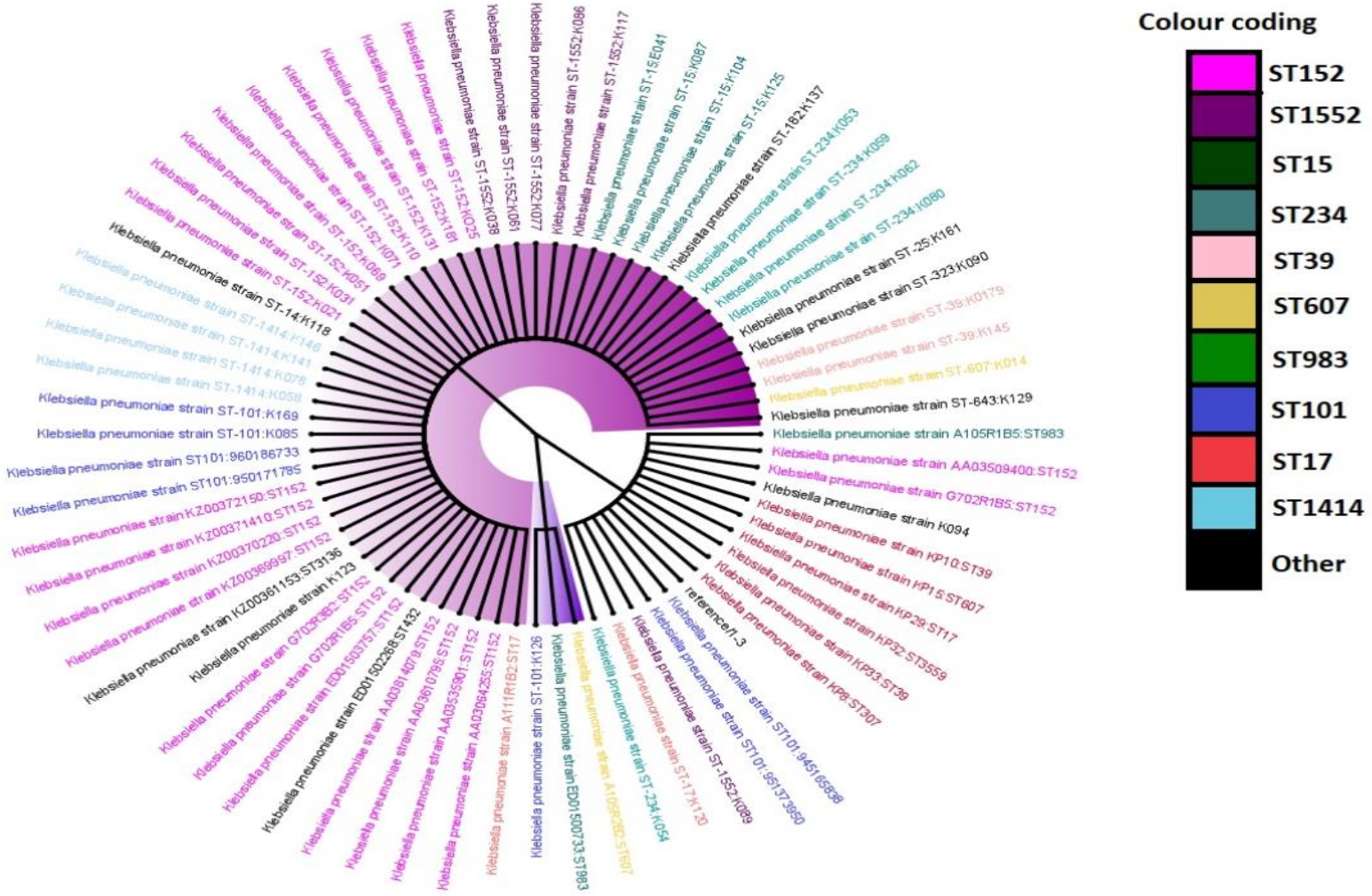


Figure 3.10: Phylogeny tree showing relatedness of *K. pneumoniae* strains in South Africa. Each strain is expressed in specie name, strain and the sequence type. Different sequence types are differentiated with different colour coding as shown in legend.

Table 3.6: Comparison of closely related *K. pneumoniae* strains on the phylogeny tree reported in different countries

Strain	Country	Year	Sequence Type (ST)	Resistance determinants	Plasmids replicons
KP33_1	Thailand	2015	ST14	**NDM-1**, CTX-M-15, *aadA2*, *armA*, *aph(3')-VlA*, *msr*(E), *mph*(E), *sul1*, *dfrA12*, *dfrA1*, *dfrA14*, *aac(6')-Ib*, *aac(6')-Ib-cr*, *cat*, *qnrB1*, *tet(D)*.	IncHI1B
KP64	Thailand	2015	ST14	**NDM-1**, CTX-M-15, OXA-1, SHV-28, TEM-1B, *aadA2*, *armA*, *aph(3')-Vla*, *msr*(E), *mph*(E), *sul1*, *dfrA12*, *dfrA1*, *cat*, *aac(6')-Ib-cr*, *aac(6')-Ib*, *fosA*, *oqxAB*,	IncR
CR-HvKP1	China	2016	ST11	**KPC-2**, CTX-M-65, TEM-1B, *rmtB*, *catA2*, *fosA14*	IncFII/R, Incl1
CRKP-1215	South Korea	2014	ST147	**NDM-5**, **OXA-181**, CTX-M-15, TEM-1B, SHV-11, OXA-1, *aadA3*, *aac(6')-Ib-cr*, *aacA4*, *dfrA23*, *sul1*, *mph*(A), *rmt*(B), *erm*(B), *fosA*, *oqxAB*	IncFII
CN1	United States	2013	ST392	**KPC-2**, CTX-M-15, OXA-1, TEM-1B	-
G702R3B2	South Africa	2016	ST152	CTX-M-15, OXA-1, TEM-1B, *aadA16*, *aac(6')-lb-cr*, *aac(3')ll-a*, *aph(6)-ld*, *aph(3')-lb*, *sul1*, *sul2*, *tetA*, *tetB*, *tetD*, *dfrA14*, *dfrA27*, *oqxA*, *oqxB*, *catA1*, *catB4*, *fosA*, *arr-3*, *gyrA*, *parC*	IncFIB, IncFII, IncFII(k), IncN, IncQ1, ColpVC, ColRNAI
KP33 (This study)	South Africa	2018	ST39	**NDM-1**, OXA-1, CTX-M-15, SHV-40, SHV-56, SHV-79, SHV-85, SHV-89, TEM-1B, *aac(6')-lb-cr*, *aph(3')-lb*, *aph(6)-ld*, *rmtC*, *aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB1*, *tet(A)*, *fosA*, *catB3*, *dfrA14*, *sul1*, *sul2*	IncFIB (k), IncFII (k), IncFII (yp)

CHAPTER 4

CONCLUDING REMARKS

4.1 Conclusion

Carbapenem-resistant *K. pneumoniae* (CRKP) has been listed by the WHO as one of the critical priority 1 pathogens. This is due to their high attributable morbidity and mortality, ranging from 33.24% to 50.06% in America, Europe, and Asia. CRKP strains are highly resistant to almost all β-lactams antibiotics including "last resort" carbapenems, limiting treatment options. Carbapenem resistance is mediated by production of carbapenemases harboured on plasmids that can be transferred from one bacterial species to another via HGT.

In this study, fifty-six *K. pneumoniae* isolates were collected from the national laboratory in Pretoria, South Africa. PCR assays and WGS techniques were used to detect carbapenemase production, describe the molecular epidemiology of *K. pneumoniae* isolates and characterize plasmids associated with CRKP.

The main findings of the study were:

1) Plasmid-mediated carbapenemase genes ($bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$-like) were the most predominant genes in *K. pneumoniae* isolates in 2018 in Pretoria.
2) Four major strains in this study were determined, with one major group comprising of ST307 *K. pneumoniae* strains.
3) Additionally, four other sequence types including ST39, ST17, ST607, and ST3559 were determined.
4) The detected carbapenemase genes in *K. pneumoniae* in this study were associated with multiple plasmid replicons including IncL/M, IncX3, IncA/C, IncFII, IncFIB, IncR and ColKP3 replicon groups.
5) A few of the carbapenem-resistant *K. pneumoniae* might represent hypervirulent strains because they were positive for KL2 during capsule serotyping.
6) Whole-genome sequencing proved to be superior to PCR-based replicon typing technique in detection of plasmid replicon types in that, the PBRT missed the IncX3 plasmids but WGS was able to identify these plasmids using PlasmidFinder.

The results of this study are similar to studies reported in South Africa reporting on the prevalence of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$-like genes in private hospital sectors. The plasmid types reported in this study, particularly IncX3 and IncFIB have been previously reported to be

associated with *bla*$_{\text{OXA-48}}$-like and *bla*$_{\text{NDM-1}}$, respectively. The *K. pneumoniae* isolates reported in this study showed multidrug resistance profiles, which are the same characteristics previously reported in private hospitals and public hospital sewage water. Thus, without proper infection control in these hospitals, there will be continued dissemination of MDR and hypervirulent *K. pneumoniae* and/or resistance and virulence plasmids to other susceptible *Enterobacteriaceae* species.

4.2 Recommendations

The increase in the prevalence of carbapenemase genes such as *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$-like genes in South African hospitals raises concerns about control measures already in place.

Antimicrobial resistance surveillance studies are important because they give an indication of what is happening in the clinical context. This will assist with the implementation of effective prevention and control strategies and measures. Strict control strategies are needed in all private and public hospital sectors in order to control escalation of MDR *Enterobacteriaceae* species. For better control and containing of MDR *K. pneumoniae* strains and resistance plasmids, regular screening of patients, proper education about prevention measures and proper precautions need to be adopted.

It is important to study the molecular characteristics and epidemiology of MDR isolates including resistance plasmids associated with them. This will help clinicians to decide on suitable treatment options and limiting the use of inactive antimicrobials.

In addition to studies focusing on surveillance of antimicrobial resistance and molecular characteristics of MDR *K. pneumoniae* strains, studies focusing on treatment markers and prevention strategies need to be conducted.

Whole-genome sequencing and bioinformatics are advantageous to other molecular methods because of the amount of information provides simultaneously. The use of this technique will be beneficial when adopted by South African hospitals for screening and characterising MDR *Enterobacteriaceae* species.

4.3 Future Research

Research has shown that within plasmids, there are other mobile genetic elements (MGEs) that play a major role in recruiting genes in the plasmid variable region. Studies looking at NDM-1 plasmids (IncX3), reported that insertion sequences (IS) and/or transposons play an essential role in acquisition of resistance genes into the plasmids. For instance, Qu and colleagues

reported that MGEs such as IS*3000*, ISCR*3*, and IS*26* groups usually found upstream the NDM-1 gene acts as a promoter region and mediate the spread of this gene intra- and interspecies. This is usually different in other NDM-1 plasmids other than IncX3 plasmids. Different genetic environments in A/C plasmids have been reported, suggesting different genetic events resulting in the acquisition of the antimicrobial resistance genes. Therefore, it is essential for studies in South Africa to focus not only on plasmids but comparison of the genetic environments of antimicrobial resistance genes. This will reveal whether plasmids mediating carbapenem resistance in South Africa carry the same MGEs or modified MGEs in the plasmid variable region. Moreover, it will increase our knowledge on the effect of these additional MGEs on plasmids.

There is limited information about the genetic environment of OXA-181 genes on IncX3 plasmids. This is an essential aspect of future research in South Africa focusing on OXA-181 plasmid (InX3) genetic environments.

ANNEXURE A

REAGENTS, BUFFERS, GELS AND EXPERIMENTAL PROCEDURES

A) Reagents, buffers and gels

1. Ethylenediaminetetraacetate (EDTA) (0.5 M; pH 8.0)

EDTA, disodium salt (Sigma-Aldrich, Missouri, USA)	186.1 g
Ultrapure water (Purite Select HP, Pocklington, UK)	800 mL
Sodium hydroxide (NaOH) pellets (Merck, Darmstadt, Germany)	20 g

Dissolve 186.1 g EDTA in 800 mL ultrapure water, adding the NaOH pellets until the solution dissolves completely. Bring the volume to 1 L and autoclave at 121°C for 15 min and store at room temperature (25°C ± 5°C).

2. Tris (1 M; pH 8.0)

Tris-base (Sigma-Aldrich, St Louis, Missouri USA)	121.1 g
Hydrochloric acid (HCl) (Merck, Darmstadt, Germany)	40 mL
Ultrapure water (Purite Select HP, Pocklington, UK)	800 mL

Dissolve the 121.1 g Tris-base in ultrapure water. Add the 40 mL of HCl and mix the solution. Bring the volume to 1 L and autoclave at 121°C for 15 min.

3. TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; pH 9.0)

1 M Tris (pH 8.0) (Sigma-Aldrich, Missouri, USA)	10 mL
0.5 M EDTA (pH 9.0) (Sigma-Aldrich, Missouri, USA)	200 µL
Sterile distilled water	800 mL

Dissolve 10 mL of Tris and 200 µL of EDTA in 800 mL of sterile distilled water. Adjust the pH to 9.0 and bring the volume to 1 L. Autoclave at 121°C for 15 min. Store at room temperature (25°C ± 5°C).

4. 5x Tris-boric EDTA (TBE) buffer

Tris-base (Sigma-Aldrich, Missouri, USA)	54 g
Boric acid (Sigma-Aldrich, Missouri, USA)	27.5 g
0.5 M EDTA (pH 8.0)	20 mL
Ultrapure water (Purite Select HP, Pocklington, UK)	800 mL

Dissolve 54 g of Tris and 27.5 g of Boric acid into 800 mL of ultrapure water. Add 20 mL of EDTA and adjust pH to 8.3 by adding concentrated HCl. Bring volume to 1 L and autoclave at 121°C for 15 min and store at room temperature.

To make 1× TBE, dilute 200 mL 5× TBE with 800 mL distilled water.

5. Agarose gel (1.5% m/v)

SeaKem LE agarose powder (SeaKem® Lonza, Rockland, USA)	1.5 g
1X TBE buffer	100 mL
Ethidium bromide [10 mg/mL (Sigma-Aldrich, St Louis, Missouri USA)]	5 µL

Add 1 g of SeaKem LE agarose powder to 100 mL 1X TBE buffer. Microwave the solution on medium heat for 2 to 3 min, stopping to swirl the solution at intervals. Allow to cool down to 50°C and add 5 µL ethidium bromide. Pour into a clean casting tray, add comb and allow to set for 30 min.

6. Brain Heart Infusion (BHI) broth

Brain Heart Infusion broth (Lab M Limited, Lancashire, UK)	37 g
Ultrapure water (Purite Select HP, Purite Ltd, Pocklington, UK)	1 L

Dissolve 37 g of BHI broth into 1 L of ultrapure water. Autoclave at 121°C for 15 min. the prepared media can be stored up to 3 months in a capped bottle at 15-20°C.

B) Experimental procedures

DNA extraction using the boiling method

1. 1000 µL of each overnight broth culture bacterium was transferred into a 1.5 mL sterile microcentrifuge tubes (Merck, Modderfontein, South Africa).
2. Microcentrifuge tubes (Merck, Modderfontein, South Africa) were centrifuged (Spectrafuge 24D, Labnet International, New Jersey, USA) at 5000 x *g* for 5 minutes at room temperature (22°C to 24°C).
3. Supernatant was discarded without disturbing the pellet
4. The pellet was resuspended into 1000 µL of Phosphate buffered saline (PBS; pH 7.2) (Gibco by Life Technologies, Thermo Fisher Scientific, Massachusetts, USA), followed by gentle vortexing (VX100, Labnet International, New Jersey, USA) for 3 seconds.
5. Microcentrifuge tubes (Merck, Modderfontein, South Africa) were centrifuged (Spectrafuge 24D, Labnet International, New Jersey, USA) at 5000 x *g* for 5 minutes at

room temperature (22°C to 24°C) and following centrifugation, the supernatant was discarded without disturbing the pellet.

6. The pellet was resuspended in 50 µL of PBS and incubated at 95°C for 15 minutes in an Accublock Digital Dry Bath (Labnet International, New Jersey, USA).
7. The solution was then incubated in an untrasonic bath (Transsonci T460, Elma, Germany) at 95°C for 15 minutes at room temperature (22°C to 24°C).
8. The solution was centrifuged at maximum speed (13 000 x *g*) for 5 minutes at room temperature (22°C to 24°C).
9. The resulting supernatant was transferred to a sterile 1.5 mL microcentrifuge (Merck, Modderfontein, South Africa) tube and stored at -20°C freezer (Defy Ltd, Durban, SA) until required for PCR assays.

DNA extraction using the Zymo Research fungal/bacterial miniprep kit

Plasmid DNA extraction using the Qiagen® plasmid midi kit

1. Overnight bacterial culture cells (100 mL of *K. pneumoniae* isolates grown in Luria Bertani broth (LB) (VWR International, Pennsylvania, USA)) was harvested by centrifugation (Eppendorf, Merck, Modderfontein, South Africa) at 6000 x *g* for 15 minutes at 4°C.
2. The resulting bacterial pellet was resuspended in 4 mL of Buffer P1.
3. 4 mL of Buffer P2 was added and mixed by vigorous shaking 4-6 times and incubated at room temperature (15°C to 25°C) for 5 minutes. (Observe a colour change from colorless to blue).
4. 4 mL of prechilled Buffer P3 was added and mixed by vigorous shaking 4-6 times and incubated on ice for 15 minutes. (Observe a colour change from blue to colorless).
5. The solution was centrifuged (Sorvall LYNX 4000, Thermo Fisher Scientific, Massachusetts, USA) at 20 000 x *g* for 30 minutes at 4°C.
6. A 100 QIAGEN-tip was equilibrated by applying 4 mL of Buffer QBT and the column was allowed to empty by gravity flow.
7. The resulting supernatant from step 5 was added to the QIAGEN-tip and allowed to empty by gravity flow.
8. The QIAGEN-tip was washed twice with 10 mL of Buffer QC.
9. 5 mL of Buffer QF was used to elute DNA into a clean 15 mL tube.
10. The DNA was precipitated with 3.5 mL room temperature isopropanol (Sigma-Aldrich, Missouri, USA) and centrifuged (Sorvall LYNX 4000, Thermo Fisher Scientific, Massachusetts, USA) at 15 000 x *g* for 30 minutes at 4°C. (Supernatant was discarded).

11. DNA pellet was washed with 2 mL room temperature 70% ethanol and centrifuged (Sorvall LYNX 4000, Thermo Fisher Scientific, Massachusetts, USA) at 15 000 x *g* for 10 minutes. (Supernatant was discarded).
12. The pellet was air-dried for 5 minutes and dissolved in 2 mL of Tris-EDTA (TE; pH 8.0) buffer and stored at -20°C freezer (Defy Ltd, Durban, SA).

ANNEXURE B

CLINICAL DATA OF PATIENTS

Table 1: Demographic features of patients from which *K. pneumoniae* was isolated

Isolate Number	**Episode/Laboratory Number**	**Ward**	**Hospital**	***Species***	**Source**	**Age** (yrs)	Gender
Kp2	RH01134398	ward 15	Kalafong	*K. pneumoniae*	Urine	38	M
Kp6	MG02977244	Neurology ward	Steve Biko academic	*K. pneumoniae*	Blood culture	31	F
Kp7	MG02965309	Medical & Pulmonary ICU	Steve Biko academic	*K. pneumoniae*	Aspirate	58	M
Kp8	RE00194731	Gynaecology ward 5	Mamelodi Hospital	*K. pneumoniae*	Pus swab	42	F
Kp9	MG03008099	Surgery ICU	Steve Biko academic	*K. pneumoniae*	Tracheal aspirate	40	M
Kp10	RH01139663	Ward 4	Steve Biko academic	*K. pneumoniae*	Urine	14 days	_
Kp11	MG03017849	Neurosurgery ICU	Steve Biko academic	*K. pneumoniae*	Tracheal aspirate	21	M
Kp12	RH01153696	Ward 4	Kalafong	*K. pneumoniae*	Rectal swab	1	M
Kp13	MG03171906	Neonatal ICU	Steve Biko academic	*K. pneumoniae*	Rectal swab	3m	F
Kp14	MG03040426	Medical & Pulmonary ICU	Steve Biko academic	*K. pneumoniae*	Blood culture	62	F
Kp15	MG03391928	Vascular Surgery ward 4	Steve Biko academic	*K. pneumoniae*	Catheter tip	57	M
Kp16	MG03335530	High Care Multidiscipline	Steve Biko academic	*K. pneumoniae*	Rectal swab	54	F
Kp17	MG03387777	Vascular Surgery ward 4	Steve Biko academic	*K. pneumoniae*	Blood culture	57	M
Kp18	MG03335487	Main Casualty	Steve Biko academic	*K. pneumoniae*	Blood culture	70	M

Kp19	IL01785517	Ward 15	Tembisa	*K. pneumoniae*	Tissue	82	F
Kp20	MG03343926	Spinal A	Tshwane Rehabilitation Centre	*K. pneumoniae*	Urine	56	F
Kp21	MG03382351	Medical & Pulmonary ICU	Steve Biko academic	*K. pneumoniae*	Catheter tip	64	M
Kp22	RH01258610	Ward 27	Kalafong	*K. pneumoniae*	Urine	1 m	M
Kp23	RH01217536	Ward 27	Kalafong	*K. pneumoniae*	Blood culture	5 days	M
Kp24	MG03240940	Nephrology ward	Steve Biko academic	*K. pneumoniae*	Blood culture	49	F
Kp25	MG03264044	Urology ward	Steve Biko academic	*K. pneumoniae*	Urine	38	M
Kp26	IL01741380	Ward 16	Tembisa	*K. pneumoniae*	Catheter tip	27	F
Kp27	MG03256440	_	Olievenhoutbosch Clinic	*K. pneumoniae*	Rectal swab	23	F
Kp28	RH01216530	Ante-natal Clinic	Kalafong	*K. pneumoniae*	Urine	31	F
Kp29	MG03254308	Neurology ward	Steve Biko academic	*K. pneumoniae*	Swab	50	F
Kp30	MG03228521	Surgery ICU	Steve Biko academic	*K. pneumoniae*	Catheter tip	29	M
Kp31	MG03291628	Urology ward	Steve Biko academic	*K. pneumoniae*	Tissue	59	M
Kp32	MG03321871	High Care Multidiscipline	Steve Biko academic	*K. pneumoniae*	Urine	73	F
Kp33	MG03303236	Spinal B	Tshwane Rehabilitation Centre	*K. pneumoniae*	Urine	55	M
Kp34	MG03284022	Transplant unit	Steve Biko academic	*K. pneumoniae*	Urine	33	M
Kp35	MG03277827	Surgery ICU	Steve Biko academic	*K. pneumoniae*	Aspirate	62	M
Kp36	MG03326742	General Surgery Male Ward	Steve Biko academic	*K. pneumoniae*	Urine	64	M
Kp37	MG03193712	General Surgery Female Ward	Steve Biko academic	*K. pneumoniae*	Tissue	37	F
Kp38	MG03474390	Paediatric Surgery Ward	Steve Biko academic	*K. pneumoniae*	Catheter tip	20 days	M
Kp39	IL01724988	Ward 10	Tembisa	*K. pneumoniae*	Urine	34	M
Kp40	RH01194844	Ward 7	Kalafong	*K. pneumoniae*	Blood culture	45	F
Kp41	RH01193524	Ward 28	Kalafong	*K. pneumoniae*	Urine	16 days	M
Kp42	RH01171870	ICU	Kalafong	*K. pneumoniae*	Rectal swab	36	F

Kp43	MG03080940	Urology & Gynaecology Ward	Steve Biko academic	*K. pneumoniae*	Urine	51	F
Kp44	MG03091513	Neonatal ICU	Steve Biko academic	*K. pneumoniae*	Blood culture	13 days	M
Kp45	IL01660158	Ward 19	Tembisa	*K. pneumoniae*	Pus swab	31	M
Kp46	RH01164494	Ward 27	Kalafong	*K. pneumoniae*	Blood culture	22 days	F
Kp47	MG03116667	Medical & Pulmonary ICU	Steve Biko academic	*K. pneumoniae*	Catheter tip	48	M
Kp48	MG03155183	Medical & Pulmonary ICU	Steve Biko academic	*K. pneumoniae*	Blood culture	42	F
Kp49	MG03137523	Oncology Ward	Steve Biko academic	*K. pneumoniae*	Blood culture	11	F
Kp50	MG03136682	Main Casualty	Steve Biko academic	*K. pneumoniae*	Blood culture	38	M
Kp51	MG03144979	Nephrology ward	Steve Biko academic	*K. pneumoniae*	Catheter tip	42	M
Kp52	MG03165489	High Care Multidiscipline	Steve Biko academic	*K. pneumoniae*	Blood culture	31	M
Kp53	MG03157767	Admissions	Steve Biko academic	*K. pneumoniae*	Rectal swab	49	M
Kp54	MG03159581	Surgery ICU	Steve Biko academic	*K. pneumoniae*	Blood culture	70	M
Kp55	MG03170657	Admissions	Steve Biko academic	*K. pneumoniae*	Rectal swab	41	F
Kp56	MG03163184	Spinal B	Tshwane Rehabilitation Centre	*K. pneumoniae*	Urine	46	M
Kp57	RH01199984	Ward 27	Kalafong	*K. pneumoniae*	Blood culture	4 days	M
Kp58	IL01718840	Ward 10	Tembisa	*K. pneumoniae*	Blood culture	43	M
Kp59	MG03159639	Haemodialysis Unit	Steve Biko academic	*K. pneumoniae*	Pus swab	39	M
Kp60	MG03216634	High Care Multidiscipline	Steve Biko academic	*K. pneumoniae*	Blood culture	49	F

ANNEXURE C

RESEARCH ETHICS APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

9 October 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 209/2018
Title: Characterization of plasmids mediating carbapenem resistance in Klebsiella pneumoniae in Pretoria, South Africa

Dear Miss K Kopotsa

The **Annual Renewal** as supported by documents received between 2019-09-03 and 2019-10-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-10-09.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-10-09.
- Please remember to use your protocol number (209/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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