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THE ROLE OF PLASMIDS AND CLONES IN THE EMERGENCE OF CARBAPENEM RESISTANT ENTEROBACTERIACEAE IN THE UNITED ARAB EMIRATES

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Dr. Agnes Sonnevend

May 2019

Declaration of Original Work

I, Shaimaa Fekry Mahmoud Mouftah Ali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*The Role of Plasmids and Clones in the Emergence of Carbapenem Resistant Enterobacteriaceae in the United Arab Emirates*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Dr. Agnes Sonnevend, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, nor formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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Abstract

Resistance to carbapenems, the ultimate beta-lactam antibiotics used to treat life-threatening Gram-negative infections associates with very high mortality. Consequently, carbapenem resistant *Enterobacteriaceae* (CRE), which are usually multi- or extremely drug resistant, are considered a critical pathogen. To help controlling their spread, we investigated the molecular epidemiology of CRE in the United Arab Emirates (UAE) and its neighboring countries. CRE isolated in the UAE were screened for IncX3 incompatibility type plasmids carrying carbapenemases. The complete sequence of the IncX3 plasmids identified was established. Thirty CRE carried $bla_{\text{NDM-1}}$, $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$, $bla_{\text{NDM-7}}$, $bla_{\text{OXA-181}}$ and $bla_{\text{KPC-2}}$ on IncX3 plasmids. These CRE belonged to 16 sequence types of five different species. Phylogenetic analysis of the conserved regions of local IncX3 plasmids and of those described globally clustered them according to the carbapenemase genes carried, suggesting that they do not evolve locally, rather, are imported from other regions. Furthermore, we investigated members of the *Klebsiella pneumoniae* ST14 clone, which was found to be significantly associated with NDM- and OXA-48-like double carbapenemase production, with extreme drug resistance, and with being isolated from Emirati patients in Dubai. To gain a deeper insight into the molecular features of this clone, 39 CRKP-ST14 selected from five cities of the UAE, from Bahrain and from Saudi Arabia were subjected to whole genome sequencing, and their resistome, virulome and core genome MLST was assessed. cgMLST revealed three clusters of 16 isolates from five UAE cities (C1), 11 isolates from three UAE cities and Bahrain (C2) and the 5 isolates from Saudi Arabia (C3), respectively, and seven singletons. Resistance gene profile and carbapenemase carrying plasmid types were variable in both C1 and C2 clusters. Cluster 2 exhibited a capsular switch from K2 to K64. The successful dissemination of the CRKP-14 clone could be explained by the genetic flexibility demonstrated. Our data show that the emergence of CRE in the United Arab Emirates is a complex phenomenon of likely international transfer of successful plasmids, and also of

countrywide clonal transmission of a genetically flexible high-risk *Klebsiella pneumoniae* clone.

Keywords: *Enterobacteriaceae*, carbapenem resistance, horizontal gene transfer, IncX3 plasmid, *Klebsiella pneumoniae* ST14 clone.

Title and Abstract (in Arabic)

دور البلازميدات وبكتريا المكورات المعوية في حالة انتشار مقاومة الكاربابينيم في دولة الإمارات العربية المتحدة

الملخص

اصبحت مقاومة البكتيريا لمضادات الكاربابينيم Carbapenems والتي تعتبر المضادات المثالية المستخدمة في علاج العدوى للبكتيريا سالبة الغرام Gram Negative، ترتبط بمعدل وفيات مرتفع. ولذلك، تعتبر بكتريا المكورات المعوية المقاومة للكاربابينيم CRE، والتي تكون مقاومة بشدة للأدوية، مسبب اساسي للأمراض. وللمساعدة في السيطرة على انتشارها، قمنا بدراسة مستفيضة لهذه البكتيريا باستخدام علم الأوبئة الجزيئي في دولة الإمارات العربية المتحدة والدول المجاورة. تم فحص مجموعات من CRE والتي تحتوي على بلازميد IncX3 الحامل لجينات ال carbapenemases والتسلسل الكامل لبلازميدات IncX3 وجدنا ثلاثين CRE حمل جينات الblaNDM-1، blaNDM-4، blaNDM-5، blaNDM-7، blaOXA-181وblaKPC-2 على بلازميدات IncX3. وتنتمي مجموعات ال CRE إلى 16 نوع تسلسلي (ST) لخمسة أنواع بكتيرية .التحليل النسجي للمناطق المحفوظة من بلازميدات IncX3 المحلية ومقارنتها بتلك التي تم وصفها عالمياً من حيث جينات ال carbapenemases التي تحملها، اشارت إلى أنها لا تتطور محليًا، بل يتم جلبها من مناطق أخرى.

لقد قمنا ايضا بدراسة نوع معين من بكتيريا الكلبسية الرئوية *Klebsiella pneumoniae* ST14، والذي وجد أنه يرتبط بإنتاج ثنائي ال NDM carbapenemases وOXA-48 ، مع مقاومة شديدة للأدوية، وقد تم عزل العينات من المرضى الإماراتيين في مستشفيات دبي. وللحصول على نظرة أعمق للسمات الجزيئية لST14، تم إخضاع 39 عينة من هذا النوع تم اختيارها من خمس مدن في الإمارات، من البحرين ومن السعودية، إلى فحص تسلسلي كامل للجينوم الخاص بهذا النوع، وتم تقييم مقاومتها، virulome و MLST. كشفت تقنية ال cgMLST عن ثلاث مجموعات، تتكون الاولى من 16 عينة من خمس مدن إماراتية

(C1)، والثانية من 11 عينة من ثلاث مدن اماراتية بالإضافة الى البحرين(C2) ، اما الثالثة فتتكون من 5 عينات من السعودية(C3) ، على التوالي وسبعة عينات فردية.

لقد كان التوصيف الجيني لمقاومة المضادات وانواع البلازميدات التي تحمل جينات ال carbapenemase متغيرين في كلا المجموعتين C1 وC2، ولكن نوع ال K2 capsular و K64كانا مرتبطان بشكل واضح مع C1 وC2على التوالي .هذه المرونة الجينية في-CRKP ST14 قد تعزز من سرعة انتشار هذا النوع. تشير بياناتنا إلى أن ظهور ال CRE في دولة الإمارات هو ظاهرة معقدة تتمثل في احتمال انتقال دولي للبلازميدات، وإلى انتقال على نطاق الدولة لبكتيريا ال *Klebsiella pneumoniae*عالية الخطورة والمرنة وراثياً.

مفاهيم البحث الرئيسية: بكتريا المكورات المعوية - مقاومة ال carbapenem - نقل الجينات الأفقي - بلازميد الIncX3 - بكتيريا الكلبسية الرئوية ST14

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Dedication

To my beloved parents, brother and husband to be

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List of Abbreviations

AbR	Antibiotic Resistance
AG	Aminoglycoside
AK	Amikacin
AMEs	Aminoglycoside-Modifying Enzymes
AMR	Antimicrobial Resistance
AZT	Aztreonam
β-lactam	Beta-Lactam
bla	Beta-Lactamase Gene
Bp	Base Pair
BL+BLI	Beta-Lactam + Beta-Lactamase Inhibitor
BSI	Blood Stream Infection
CAZ	Ceftazidime
°C	Celsius
CDC	Centers For Disease Control and Prevention
CFU	Colony Forming Unit
cgMLST	Core Genome MLST
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COL	Colistin
CPE	Carbapenemase Producer *Enterobacteriaceae*

CPS	Capsular Polysaccharide Synthesis
CRE	Carbapenem Resistant *Enterobacteriaceae*
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
CRKP-ST14	Carbapenem Resistant *Klebsiella pneumoniae* Sequence Type 14
CTX	Cefotaxime
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ERT	Ertapenem
ESBLs	Extended-Spectrum Beta-Lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOS	Fosfomycin
GES	Guiana Extended Spectrum B-Lactamases
GN	Gentamicin
GNB	Gram-Negative Bacteria
HAIs	Hospital Acquired Infections
HiR	High-Risk
HvKP	Hypervirulent *Klebsiella pneumoniae*
ICEs	Integrative and Conjugative Elements
IMP	Imipenem

Inc	Incompatibility Type
IR	Inverted-Repeat Sequences
IRT β-lactamases	Inhibitor-Resistant TEM-Beta-Lactamase
IS	Insertion Sequences
K. pneumoniae	*Klebsiella pneumoniae*
Kb	Kilobase Pair
KPC	*Klebsiella pneumoniae* Carbapenemase
MCR	Mobile Colistin Resistance
MDR	Multidrug Resistant
MDR-KP	Multidrug Resistant *Klebsiella pneumoniae*
MDROs	Multidrug Resistant Organisms
MEM	Meropenem
MGEs	Mobile Genetic Elements
MHT	Modified Hodge test
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MPF	Mating Pair Formation
MRSA	Methicillin Resistance *Staphylococcus aureus*
MST	Minimum Spanning Tree
MBLs	Metallo-B-Lactamases
NDM	New Delhi Metallo- B-Lactamase
NMCA	Non–Metallo-Carbapenemase-A
NSSTI	Necrotizing Skin and Soft Tissue Infections

OM	Outer Membrane
Ori	Origin of Replication
Orit	Origin of Transfer
P. aeruginosa	*Pseudomonas aeruginosa*
pAmpC	Plasmid Mediated Ampc Genes
PBPs	Penicillin Binding Proteins
PBRT	PCR- Based Replicon Typing
PCR	Polymerase Chain Reaction
PDR	Pan-Drug Resistant
PFGE	Pulsed Field Gel Electrophoresis
PMQR	Plasmid Mediated Quinolone Resistance
pMLST	Plasmid Multilocus Sequence Typing
RMTASE	Rna Methyltrasnferases
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulfate
SFC	*Serratia fonticola* Carbapenemase
SME	*Serratia marcescens* Enzyme
ST	Sequence type
SXT	Trimethoprim-Sulphamethoxazole
T4SS	Type 4 Secretion System
TBE	Tris/Borate/Edta
TC	Transconjugant
TE	Tris/EDTA

TET	Tetracycline
TF	Transformant
TGC	Tigecycline
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UAE	United Arab Emirates
UAEU	United Arab Emirates University
USA	United States of America
UTI	Urinary Tract Infections
UV	Ultraviolet
VIM	Verona Integron–Encoded Metallo-B-Lactamase
WGS	Whole Genome Sequence
WHO	World Health Organization
WT	Wild Type
XDR	Extremely Drug Resistant
Ybt	Yersiniabactin

Chapter 1: Introduction

1.1 The international problem of antibiotic resistance

With the discovery of the first antibiotics some hundred years ago, the danger of infectious diseases caused by bacteria diminished, or at least humanity perceived so. Unfortunately, antimicrobial agents, which used to have the ability to treat and prevent various infectious diseases are becoming less, or even completely un-effective against different micro-organisms due to the occurrence of antibiotic resistance. As a consequence of the extensive use and often misuse of antibiotics for more than 70 years, different new mechanisms of antibiotic resistance arise in a dangerously high rate, and spread globally. As a result, without effective antibiotic agents available, we face difficulty in treating common and serious infectious diseases. Furthermore, in the near future, medical procedures requiring antibiotic prophylaxis, *e.g.* major surgical procedures or cancer chemotherapy, would be significantly compromised; leading to prolonged illness, disability and high mortality rates.

The World Health Organization (WHO) has stated that antimicrobial resistance (AMR) is a "global health security threat that requires action across government sectors and society as a whole" [1]. The Centers for Disease Control and Prevention (CDC) has valued the excess direct healthcare costs associated with AMR to be as high as $20 billion, and extra costs to society for lost productivity as high as $35 billion a year in the United States alone [2].

The resistance to antibiotics is a complex multifactorial process [3]. From the bacterial side it reflects evolution in action, due to continuous exposure to antibiotics, where selective pressure gives rise to the emergence of numerous resistance mechanisms [4]. This persistent evolution over the years has led to the emergence of multidrug resistant (MDR) and extremely drug resistant (XDR) strains. Historically, multidrug resistant organisms (MDROs) have affected patients in hospital settings, where exposure to antibiotics, frequent and/or long-term hospitalization, use of in-dwelling devices, and host factors were the risks for acquisition [5, 6]. Over the last two decades, the difference between MDR healthcare-acquired and community-onset bacterial infections has become blurred, with a surge of antibiotic resistance genes located on mobile genetic elements (MGE), which spread efficiently between bacteria within hosts in and out of hospitals [7]. This trend was observed in *Enterobacteriaceae*, a family of Gram-negative bacteria (GNB), which are especially important, as they are common cause of community-associated as well as healthcare-associated infections [8]. Carbapenem resistant *Enterobacteriaceae* (CRE) are listed among the most urgent antibiotic resistance threats, both by the WHO and by the CDC [1, 2].

1.2 The Problem of CRE in the United Arab Emirates (UAE)

As the only available antibiotic resistance surveillance data from the country (covering Abu Dhabi emirate only) revealed the UAE has seen the emergence of CRE in the past 8 years, especially in resistance to carbapenems in *Klebsiella pneumoniae* (*K. pneumoniae*), as percent of resistant isolates increased from 0.5% in 2010 to 4.8% in 2016

(https://www.haad.ae/HAAD/LinkClick.aspx?fileticket=8CUkors50vA%3d&tabid=1177). The situation is probably even worse in other parts of the country, as for 2017 the CDDEP resistance map projects the level of carbapenem resistance in *K.pneumoniae* in the UAE between 10-16% (https://resistancemap.cddep.org/CountryPage.php?countryId=95&country=United+Arab+Emirates). Therefore, to potentially decrease transmission by helping infection control decisions, we investigated the modes of CRE spreading in the UAE, to explain whether this emergence is due to horizontal gene transfer or clonal spread. We studied the genetic background of carbapenemase genes, the plasmids carrying them, and the molecular features of a commonly encountered carbapenem resistant clone of *K. pneumoniae* by whole genome sequencing (WGS).

1.3 *Enterobacteriaceae*

The family of *Enterobacteriaceae* belongs to the order of *Enterobacteriales*, which is a large diverse group of rod shape Gram negative, facultative anaerobic bacteria [9]. *Enterobacteriaceae* encloses several genera such as *Escherichia* [10], *Citrobacter* [11], *Enterobacter* [12], *Klebsiella* [13], *Salmonella* [14], *Shigella* [15]. All genera in this group are catalase-positive and oxidase-negative. *Enterobacteriaceae* are ubiquitous organisms, these organisms are inhabitants of the intestinal flora of most animals, including man, and can be found also in water, soil and plants [16, 17].

1.3.1 *Klebsiella pneumoniae* as a human pathogen

Klebsiella pneumoniae is a significant member of the *Klebsiella* genus that belongs to the *Enterobacteriaceae* family. It is described as a Gram-negative,

lactose-fermenting, rod-shaped bacillus with a prominent capsule. *K. pneumoniae* has a ubiquitous distribution among animals, humans, plants, soil and both salt and fresh water environments [18]. *K. pneumoniae* is a natural inhabitant of the normal microbiota of the animal gut, whereas rate of colonization in healthy human has been reported to be around 6 % [19, 20], while the rate was higher in cows reaching ~44% [21]; *K. pneumoniae* has also been recovered and cultured from other domestic and agriculture animals, earthworms, insects and even fish and shellfish [22].

After *Escherichia coli* (*E. coli*), *K. pneumoniae* is the most reported Gram negative pathogen causing a wide range of human infections [23], it has been considered as an opportunistic pathogen, since it affects patients with compromised immune system or those who are debilitated by other infections [18]; these opportunistic *K. pneumoniae* are the causative agents of multiple infections in humans, including pneumonia, urinary tract infections (UTI), surgical wound infections and even more severe, life-threatening infections such as bloodstream infection and endocarditis [24, 25].

Klebsiella species have been reported as one of the leading cause of nosocomial infections, or hospital acquired infections (HAIs) [25, 26]. These infections caused by *K. pneumoniae* tend to be critical for two main reasons: first, *K. pneumoniae* biofilms created in vivo guard the pathogen from attacks of the host immune responses and antibiotics [27, 28]; second, nosocomial isolates of *K. pneumoniae* are often multidrug-resistant, thus it is challenging to find suitable antibiotics for treatment [29, 30].

Moreover, *K. pneumoniae* is reported as the second leading cause of bloodstream infection (BSI) right after *E. coli* [25]. BSI occurs often as a secondary infection due to dissemination from a known source into the bloodstream, *e.g.* from the gastrointestinal tract, respiratory sites, the urinary tract or from an intravenous or urinary catheter [31].

Currently, two forms of *K. pneumoniae* strains are evolving: The classical *K. pneumoniae*, which is often multidrug resistant (MDR-KP) or even carbapenem-resistant (CRKP) causing opportunistic, frequently HAIs; and the other form is the hypervirulent *K. pneumoniae* (hvKP). The hvKP is highly invasive and usually affects previously healthy people causing life-threatening invasive infections often acquired in the community, *e.g.* meningitis, severe pneumonia, endophthalmitis, necrotizing skin and soft tissue infections (NSSTI) and pyogenic liver abscess [32, 33]. HvKP infections are now being extensively reported around the Globe [34-39]**.** CRKP strains and hvKP types rarely overlap; CRKP is mostly of low virulence and not frequently linked to invasive infections [40]. However, few cases have been reported where CRKP caused NSSTI in liver transplant patients [41]. Recently, the emergence of hypervirulent CRKP (hv-CRKP) was reported mostly from Asia [42-44], and lately, a hv-CRKP causing NSSTI was reported from USA as well [45].

Numerous factors ameliorate *K. pneumoniae* virulence, such as its ability to produce siderophores (scavenge essential iron), lipopolysaccharide (provide serum resistance) and extracellular polysaccharide capsule (for evasion and inhibition of phagocytosis) [18]; The striking ability of hvKP strains to cause severe infections is highly associated with presence of unique virulence factors; particularly with the

overproduction of capsular polysaccharide [46]. Genes encoding the synthesis of capsules in *K. pneumoniae* are located on the capsular polysaccharide synthesis locus (*cps*). Expression of the *cps* genes and hypermucoviscous phenotype has been reported to be associated and regulated by various proteins among which are the RmpA and RmpA2 proteins; those proteins are reported to be highly associated with HvKP especially in Asia [47].

The biochemical characteristics and complexity of the capsule leads to the creation of strain specific antigenic types of the capsular material. So far, 78 capsular serotypes (K antigen) have been recognized in *K. pneumoniae*, those antigens have been used to differentiate between strains during clinical infections [48-50]. However, only a small subset of hvKP serotypes (mostly K1 and K2) have a distinctive hypermucoviscous phenotype, due to the increased production of capsular polysaccharide, which is considered to be one of the most important virulence factor of *K. pneumoniae* [32, 33, 51].

Siderophores, another important virulence factor secreted by wide variety of bacteria including *K.pneumoniae*, are high affinity iron chelating molecules; they aid in iron acquisition since iron is vital for bacterial replication and growth, and plays a crucial role in the establishment of infection [52]. *K. pneumoniae* may encode a number of siderophores, such as aerobactin, yersiniabactin, enterobactin and salmochelin; expression and influence of each siderophore to virulence differ, as well as, their affinity to iron, with aerobactin having the lowest affinity while enterobactin having the highest [46]. Yersiniabactin (Ybt), which was first detected in *Yersinia* species, is an important virulence factor that is mostly associated with hvKP clinical

isolates, playing a crucial role in pathogenesis [53]. ICE*kp* which is an integrative conjugative element, is responsible for the mobilization of *ybt* locus in *K.pneumoniae*, it encodes *g*enes essential for the regulation, biosynthesis and transport of yersinibactin siderophore and its receptors [54, 55].

1.4 Mechanism of action of antibiotics

The mechanisms of action by which antimicrobial agents inhibit the growth or cause bacterial death are diverse, and depend on the targets affected; for example, antibiotics can interfere with microbial metabolism either by affecting the plasma membrane integrity or by inhibiting cell wall synthesis, nucleic acid synthesis, protein synthesis or folate synthesis.

Anti-Gram-negative antimicrobial agents belong to several classes of antibiotics: beta-lactam (β-lactam) antibiotics, including penicillin and derivatives, cephalosporins, monobactams and carbapenems, which inhibit peptidoglycan synthesis. Polymyxin B and polymyxin E (colistin) cause cell leakage by disrupting the bacterial outer membrane. Quinolones inhibit DNA replication by binding to bacterial complex of DNA-DNA topoisomerase II and IV. Rifampin, on the other hand, bind to DNA directed RNA polymerase thus blocks RNA synthesis. Some other antibiotics such as aminoglycosides, chloramphenicol and tetracycline all interfere with ribosome function and block protein synthesis. Sulfonamides and trimethoprim inhibit the synthesis of the folate needed for DNA synthesis [56].

1.5 Mechanisms of acquired bacterial resistance to antibiotics

Bacterial resistance can be either natural or acquired. Acquired resistance may develop by mutations, which lead to modification of the target molecule of the antibiotic, thus reduces the antibiotic affinity to its target. Mutations in permeability channel proteins or their expression lead to reduction in the porin production. Mutations can also increase expression of efflux pump systems that also leads to decreased concentration of antibiotics at the target site. Furthermore, mutations can also change the substrate spectrum of degrading enzymes that render the antibiotic less active or inactive [57].

Generally, bacteria overcome the inhibitory action of antibiotics by three primary mechanisms that frequently operate simultaneously with each other. These mechanisms are: decrease in the uptake of the drug [58, 59], inactivation of the drug [60] and target modification [61]. Bacterial resistance develops either by spontaneous mutations in existing genes, or by the acquisition of genes encoding new degrading enzymes, or a molecule protecting the target, or a new efflux pump [56]. The survival of resistant mutants is a matter of cost of fitness in a given environment [62, 63].

1.6 Antimicrobial resistance mechanisms of bacteria

1.6.1 Decreased uptake of antibiotics

Antibiotics should penetrate the outer membrane (OM) of bacteria in order to reach their targets and exert their effect. The OM of Gram-negative bacteria is comprised of a lipopolysaccharide-phospholipid bilayer and of porins [64] . Bacteria overcome the accumulation of antibiotics on their targets either by decreasing the

absorption of these molecules or increasing their discharge, or by using both mechanisms together.

In theory, hydrophilic antibiotics, such as beta-lactams, pass through porins, while hydrophobic antibiotics, as quinolones and macrolides, pass through the lipid bilayer [64, 65].

The OM porins of bacteria may be modified via the substitution of only one or two amino acids to act as a permeability barrier for antibiotics. This, in addition to upregulation of efflux molecules may work together significantly increasing the discharge of antibiotics, thus avoiding accumulation on target [59]. During the 1970s, detection of efflux-mediated resistance against tetracycline was first discovered among *E. coli* isolates [66, 67]. Since then various structures functioning as efflux pumps have been reported. Efflux pumps are recognized as one of the main mechanisms of MDR in bacteria, mostly among Gram-negative bacteria [68, 69]. The substrate specificity of efflux pumps widely differs, and some of them were found to have an extraordinarily broad spectrum [68].

1.6.2 Target modification and protection

In order for the bacteria to avoid the harmful effect of antibiotics, it modifies or even replaces target molecules [70]. In the 1960s, methicillin resistance among *Staphylococcus aureus* (MRSA) was first observed, which emerged through the replacement of the target molecule [71]. β-lactam antibiotics act on the cell wall synthesis, and inactivate penicillin binding proteins (PBPs) initiating dysregulation of the peptidoglycan synthesis, and activating a series of events that eventually lead to bacterial death [72]. MRSA produces PBP-2a, a homolog enzyme but with a

reduced affinity to beta-lactam antibiotics. PBP-2a is fully active, and able to restore the vital functions of inactivated PBPs [73].

Plasmid-mediated quinolone resistance (PMQR) is a prominent example of target protection. Several unique oligopeptides encoded on genes known as “qnr”, are capable of protecting gyrase from the inhibition by quinolones [74]. These proteins primarily spread on multi-resistance plasmids, mostly along with extended-spectrum beta-lactamases (ESBLs) or carbapenemases [75].

Another form of modification, which cause an extreme level of resistance to aminoglycoside (AG), is achieved by the modification of AG binding site enzymatically (methylation of bacterial 16S rRNA) by 16S ribosomal RNA methyltransferases (RMTase) [76, 77]. So far, 10 types of 16S rRNA methylases have been reported from clinical isolates, these are ArmA, RmtA, RmtB, RmtC, RmtD, RmtE, RmtF, RmtG, RmtH and NpmA [78-80]. The first reported 16S rRNA methylase gene *armA* was located on a plasmid from a clinical *K. pneumoniae* strain in France [81]. Sequencing of the genetic surrounding of *armA* showed that this gene is mostly located on a transposon giving it a great advantage in dissemination [81, 82].

Polymyxin resistance is acquired by altering the lipid A component of the OM via spontaneous mutations [83]. Lately, plasmid mediated colistin resistance due to lipid A modifying enzymes (mcr-1 to -8) has been reported to insidiously disseminate among the members of *Enterobacteriaceae* [84, 85].

Resistance to sulfonamides and trimethoprim in Gram negatives is mainly due to plasmid mediated *sul* and *dfr* genes, which are encoding nonallelic and drug-resistant variants of the chromosomal target enzymes dihydropteroate synthase and dihydrofolate reductase. Therefore, acquisition of these renders bacteria resistant to folate pathway inhibitors [86].

1.6.3 Enzymatic inactivation of antibiotics

Aminoglycosides resistance emerged between numerous species of bacteria, predominantly among Gram-negatives, Gram-positives, and *Mycobacterium*, due to the dissemination of aminoglycoside-modifying enzymes (AMEs) carried on mobile genetic elements [87]. AMEs resemble the rRNA targets of aminoglycosides, thus have the ability to bind to aminoglycosides instead of the target molecule and inactivate them [88]. Beta-lactamase (β-lactamase) mediated resistance is another form of bacterial resistance by enzymatic inactivation of antibiotics.

1.6.3.1 Beta-lactam resistance genes

Beta-lactam antibiotics are natural products of some microorganisms. Thus, in nature, even before the human history, microorganisms produced beta-lactamases and survived in the presence of antibiotic producers [89].

Since 1940s, β-lactam antibiotics were used and prescribed in human medicine, and they are considered as a major, probably the most important class of antibacterial agents. Subsequently, after their introduction, various β-lactamases have been produced by enteric pathogens, including *K. pneumoniae*, reaching an alarming number (>2000) and diversity [90].

Two main schemes are employed to categorize β-lactamases; the first scheme is based on molecular discrimination (proposed by Ambler) depending on amino acid sequences and functional differences. The other scheme suggested by Bush-Jacoby-Medeiros divides enzymes according to their substrate and inhibitor specificities [91]. The Ambler scheme is more commonly used, with each β-lactamase class grouping proteins according to apparent features, such as conserved amino acid motifs or molecular weight [92].

Due to the extremely large number and variety of beta-lactamases, in the following paragraphs mainly beta-lactamases produced by *K. pneumoniae*, *i.e.* the subject of my thesis, will be discussed.

1.6.3.1.1 Broad-spectrum and extended-spectrum β-lactamases

The first described β-lactamase gene, $bla_{\text{TEM-1}}$ (named after Temoniera) was discovered in *E. coli* in 1963 in Greece [93]. This enzyme had a narrow hydrolytic profile compared to the extended-spectrum β-lactamase (ESBL) enzymes discussed below, although $bla_{\text{TEM-1}}$, similarly to the majority of ESBLs, belong to the Ambler class A of beta-lactamases.

Twenty years later, in 1985, the first extended spectrum β-lactamase (ESBL) gene, $bla_{\text{SHV-2}}$, was identified in a clinical isolate of *K. pneumoniae* in Germany [94]. This β-lactamase gene showed an extended-spectrum activity against penicillin derivatives, as well as against third-generation cephalosporins and monobactams. Soon after, $bla_{\text{TEM-3}}$, a new plasmid-mediated ESBL variant was described from this pathogen in France [95].

Since the occurrence of ESBL in *K. pneumoniae*, this pathogen has become the major ESBL-carrying pathogen related to nosocomial outbreaks [96, 97]. Moreover, occurrences of *K. pneumoniae* strains in different countries harboring various alleles of TEM and SHV β-lactamases were reported throughout 1990-2000s [98, 99].

Later in the 2000s, identification of plasmids and transposons encoding $bla_{\text{CTX-M-type}}$ ESBLs lead to a shift in the type of ESBLs present in *K. pneumoniae* associated with hospital outbreaks, and since then we experience the dominance of CTX-M producing strains [100].

Combination therapy with beta-lactams and classical β-lactamase inhibitors (e.g. clavulanic acid, sulbactam and tazobactam) (BL+BLI) is a successful strategy to overcome non-ESBL TEM-type mediated resistance. However, it was reported that the susceptibility of some *Enterobacteriacea* isolates to β-lactamase inhibitors can be altered by the modification of the outer membrane proteins or by the hyperproduction of unmodified TEM-type β-lactamase [101, 102], or by both mechanisms [103]. Resistance to BLI may also be owed to production of OXA-type enzymes, or to high level production of a cephalosporinase [104].

Since 1990, the effect of β-lactamase inhibitors has been compromised by the occurrence of mutant TEM-type β-lactamases [105, 106], termed as inhibitor-resistant TEM or IRT β-lactamases, these are enzymes that are only partially inhibited by clavulanic acid and tazobactam [90].

The prevalence of ESBL producing *K. pneumoniae* is increasing worldwide, being epidemic in many countries [100], although the exact figures and ESBL gene content differ between different geographical regions. According to the WHO, the prevalence of ESBL-producing *K. pneumoniae* has reached now endemic rates of up to 50% in many parts of the world, and up to 30% in the community indicating the expansive nature of this resistance [1].

1.6.3.1.2 Plasmid-mediated AmpC genes (pAmpC)

During the late 1980s and early 1990s, bla_{AmpC} gene families have emerged. They are also serine beta-lactamases, but members of the Ambler class C. The most abundant bla_{AmpC} gene families in *K. pneumoniae* belong to the CMY, DHA, FOX and MOX types. Plasmid-encoded bla_{ACT}, bla_{MIR}, bla_{ACC} and bla_{LAT} appear to be very rare AmpC genes in this species [107].

In parallel with the explosion of ESBL genes, emergence and spread of plasmid-mediated AmpC-like cephalosporinases in *K. pneumoniae* was reported, [107, 108] and they were entirely plasmid-born in this species [107]. Nevertheless, as a manifest to the species evolutionary propensity to integrate resistant genes into its chromosome, the first chromosomal AmpC, $bla_{\text{CMY-2}}$ was identified in *K. pneumoniae* in 2015 [109]. Acquisition of AmpC enzymes combined with porin loss or increased efflux resulted in enhanced resistance to β-lactams in *K. pneumoniae*. These genes can be overexpressed on plasmids due to high copy number, or increased promoter strength of plasmid genes, and can even initiate carbapenem resistance [107].

1.6.3.1.3 Carbapenem resistance

Carbapenems are antimicrobial agents which are extremely stable to degradation by ESBLs and pAmpC, thus used to treat severe infections caused by ESBLs and pAmpC producers [110]. However, the MDR phenotype characteristics of these ESBL-producing *K. pneumoniae* strains have led to a remarkable increase in carbapenem use, which was the last resort to treat ESBL producing *K. pneumoniae* [111]. As a result of such extensive use of carbapenems, emergence of plasmid-mediated carbapenemases occurred with the ability to hydrolyze all β-lactams including the last-line carbapenems [112].

In 1988, the first plasmid-coded carbapenemase, IMI-1, was described in a Japanese *Pseudomonas aeruginosa* (*P. aeruginosa*) isolate [113]. The first carbapenemase produced by *Enterobacteriaceae*, NmcA was identified in a clinical isolate of *Enterobacter cloacae* in 1993 [114].

Their appearance in *Enterobacteriaceae* led to carbapenem resistant *Enterobacteriaceae* (CRE). *Klebsiella* turned out to be the major CRE to have spread worldwide, probably due to its hospital association [115], posing a frequent therapeutic challenge and a major public health threat [116].

CRKP strains are now relatively common and because patients infected with these strains frequently receive inadequate empiric and definitive antibiotic therapy, they experience mortality rates of 23-75% [117, 118].

A. Mechanisms of resistance to carbapenems in *Enterobacteriaceae*

Carbapenem resistance was first noted in *Enterobacteriaceae* due to overproduction of AmpC β-lactamases or ESBLs associated with permeability alterations due to porin loss and/or over expression of efflux pumps [119, 120].

The carbapenemase genes in *Enterobacteriaceae* have been shown to be associated with mobile genetic elements such as plasmids or transposons, thereby facilitating their dissemination into the community and the environment [112]. While these plasmid-encoded carbapenemases have been increasingly reported worldwide, their prevalence varies geographically [121, 122].

Among the four classes of β-lactamases defined by the Ambler classification system, the carbapenemases belong to three [112, 123]: the Ambler class A (serine carbapenemases), class B (metallo-β-lactamases), and class D, *i.e.* OXA-type β-lactamases [112]. Furthermore, rare chromosomally encoded cephalosporinases (Ambler class C) produced by *Enterobacteriaceae* may possess minor extended activity toward carbapenems [112, 124].

I. Class A carbapenemases

Class A β-lactamases includes: *Klebsiella pneumoniae* carbapenemase (KPC), Non–metallo-carbapenemase-A (NmcA), imipenemase (IMI), *Serratia marcescens* enzyme (SME), *Serratia fonticola* carbapenemase (SFC) and Guiana extended spectrum β-lactamases (GES); these enzymes are inhibited *in vitro* by clavulanic acid or boronic acid [112].

Carbapenemases of the KPC family have the most extensive global

distribution of all carbapenemases associated with *Enterobacteriaceae*. In 1996, the first KPC- producing *K. pneumoniae* was isolated from a patient in North Carolina in the United States of America (USA) [125]. bla_{KPC} genes are generally encoded on plasmid and has been extensively described in *K. pneumoniae* [30]*;* these enzymes provide resistance to penicillins, cephamycins, cephalosporins, carbapenems and monobactams and can be weakly inhibited by classical β-lactamase inhibitors such as tazobactam or clavulanic acid [112]. Moreover, strains harboring bla_{KPC} often have acquired resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole, creating MDROs [126]. As a consequence, increased mortality rates were shown for infections caused by KPC-producing *K. pneumoniae* [30]. Although, more than 22 different variants of KPC have been reported [127], KPC-2 and KPC-3 are the most commonly identified variants [128], and these latter are the highly prevalent ones in strains involved in hospital outbreaks in many countries [129].

The main force for international dissemination of these genes was, and still is clonal expansion of *K. pneumoniae* of sequence type 258 (ST258), which, since its first description has become endemic in many countries around the world [30, 130-132]. Further to clonal spread, bla_{KPC} genes mostly reside in distinctive Tn*4401* transposon variants [133-135], and can be inserted into plasmids of various replicon types, which eases the dissemination of the gene to other bacterial species [136-138]. In addition to the *K. pneumoniae* ST258, several sequence types harboring bla_{KPC} on a variety of plasmids have been found worldwide [116, 139, 140].

As stated earlier, the first KPC-producing *K. pneumoniae* was reported from USA in 1996 [125]. By 2001, there was a tremendous escalation of KPC-producing bacteria in hospitalized patients in the North-Eastern United States [30, 141]. The increasing prevalence and extensive geographical spread of KPC-producing bacteria across the USA is obvious: as of April 2016, the CDC stated that KPC producing bacteria have been recognized in 48 member states. Nevertheless, the endemicity of KPC producing bacteria is still focused in regional hot spots within the USA [142, 143]. Outbreaks of KPC-2-producing *K. pneumoniae* were caused by several *K. pneumoniae* clones including ST37, ST392, ST395 and ST11, suggesting the horizontal transfer of $bla_{\text{KPC-2}}$ gene between different *K. pneumoniae* clones [144].

Apart from North America, KPC-producing CRE are also prevalent in South and Central America, where KPC-producing bacteria spread throughout Colombia after the discovery of *K. pneumoniae* harboring $bla_{\text{KPC-2}}$ in patients with no travel history [145-147]. In Colombia, an outbreak of *K. pneumoniae* carrying $bla_{\text{KPC-3}}$ was also recorded, which when traced back, was linked to a patient who had a prior travel to Israel [147, 148].

In Latin America, outside of Colombia, the highest occurrence of bla_{KPC} carrying bacteria is in Brazil, with reports of KPC-producing isolates spreading in all states [149]. The dissemination in Brazil has been linked mostly to clonal complex 258 (CC258) *K. pneumoniae*, containing ST258, ST11 and ST437; these strains bearing a $bla_{\text{KPC-2}}$ gene located in *Tn4401b* transposon and in multiple plasmids types (IncFII, IncL/M, and IncN) [130, 150, 151].

In Europe, the highest occurrence of KPC-producing *Enterobacteriaceae* is in the Mediterranean countries, specifically in Greece and Italy [152]. These were the only two European countries reported to have an "endemic situation" for KPC in 2014–2015 [152]. Although other carbapenemases are present in Greece and Italy, KPC remains the most common reason of carbapenem resistance [152].

In the Middle East, a significant epidemic of KPC-producing *Enterobacteriaceae* has evolved in Israel, which was the second country (after USA) to report outbreaks of infection due to KPC-producing *K. pneumoniae*. During 2004–2006 a study of CRKP revealed epidemic $bla_{\text{KPC-2}}$ or $bla_{\text{KPC-3}}$ carrying strains. However, the peak of the outbreak was in 2007 [153, 154]. Although other carbapenemase genes were also reported in 2008 (including bla_{NDM} and $bla_{\text{OXA-48-like}}$), KPC producing *K. pneumoniae* remained the predominant CRE, with an increasing proportion of ST258 *K. pneumoniae* strains [155]. In the Arabian Peninsula, the occurrence of KPC-producing *K. pneumoniae* is low compared to other carbapenemases [156]. The first documented KPC-producer *K. pneumoniae* were two *K. pneumoniae* ST14 strains harboring $bla_{\text{KPC-2}}$ in the United Arab Emirates (UAE) in 2015 [157].

II. Class B Metallo-β-Lactamases

Metallo-β-lactamases (MβLs, Ambler class B) are zinc dependent enzymes. These metal ions attract water molecules that render them nucleophiles, thus hydrolyze the cyclic amide bond of the β-lactam ring, rendering the β-lactam biologically inactive [158, 159]. The clinically important MBL types in *K. pneumoniae* are New Delhi metallo- β-lactamase (NDM) type [112, 160], IMP

(active-on-imipenem), and Verona integron–encoded metallo-β-lactamase (VIM) type enzymes [159, 161].

MBLs genes are a complex group of enzymes hydrolyzing all β -lactams with the exception of aztreonam [162]. Their activity is inhibited in vitro by EDTA and dipicolinic acid, but not by any commercially available β-lactamase inhibitors such as clavulanic acid [160, 163, 164]. The spread of MBLs herald the emergence of pan-resistant bacteria and considered as an imminent health-care crises worldwide [165].

In 1993, the first transmissible carbapenemase gene, *bla*$_{\text{IMP-1}}$ was identified in a clinical *Serratia marcescens* isolate in Japan; this MBL gene was found on an integron and was associated with a plasmid-mediated outbreak. Extensive spread of *bla*$_{\text{IMP-1}}$ harboring *Enterobacteriaceae* all over Japan followed this event [166].

So far, at least 52 variants of IMP genes have been identified in various species from different regions around the world; however, IMP type MBL-containing *Enterobacteriaceae* are endemic only in Taiwan and Japan [167]. Reports from Europe and other countries showed mostly sporadic outbreaks or single occurrence of IMP-type MBLs [90, 115, 139].

In 1996 and 1997, VIM-type MBLs were identified in *P. aeruginosa* from Verona, Italy (VIM-1), and France (VIM-2) [168, 169]. After its initial discovery, bacteria producing VIM enzymes have been identified globally [167]. VIM-producing *Enterobacteriaceae* are commonly reported from *K. pneumoniae, E. coli* and *Enterobacter species* [112, 117, 160]. So far, at least 46 *bla*$_{\text{VIM}}$ variants have been described; studies showed that VIM genes are often located within class 1

integrons that facilitate their inter species dissemination [129].

In Spain, Italy and Hungary, “inter-regional spread” of VIM producers has been reported [152]. Emergence of multidrug-resistant *K. pneumoniae* isolates producing VIM-4 metallo-beta-lactamase was reported in 2005 in 11 patients from Tunisia [170]. VIM-producing CRE was reported as well from Nigeria and South Africa [171]. A plasmid-mediated outbreak of VIM-4 producer *Enterobacteriaceae* was reported from Kuwait [172], where otherwise only sporadic occurrence of VIM-producer *Enterobacteriaceae* was reported earlier [173].

In 2009, MBL-producing *Enterobacteriaceae* became the center of attention, as *K. pneumoniae* ST14 with a new MBL gene, New-Delhi-metallo-beta-lactamase ($bla_{\text{NDM-1}}$) was identified from a Swedish patient who received healthcare in New Delhi, India [174]. Subsequently, there has been an international dissemination of NDM-type MBLs with rapid gene transfer between species. Twenty variants of NDM enzyme have been described so far, most of them originated in Asia [175-177].

Most of NDM producing bacteria also carry a diversity of other resistance mechanisms [167]. These extra mechanisms include the following: plasmid-mediated AmpC β-lactamases (especially CMY types), ESBLs (especially CTX-M-15), different carbapenemases (e.g., OXA-48, VIM, and KPC types), 16S rRNA methyltransferases, PMQR determinants, macrolide-modifying esterases, and rifampin-modifying enzymes. Consequently, most of *Enterobacteriaceae* with NDM-type enzymes remain susceptible to colistin, fosfomycin, and tigecycline, only [176, 178, 179].

Despite the resemblance between the high rate of spread of NDM-producing *Enterobacteriaceae* and that of KPC-producing *Enterobacteriaceae*, the dissemination of NDM-type MBLs does not appear to be related to a specific dominating clone; rather it is facilitated by dissemination of plasmids of various incompatibility (Inc) types [180, 181]. Nevertheless, NDM-type MBL genes have been reported in some epidemic clones, including *K. pneumoniae* ST147 and ST11 and *E. coli* ST101 and ST131, which are well-known to carry other β-lactamase genes and antibiotic resistance determinants [139, 182, 183].

In Europe, NDM producers have been commonly identified in Poland, Denmark, and Romania where "inter-regional spread" is thought to be present [152].

Although the MBL production as a reason of carbapenem resistance remained uncommon in *Enterobacteriaceae* isolates of the USA and Canada [184], in 2012 an outbreak of $bla_{\text{NDM-1}}$ producing *K. pneumoniae* was reported from Denver [185]. Later, in 2016, 157 NDM-producing CRE were reported from 25 states to the CDC [143].

On the other hand, several outbreaks were reported from South Africa, raising concerns that NDM-producing CRE are moving towards endemicity [186, 187]. Furthermore, NDM-producing CRE have been described from other African countries including Kenya, Cameroon, Algeria, Egypt, Morocco and Tanzania [188-193].

In the Middle East, NDM-producing *Enterobacteriaceae* are endemic, and this enzyme is one of the most frequent cause of carbapenem resistance in this family of bacteria [156, 194, 195].

III. OXA-48-like Class D carbapenemases

Class D β-lactamases (oxacillinases) are enzymes that powerfully hydrolyze narrow-spectrum β–lactams, weakly or not hydrolyze cephalosporins, and are not inhibited by classical inhibitors. The OXA-48-like subgroups of this enzyme family also hydrolyze, albeit weakly, carbapenems [167, 196, 197]. If isolates producing these enzymes also produce an ESBL enzyme and/or have a permeability defect, they may be resistant to all β-lactam antibiotics [198].

CRE-producing OXA-48-like enzymes may be hard to recognize [122], because of the heterogeneity of hydrolysis of carbapenems, broad-spectrum cephalosporins, aztreonam and absence of inhibition by EDTA or clavulanic acid, thus the prevalence of these enzymes is most likely underestimated [167, 199].

The first OXA-48 producer was isolated from *K. pneumoniae* strain, from a 54-year-old man with a urinary tract infection and skin burns from Istanbul, Turkey in 2001 [200]. Since then OXA-48-like producing *K. pneumoniae* clones have persisted as a source of nosocomial infection reaching the highest epidemiologic level in Turkey [201-204]. Nevertheless, OXA-48-like and its variants became clinically significant worldwide [121]. It has been described in all members of the *Enterobacteriaceae* family; however, it is mostly found in isolates of *E. coli* of community origin and *K. pneumoniae* commonly of nosocomial origin.

Among carbapenems, OXA-48 has a weak hydrolytic activity for both imipenem and meropenem compared to ertapenem, which is considered the best substrate for this enzyme [205].

Several variants have emerged that differs from OXA-48 by one to five amino acid substitutions and/or by a four-amino- acid deletion, which lead to a modification in the β-lactam hydrolysis spectrum [198, 200, 206]. These include OXA-162 (single replacement at Thr213Ala) described in Turkey from *K. pneumoniae* isolates [207]; OXA-181 (four substitutions at Thr104Ala, Asn110Asp, Glu168Gln, and Ser171Ala) emerged in a *K. pneumoniae* isolate from India [208]; OXA-163 (with single substitution at Ser212Asp and four deletions at Arg214, Ile215, Glu216, and Pro217) which was collected from *Enterobacter cloacae* and *K. pneumoniae* isolates in Argentina [209]; OXA-204 (two substitutions at Gln98His Thr99Arg) was reported from *K. pneumoniae* isolates isolated from patients having a connection with North Africa [210]; OXA-232 (single replacement at Arg214Ser) was first detected in France in a *K. pneumoniae* isolate recovered from a patient who had been transferred to Mauritius from India [211]; OXA-247 (two substitutions at Tyr211Ser and Asp212Asn) was identified in a *K. pneumoniae* isolate recovered in Argentina [212]; OXA-244 (single substitution at Arg214Gly) and OXA-245 (single substitution at Glu125Tyr) were found in *K. pneumoniae* isolates in Spain [213]; OXA-370 (single substitution at Gly220Glu) was reported from Brazil [214] and OXA-405 (four deletions at Thr213 to Glu216) was identified in France [206].

Some OXA-48-like variants such as OXA-48, OXA-232, OXA-181, OXA-204 and OXA-162 have been stated to have a very similar resistance pattern [209,

211]. Remarkably, OXA-163 and OXA-405 have marginal hydrolytic activity against carbapenems, but displayed capacity to hydrolyze aztreonam and ceftazidime, and they both show an increased ability to hydrolyze cefotaxime and cefepime over OXA-48, making these enzymes resemble ESBL enzymes more than carbapenemases [209, 211].

The dissemination of the OXA-48 enzyme is facilitated by the rapid spread of a conjugative broad host-range plasmid harboring the *bla*$_{\text{OXA-48}}$ gene located on a composite transposon, namely Tn*1999* [142, 198, 199, 201, 215, 216]. The *bla*$_{\text{OXA-48-like}}$ genes are commonly observed on IncL/M plasmids, which do not harbor extra resistance genes, and are 60–70 Kilobase pair (Kb) in size [217]. The IncL/M plasmids are considered a major resistance plasmid family recognized in clinically significant *Enterobacteriaceae* [140]. The high transfer ability of the epidemic IncL/M plasmid is the reason suggested for the successful dissemination of *bla*$_{\text{OXA-48}}$ in *Enterobacteriacea* [121, 218].

On the other hand, the international spread of OXA-48-type carbapenemases is attributed as well to the success of OXA-48–type-producing clones, and, to a lesser extent, to certain regions (*e.g.* the spread of OXA-181 in the Indian subcontinent) [167, 199, 215]. *bla*$_{\text{OXA-48-like}}$ genes were also reported to spread in strains belonging to different *K. pneumoniae* STs (ST101, ST11, and ST14) endorsing the ability of high-risk clones to accumulate resistance determinants [219-221].

There is an inter-regional spread of OXA-48-producing *K. pneumoniae* rendering it endemic in a number of European countries: Spain, France, Belgium and Romania [200, 203]. In addition to Europe, OXA-48-like enzymes have been

described globally in *Enterobacteriaceae*. Dissemination of OXA-48-like producing CRE in the Middle East was reported from Lebanon, Saudi Arabia, the United Arab Emirates and Israel [155, 222, 223]. In Africa, it was reported from Egypt, Algeria, Libya and South Africa [224-227]. OXA-48-like producer *Enterobacteriaceae* were also encountered in several Asian countries: India, China, Taiwan, Thailand and Russia [228-232]. OXA-163 is especially prevalent in South America [233, 234].

1.7 The role of *Klebsiella pneumoniae* clones in spreading carbapenem resistance

As outlined earlier, the spread of antibacterial resistance has two main routes: dissemination of resistant bacterial clones and horizontal gene transfer. In the following section, the role of high-risk (HiR) clones of *Klebsiella pneumoniae* will be discussed, as they play a major role in the global dissemination of CRE [235]. The significance of HiR bacterial clones in the setting of worldwide spread of antibiotic resistance was first documented by Woodford *et al.* [235]. A 'high-risk clone' was defined as group of isolates that share the same ST established by multilocus sequence typing (MLST), even if isolates have been collected from distinct geographic locations and time. HiR clone have a high ability to spread, colonize, and persist in a range of ecological niches [236]. The HiR clones have attained definite adaptive traits that increase their pathogenicity and existence, apart from the acquisition of antibiotic resistance determinants. Such clones have contributed to the global spread of antibiotic resistance, and they are also considered as a source of the propagation of genetic components of antimicrobial resistance such as transposons, genes, integrons and plasmids, especially among Gram-negative bacteria [235].

Furthermore, it was reported that some definite *K. pneumoniae* STs are considered to be outbreak-causing infectious agents more than others [235, 237]. The frequency of outbreaks caused by a bacterial clone signifies its epidemic potential. Therefore, these are the clones that should be recognized, examined and controlled. The definition of HiR clone has been laid out by Navon-Venezia *et al.*: clones that caused at least four known outbreaks and were reported from ≥10 countries [238]. Accordingly, nine HiR worldwide disseminating MDR and XDR *K. pneumoniae* clones were recognized : CC258 (ST258, ST11, ST512), CC15 (ST15, ST14), ST147, ST37, ST101 and ST17, all of which were known to be carbapenem resistant *K. pneumoniae* from earlier studies [238]. All nine sequence types demonstrated international spread in three to five continents. The most significant of them causing 68% of all outbreaks, was CC258 which consists of three STs: ST258, ST11 and ST512. The second most prevalent CC, responsible for about 20% of all outbreaks, was CC15, consisting of ST14 and ST15. Other STs were epidemic but to a lower degree [238].

1.7.1 Dissemination of *Klebsiella pneumoniae* ST258 clone

The emergence and worldwide dissemination of *K. pneumoniae* ST258, and reports showing that this clone has propagated as a result of recombination and replacement of the *cps* locus, raise the question of its evolutionary history. A study showed that ST11 (allelic profile 3-3-1-1-1-1-4), a single-locus variant of ST258 (allelic profile 3-3-1-1-1-1-79), and an extremely predominant multidrug-resistant clone in South America and Asia [151, 239, 240], gave rise to the ST258 clone through the acquisition of the tonB79 allele [241]. Another investigation

hypothesized that *K. pneumoniae* ST258 is a hybrid clone that was produced by a large recombination event between *K. pneumoniae* ST11 and *K. pneumoniae* ST442 [116].

Numerous reports highlighted a number of superior features of CC258 that might be linked to its successful prevalence worldwide. Genomic comparison studies revealed the existence of 50 unique genes present in ST258 clone and absent in a non-ST258 sporadic clone (ST376), those genes were linked to cell motility, secretion, DNA modification and repair [237].

A different study described an integrative conjugative element, ICEKp258.1, carried by *K. pneumoniae* ST258 and ST11, encoding a type IV secretion system, that probably assists the propagation of mobile elements [242]. Another element, ICEKp258.2, present in *K. pneumoniae* ST258 and in its descendant *K. pneumoniae* ST512, encodes a type III restriction-modification system and a type IV pilus gene cluster, which possibly improve adherence abilities and narrow the range of compatible plasmids and other mobile elements [116]. This may explain the low diversity of carbapenemases in *K. pneumoniae* ST258 and ST512 compared to *K. pneumoniae* ST11 [238]. Moreover, *K. pneumoniae* ST258 showed low susceptibility to chlorhexidine, a disinfectant used in hospitals, thus suggesting its selective advantage in the hospital environment compared to other sporadic KPC-producing clones [243].

ST258, the most prevalent clone of this CC, is considered the main driving force of worldwide spread of KPC enzyme [30, 132, 244]. ST258 outbreaks were mainly linked to $bla_{\text{KPC-3}}$ and $bla_{\text{KPC-2}}$; ST258 clone carrying bla_{KPC} genes were

reported from Greece [245], Israel [246], Finland [247], Italy [248], Germany [249], Denmark [250] and the USA [132]. *K. pneumoniae* ST512, as well, was found to be exclusively linked to *bla*$_{KPC}$ [30, 251].

In Hungary the first isolate with a KPC enzyme was found to belong to *K. pneumoniae* ST258. It was reported to harbor *bla*$_{KPC-2}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$ and *bla*$_{SHV-11}$ [252]. Worryingly, in Hungary the same KPC-2 producing clone was reported exhibiting colistin resistance, as well [252].

In 2009, the emergence of the first isolate with KPC carbapenemase producing isolates of *K. pneumoniae* ST258 clone in Norway and Sweden was documented [253, 254]. *K. pneumoniae* ST258 with KPC-2 in Poland was also documented [255], which resembled the KPC producer isolates found in Norway and Sweden [254]. These findings strongly support the prominent role of this group in the dissemination of *bla*$_{KPC}$.

Therefore, *K. pneumoniae* ST258 is considered as the dominant carbapenemase-expressing lineage in much of North America and Europe [256].

1.7.2 Dissemination of *Klebsiella pneumoniae* ST11 clone

K. pneumoniae ST11, a single-locus variant (tonB) of the international hyperepidemic ST258, is also a high risk clone [241]. *K. pneumoniae* ST11 lineage is predominant in China, South Korea, and Hungary [239, 257-259], and has also been widely documented in New Zealand, Poland, Spain, Singapore and Brazil [130, 260-264]. This clone has been linked with different ESBLs, mainly CTX-M-15 and CTX-

M-14 [252, 257, 260, 265], and later with various carbapenemases: KPC-2 [239, 254, 266], VIM [267, 268], OXA-48 [261, 269] and NDM [270, 271].

Recently in Spain, the emergence of colistin resistant *K. pneumoniae* ST11 expressing OXA-48 and CTX-M-15 was reported [261]. Another report described the prevalence of OXA-48-producing *K. pneumoniae* ST11 clone throughout Spanish hospitals. [272] A fatal outbreak of carbapenem resistant, hypervirulent *K. pneumoniae* ST11 was reported from a Chinese hospital [273], heralding the emergence of true 'superbugs', which are not only being multidrug resistant and hypervirulent, but also extremely transmissible, initiating severe and even fatal infections [273].

The success of *K. pneumoniae* ST11 clone is not fully understood. A possible explanation is that *K. pneumoniae* ST11 clone possesses seven *cps* locus types, compared to one or two loci found in other clones, thus it was proposed that the divergence in capsular content may obscure the recognition of this clone by the immune system [274].

K. pneumoniae ST11 associated with $bla_{\text{NDM-1}}$ was identified in many countries: USA [184], Australia [275], Switzerland [276], Greece [277], the Czech Republic [278], Spain [279], Thailand [280] and the UAE [281]. The Polish outbreak of NDM-producing *K. pneumoniae* ST11 was likely introduced from the Czech Republic, as the clone in Poland was similar to an isolate found in the Czech Republic in 2013 [282], thus supporting the local dissemination of this clone. ST11 has also been associated with OXA-48-like enzymes in Argentina, Turkey and Spain

[279, 283]. Furthermore, the majority of NDM-producing *K. pneumoniae* isolates found in India belonged to either ST11 or ST147 [283].

K. pneumoniae ST11 is one of the major clones spreading KPC-2 carbapenemase in China [144, 284], and was reported to be associated with dissemination of the same carbapenemase in Singapore as well [264].

Moreover, from a multicenter survey of ten university hospitals in South Korea, *K. pneumoniae* ST11 isolates were the most prevalent having at least eight different ESBL genotypes, but none had KPC enzymes, implying that ST11 was widespread independently of KPC carbapenemase. Possibly, multiple acquisition events led to the presence of multiple variants of the clone circulating in South Korea [257].

1.7.3 Dissemination of *Klebsiella pneumoniae* CC15 (ST15 and ST14)

K. pneumoniae ST15 has been recognized as a HiR *K. pneumoniae* clone disseminating ESBLs and carbapenemase enzymes worldwide. It has recently been reported to carry CTX-M-15 from various countries including Bulgaria [285], Netherlands [286], Japan [287] and Brazil [288]. This clone was also reported with all types of carbapenemase genes: bla_{NDM}, $bla_{\text{OXA-48-like}}$, bla_{VIM}, bla_{IMP} and bla_{KPC} [238]. NDM-producing members of this clone were described from Croatia [289], Canada [290], China [291], and Thailand [280]. VIM producing *K. pneumoniae* ST15 was encountered in Spain [279] and Hungary [292]. Moreover, KPC-2 producing *K. pneumoniae* ST15 outbreak was reported from Bulgaria [293].

K. pneumoniae ST14 differs from ST15 by the *infB* allele only. Previous reports presented ST14 as a high-risk clone, due to its high ability to disseminate worldwide (illustrated in Table 1) and to host multiple β-lactam resistance determinants including ESBLs (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$), AmpC cephalosporinases (*bla*$_{\text{FOX}}$, *bla*$_{\text{CMY}}$) and carbapenemases (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-48-like}}$)[157, 233, 294, 295].

On the other hand, *K. pneumoniae* ST14 clone has been mostly described as a frequent host of CTX-M enzymes and was reported as a host lineage of NDM-1 enzyme [235, 271].

In addition, *K. pneumoniae* ST14 clone have been extensively reported with capsular serotype K2, which is one of the most reported *K.pneumoniae* serotype linked to invasive diseases [296-299]. Another study that was conducted to identify *K. pneumoniae* hypervirulent clones of capsular serotype K2 proved that the most frequent clone linked to these types is ST14 [300].

Table 1: Dissemination of carbapenemase producer *K. pneumoniae* ST14 in different countries

Carbapenemase gene(s) carried	**Year**	**Country**	**No. of ST14 isolates reported**	**Reference**
bla$_{\text{NDM-1}}$	2009	India	1	[174]
bla$_{\text{NDM-1}}$	2011	Oman	1	[301]
bla$_{\text{NDM-1}}$	2011	Kenya	7	[192]
bla$_{\text{NDM-1}}$	2012	India	10	[299]
bla$_{\text{NDM-1}}$	2012	UK	2	[299]
bla$_{\text{NDM-1}}$	2012	Sweden	1	[299]
bla$_{\text{KPC-2}}$	2012	USA	2	[147]
bla$_{\text{NDM-1}}$	2012	India	2	[283]
bla$_{\text{OXA-181}}$	2012	Finland	1	[302]
bla$_{\text{OXA-181}}$	2014	South Africa	2	[303]
bla$_{\text{OXA-48}}$	2015	Germany	6	[295]
bla$_{\text{NDM}}$ *or bla*$_{\text{OXA-48}}$	2015	Ireland	2	[304]
bla$_{\text{NDM-1}}$	2015	UAE	7	[156]
bla$_{\text{NDM-1}}$	2015	Singapore	1	[305]
bla$_{\text{KPC-2}}$	2015	UAE	2	[157]
bla$_{\text{KPC-2}}$	2015	Colombia	16 out of 193 (8.2%)	[306]
bla$_{\text{KPC-3}}$	2015	USA	1	[307]
bla$_{\text{KPC-3}}$	2017	USA	1	[45]
bla$_{\text{NDM−5}}$	2017	China	1	[308]
bla$_{\text{NDM-1}}$+*bla*$_{\text{OXA-232}}$	2017	India	3	[309]
bla$_{\text{KPC-2}}$	2017	UK	1	[294]
bla$_{\text{NDM-1}}$+ *bla*$_{\text{OXA-48}}$	2018	Pakistan	3	[310]
bla$_{\text{OXA-48}}$	2018	Catalonia	1	[219]
bla$_{\text{NDM-1}}$	2018	Czech Republic	1	[311]

1.7.4 Dissemination of other high risk *Klebsiella pneumoniae* clones (ST147, ST37, ST101 and ST17)

K. pneumoniae ST147, ST37, ST101 and ST17 clones were reported to carry KPC-, NDM-, VIM- and OXA-48-like carbapenemases worldwide [238], but before the emergence of these carbapenemases, most of them were already spreading CTX-M-type ESBL enzymes [312] highlighting their outstanding ability to disseminate antibiotic resistance determinants. *K. pneumoniae* ST147 was also reported to co-harbor more than one carbapenemase genes in various combinations [313, 314], and to develop into a pan-drug resistant bacterium [314, 315]. Furthermore, like *K. pneumoniae* ST11, carbapenemase producer strains of this clone has also been reported to carry genetic determinants of hypervirulence [316].

1.7.5 Carbapenemase producer *Klebsiella pneumoniae* clones encountered in the Arabian Peninsula

NDM and OXA-48-like carbapenemases are considered the major carbapenemases found in *Enterobacteriacea* in countries of the Arabian Peninsula [156, 194, 317-319]. This is in sharp contrast to the dominance of the KPC-type enzymes experienced in many parts of the world [320]. In this region only sporadic cases of KPC-producing *Enterobacteriaceae* have been reported [157, 321, 322]. VIM carrying strains were mostly encountered sporadically too [156, 173, 322-325], however, between 2009 and 2011 there was a temporary elevation in VIM producing clonally unrelated *Enterobacteriaceae* in Kuwait [326], which was due to a plasmid outbreak [172]. Several *K. pneumoniae* sequence types have been described from the region: ST29 was the major clone responsible of an OXA-48-producing CRKP outbreak in Saudi Arabia [327]. In the same country polyclonal occurrence of CRKP

of ST199, ST152, ST37, ST48, ST340, ST15 and ST16 were detected. In that study a significant link was observed between carbapenemase genes and the clonal types of *K. pneumoniae*: ST152 was reported as a frequent carrier of the *bla*$_{\text{NDM-1}}$ gene, while ST199 isolates were positive for *bla*$_{\text{OXA-48}}$ [328].

In a collection of CRE isolates of Saudi Arabia, UAE, Oman and Kuwait three major clones of *bla*$_{\text{NDM-1}}$ producing *K. pneumoniae* were detected: ST152 from Saudi Arabia, ST14 from the UAE and ST147 from Oman, with *bla*$_{\text{NDM-1}}$ detected on HI1b incompatibility type plasmids in both the ST14 and the ST147 isolates [156]. In this study, the *bla*$_{\text{NDM-1}}$ was the most common carbapenemase, followed by non-clonal CRE, mostly *K. pneumoniae* harboring *bla*$_{\text{OXA-48}}$ [156].

In the UAE, single cases of *K. pneumoniae* ST29, ST1425, and ST340 producing OXA-48 were detected in bloodstream infections in Abu Dhabi [222]. Furthermore, the occurrence of a pan-drug resistant *K. pneumoniae* ST147 producing OXA-181, and in certain isolates also NDM-5, was reported from multiple hospitals and geographical regions of the UAE [314].

In Oman, NDM-1 producer *K. pneumoniae* was associated with five different STs: ST147, ST15, ST11, ST101, ST372, while OXA-48 producing *K. pneumoniae* belonged to ST753, ST754 [319].

K. pneumoniae strains co-producing two carbapenemases were reported from Oman [319], Saudi Arabia [317] and UAE [314, 329]. In Oman an OXA-181 and NDM-1 co-producing ST11, in Saudi Arabia OXA-48 and NDM-1 co-producing ST152, in the UAE clonal expansion of ST147 producing OXA-181 and NDM-5,

and ST14 producing OXA-232 and NDM-1 was noted [314, 329]. Furthermore, ST307 and ST1318 co-producing NDM-1 and OXA-162 or NDM-1 and OXA-48, respectively, were encountered [329].

1.8 Spread of antibiotic resistance by horizontal gene transfer

Mobile antibiotic resistance genes are confined in platforms that include plasmids, integrative and conjugative elements (ICEs) [330], integron cassettes and a range of transposons and related elements. All these are recognized as mobile genetic elements (MGEs). MGEs can transfer by a variety of molecular mechanisms, including transduction, transformation and conjugation (Figure 1).

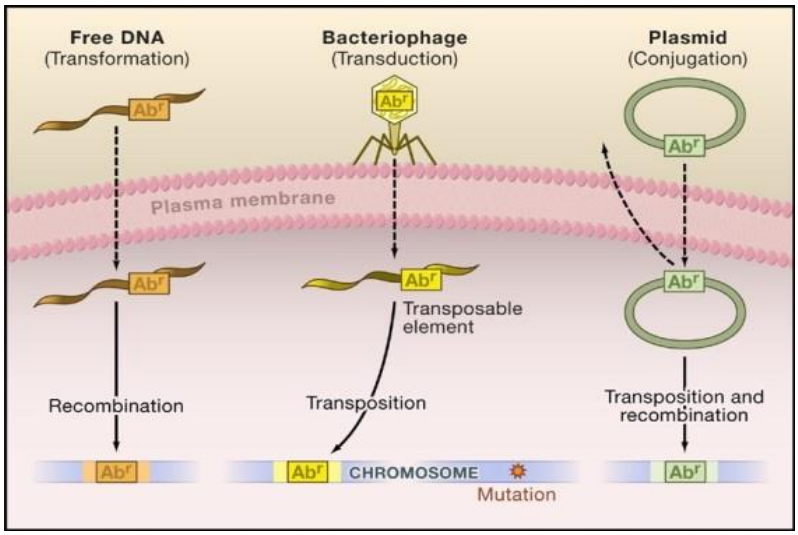

Figure 1: Acquisition of antibiotic resistance genes [331]

Transduction is mediated by a bacteriophage (bacterial virus), which facilitates the transfer of host DNA from one bacterium to another. It does not require cell to cell contact [332]. Transduction have been reported to transfer antibiotic resistance genes both intra- and interspecies. Transformation is the process where naked DNA is taken up by the cell from the extracellular environment.

Bacteria that have the ability to endure transformation is termed as “competent” [333]. Conjugation is a procedure where a conjugative element such as a plasmid or a transposon is transferred between bacteria via a contact dependent semiconservative replication process. Plasmid mediated conjugative transfer is by far the most common mechanism of horizontal gene transfer in Gram negative bacteria [334].

1.8.1 Integrative and conjugative elements

The foremost types of mobile elements currently involved in the capture and mobilization of antibiotic resistance genes found in Gram-negative bacteria are certain insertion sequences (IS) [335], gene cassettes/integrons [336] and transposons of the Tn3 [337] and Tn5053 families [338] (Figure 2). These elements have some features in common, but capture and/or move genes by different mechanisms [339].

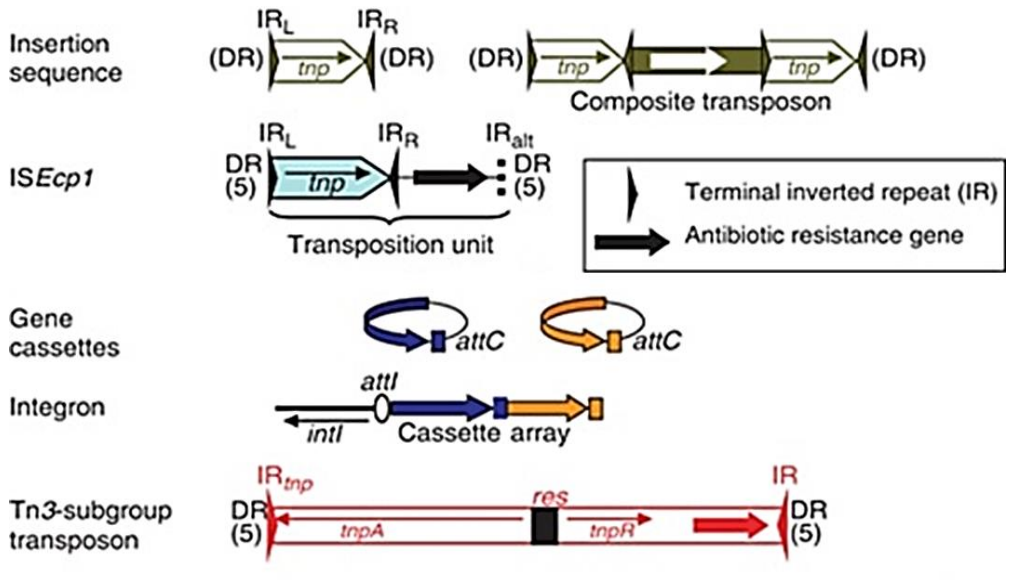

Figure 2: Characteristics of mobile elements in Gram-negative bacteria involved in the capture and mobilization of antibiotic resistance genes [339]

Insertion sequences (IS) are small transposable segments of DNA (<2.5Kb) with a simple genetic organization. Most IS elements display short terminal inverted-repeat sequences (IR) and code for a transposase, an enzyme that is essential for transposition and has recombinase activity [340]. These mobile elements play a major role in assembling of "accessory" functions in bacteria (as genes essential for catabolic pathways) and in spread of resistance genes. By inserting within a coding sequence they may render the gene inactive, or by inserting upstream of a gene they may adjust its expression. ISs may also assist the integration of plasmids into the bacterial chromosome. Some ISs are capable of mobilizing nearby DNA by a process similar to one-ended-transposition (such as IS*Ecp1*), whereas others form a composite transposon, thus being able to carry the sequence situated between the two ISs [341].

Transposons such as the large group of type II transposons of the Tn3 family and Tn7 are complex structures and contain integrons carrying multiple antibiotic resistance genes. This association of transferable elements with integrons stimulates rapid spread among clinical strains, and creates more occasions for inclusion of extra resistance determinants. A transposon is a composite form of IS, surrounded by inverted ISs on both extremes and consequently have the ability to carry more than one genetic determinant. Transposons may lead to complex DNA rearrangements and may accumulate several resistance genes to form an MDR phenotype [342].

Integron is a genetic structure which has the ability to capture and simultaneously express several antibiotic resistance genes. It is formed of an integrase gene and a promoter sequence. Gene cassette is a gene linked to a 59-base

element, which enables the gene to integrate to an integron. Integrons may carry multiple resistance determinants concurrently, and they may be located on a transposon, ensuring their mobility [343, 344].

1.8.2 Plasmids

A plasmid is an independently replicating extra-chromosomal, circular, double-stranded DNA molecule, which does not carry genes essential for the growth of host cells under non-stressed conditions [345]. However, once gained, they can become essential for bacterial survival. Plasmids have systems, which guarantee their self-directed replication (independently from the replication of the bacterial chromosome) while controlling their copy number and assuring stable inheritance during cell multiplication [346, 347]. The part of the plasmid that maintains copy number of parent plasmid with controlling the initiation of replication is termed as the basic replicon [348].

Several plasmids have accessory genes that are useful not only to themselves, but also to their host, *e.g.* genes controlling drug resistance, degradation of organic compounds, colonization of new habitats and virulence factors (including production of toxins). These types of genes are mostly located within transposons, leading to a great variation and flexibility in the composition of plasmids [217, 331].

A plasmid's success depends upon a balance of multiple factors, including horizontal transfer through the conjugation process among bacteria of different genera and kingdoms, segregation error rate (vertical transfer), post-segregation killing, fitness cost, and compensatory mutations which can occur to reduce fitness cost [349-351]. These systems ensure plasmid maintenance in the bacterial

population despite other selective pressure. Conjugation alone is considered enough to stably maintain a high fitness cost plasmid, even in the absence of any selective pressure [349, 352].

Promiscuous, broad-host-range plasmids usually have the restriction/anti-restriction mechanisms and partitioning systems, guaranteeing stable inheritance throughout cell division. A lot of plasmids encode also addiction systems based generally on toxin–antitoxin factors, which have the ability to destroy daughter cells that fail to inherit the plasmid during cell division [346]. These systems support plasmid maintenance, but do not provide any benefit to the bacterial host.

Plasmids exist in a cell in a definite number of copies, maintained and controlled by their origin of replication [353]. Introducing either a low or a high copy number plasmid into cells lacking any plasmid has revealed that the copy number is commonly fixed by controlling the initiating of replication [354, 355]. Several mechanisms to maintain the copy number of plasmids isolated from *E. coli* have been described, but all approaches use negative regulation tactics. In one set of plasmid types, the quantity of the produced initiator protein is controlled by a specific inhibitor [353]. In another mechanism, multiple binding sites compete with the origin for binding to a small pool of initiator protein. In a third strategy, an RNA molecule that is essential for initiation is prohibited from binding to the origin by a second RNA and a protein. These approaches control the initiation frequency of DNA replication.

Upon cell division, both daughter cells inherit plasmid molecules. In case of high copy number plasmids, there are enough plasmids for each daughter cell to gain

at least a few. As a result, it is not essential for plasmids with high copy number to have a strict control to segregate or partition plasmid molecules into daughter cells [353]. However, one of the issues faced by the high copy number plasmids during partitioning is the multi-number formation among plasmid copies. This will hinder the plasmid segregation, thus plasmids encoded genes are needed to maintain the stability of the plasmid. An example is the *cer* gene (present in ColE1 plasmids of high copy number), which encodes a product that converts plasmid multimers into plasmid monomers. This makes sure the independent partition of all copies of the plasmid. Although *cer* ensure the stability of ColE1 plasmids, it is not vital for maintenance of ColE1 in a population [356].

On the other hand, in case of low copy number plasmids (less than 10 copies of plasmids per chromosome) partition into daughter cells is much more critical. As there are limited number of plasmid copies, if one daughter cell receives two molecules, the other daughter may not inherit any plasmid molecule. Thus, those types of plasmids have mechanisms to ensure their right partitioning (Figure 3). An example of partitioning system is the *parAB-parS* system, where ParA and ParB proteins (produced by the plasmid) bind to the *parS* (centromere) sequence forming the parS-parA-parB complex that have the function of aligning all the of the plasmid molecules in the middle of the cell until division starts, afterward the plasmid molecules are partitioned into daughter cells by the combined action of ParA/ParB pushing the plasmids apart. As a result the low copy number plasmid is properly segregated into the daughter cells. This is a process similar to the partitioning of chromosomes during mitosis [357, 358].

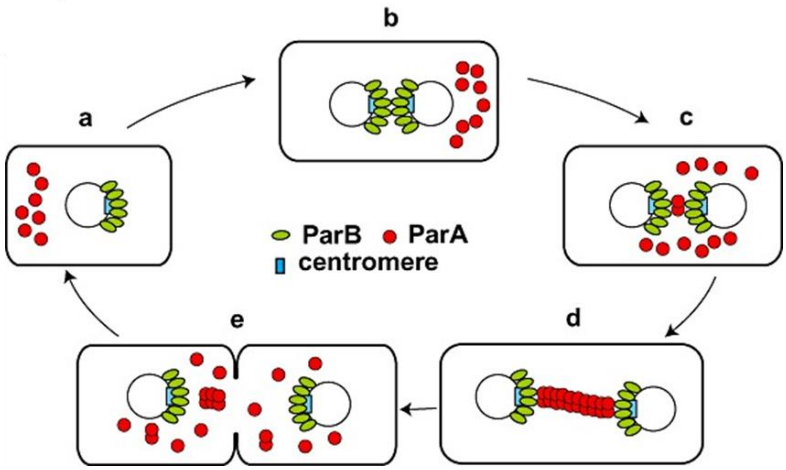

Figure 3: Partitioning of low copy number plasmids [358]

Transfer of plasmids from a donor cell to a recipient is called conjugation. Conjugation process involves two sets of plasmid genes, the mobility (MOB) genes and the mating pair formation (MPF) genes, together with an origin of transfer (*oriT*) [359]. The MOB genes code for both a relaxase and a DNA processing proteins (which are essential for the relaxosome and formation of coupling protein that links the relaxosome to the mating channel). MPF genes encode for the membrane–associated mating pair formation complex, a system of type 4 secretion system (T4SS) that offers the mating channel [359]. The early step in the conjugation process is the formation of mating pair, where the donor and recipient get cell to cell direct contact. The next step includes relaxase-mediated nicking of the plasmid at *oriT* thus creation of the relaxosome (coupling of single-stranded DNA with a protein complex). The relaxosome docks to the coupling protein, which assist to initiate the transport through the T4SS into the recipient cell, then establishment and replication of the plasmid in the recipient will follow [360].

1.8.2.1 Characteristics of the large resistance plasmids

Plasmids conferring multi-drug resistance are usually large (>50 Kb), self-conjugative and encode special mechanisms regulating their copy number and controlling the rate of replication [347]. The minimal portion of the plasmid that replicates with the characteristic copy number of the parent plasmid is called basic replicon [361]. Replicons comprise of the origin of replication (*ori*) and encode definite replication initiator proteins (Rep) that bind to the *ori* and to their regulating factors [361, 362]. The strict control of replication suggests that two plasmids that share the same replicon cannot propagate stably in the same cell line. This phenomenon is known as plasmid incompatibility [358].

1.8.2.2 Plasmid typing

Identification and classification of plasmids should be established on genetic traits that are universally present and constant. These criteria are best met by qualities concerned with plasmid maintenance, especially replication controls [363] .

A plasmid classification scheme was suggested by Datta and Hedges in 1971, it was established on the stability of plasmids during conjugation, a phenomenon called plasmid incompatibility [364, 365]. Incompatibility is a representation of the relatedness of plasmids that have common replication controls [364, 366]. Incompatibility was well defined as the incapability of two related plasmids to propagate stably in the same cell line; thus, compatible plasmids only can be stably maintained in transconjugants (TC). In other words, it is the determination of the transfer frequency of plasmid in different groups together with their stable co-existence in bacteria.

The initial incompatibility (Inc) groups were defined as follows: W (named after a reference strain received from Tsutomu Watanabe) [367]; IncI plasmids (producing type I pili while being susceptible to phage Ifl); IncN (N3-related plasmids susceptible to phage Ike); IncF (plasmids producing type F pili susceptible to phage Ff); and IncP (RP4-related plasmids susceptible to the PRR1 phage) [364, 365]. Later, updates on this scheme was done and 23 plasmid incompatibility groups were identified B, C,D, E, FI, FII, FIII, FIV, H, Ia, I2, Ic, Id, If, J, K,M, N, P, T, V,W and X . Further annotation and modification were made, e.g. plasmids that where incompatible with IncA and IncC were referred as IncA/C, former IncL were renamed as IncM, and those previously named IncL became IncL/M[368] .

Among *Enterobacteriaceae* 28 Inc groups have been recognized so far, in *Pseudomonas* 14, and around 18 in *Staphylococcus* [369]. Currently, the plasmid typing scheme frequently used is Inc/rep typing. This typing is generally consistent with the conjugation dependent scheme.

The first replicon typing scheme was established in 1988, based on Southern hybridization: a genetic plasmid typing scheme using cloned replication regions (replicons) as probes was identified by Couturier and colleagues [362]. This method efficiently offered classification for both conjugative and non-conjugative plasmids. However, it could not be easily applied to a large number of isolates as it was limited by the time consuming work. Furthermore, with the low specificity of the hybridization method, it undervalued plasmid variety due to the cross-hybridization reaction among highly related replicons (repI, repB/O, repFII, repFIC). Thus it was limited only to IncP, IncW, IncN and IncQ plasmids [370].

Since 2005, a PCR-based replicon typing (PBRT) scheme has been presented by Carattoli *et al*. [371] using a set of primers targeting different regions (*rep* gene, iterons, RNAI) of the major plasmid families found in *Enterobacteriaceae* (HI2, HI1, I1-γ, FIA, X, L/M, A/C, N, FIB, FIC, W, Y, P, T, B/O K) and also having additional PCR assays (FrepB and FIIAs PCRs), detecting the FII, FIII, FIV, and FIV variants and the FII replicon of the *Salmonella* virulence plasmids, respectively [371]. Targets for further identification of other plasmid groups have been added to the typing system by Villa *et al*. [372] and Garcia- Fernandez *et al*. [373].

With the advent of next generation sequencing and its use in bacterial genome sequencing, the data generated by this method became available for plasmid identification and typing. The web-based tool, PlasmidFinder can be used for *in silico* detection and typing of plasmids [374].

Further to that, in order to increase the sensitivity of detecting low-copy plasmid replicons, real-time PCR was proposed by Boot *et al.* [375]. Moreover, a scheme was proposed by Bousquet *et al*.[376] that can be used together with PBRT; whereas diverse partition systems located on MDR plasmids were described, which led to the design of a multiplex PCR method termed as plasmid partition gene typing (PAR-T). This technique can be used for a further plasmids classification in *K. pneumoniae*, *Salmonella enterica spp*. and *E. coli* [376].

An additional approach, known as plasmid multilocus sequence typing (pMLST) has been developed for IncN, IncA/C and IncI plasmids; it is used to increase the discriminatory power in the differentiation and characterization of plasmids within incompatibility groups. Further to that the pMLST is used to confirm

evolutionary and epidemiological relatedness [377-379].

Multi-replicon plasmids impose a challenge for plasmid replicon typing. One of the best known multi-replicon plasmid is the IncF which can have an FII, FIA and/or FIB replicon. Moreover, other plasmids can co-integrate, generating another form of multi-replicon plasmid [380-382]. These as well, are challenging for typing and understanding of plasmid epidemiology.

Francia *et al.* and Garcillan-Barcia *et al*. have offered the use of conjugative relaxases as a plasmid-typing tool. Relaxase-based typing can be applied to any plasmid (immobilizable or conjugative) [383, 384]. Another major benefit of this system is that while a plasmid can have multiple replicons, there is always only one relaxase per plasmid. Since relaxases are said to be stable markers of plasmid evolution, they represent better evolutionary relatedness.

Nevertheless, all these PCR based typing schemes have some limitations. Therefore, the most accurate method to characterize a plasmid is based on the determination of the full-length DNA sequence [140].

1.8.2.3 Resistance plasmids of *Enterobacteriacea*

Plasmids of the family *Enterobacteriaceae* have been categorized into replicon (Inc) types by PCR-based replicon typing (PBRT) [385, 386]. PBRT, in combination with other characteristics of the bacterial host, such as serotype, sequence type (ST) by MLST and resistance gene profiles, is used to demonstrate the spread of antimicrobial resistance determinants between genetically unrelated bacterial hosts [387].

In *Enterobacteriaceae*, IncHI, IncF, IncL/M, IncA/C, IncI1, and IncN plasmids are considered to be the predominant plasmid families responsible for worldwide dissemination of carbapenemase, AmpC cephalosporinase, ESBL, and PMQR genes [140, 217].

1.8.2.3.1 IncF plasmids

IncF plasmid's host range is limited to the *Enterobacteriacea* family. They have been reported in almost all species in this family. IncF-related plasmids are low copy number, conjugative with size ranging from 45-200Kb [371]. IncF plasmids are known to be typical multi-replicon plasmids, carrying the FII replicon together with the FIA and FIB replicons. Moreover, co-integration of IncI1 and IncN replicon type plasmids into IncFII has been reported [382, 388].

Based on PCR and sequencing, IncFII plasmid family are subcategorized to the FIIk (*Klebsiella*), FII (*E. coli*), FIIs (*Salmonella*) and FIIy (Yersinia) specific groups [372].

Resistance genes that have been frequently described on IncF plasmids are genes encoding ESBLs, carbapenemases, AMEs and PMQR genes [319, 389]. The international dissemination of $bla_{\text{CTX-M-15}}$ in *E. coli* and *K. pneumoniae* isolates is extensively related to IncF plasmids [390-392].

FIIk is regularly described with replicons of the FIB-type [374]. Two kinds of FIB replicons have been stated more commonly in *K. pneumoniae* plasmids, termed FIBpKPQIL and FIBpKPN. These FIB replicons differentiate plasmids pKPQIL and pKPN3 which are usually found in *K. pneumoniae* epidemic CC258 [393, 394].

These plasmids are rarely reported from other enterobacterial species, which may be due to the narrow host range features of IncFIIk plasmids limiting their spread. The occurrence of these plasmids in *K. pneumoniae* beside their role in biofilm formation and iron scavenging may be the reason for persistence and survival of *K. pneumoniae* within hospitals and patients [395].

1.8.2.3.2 IncA/C plasmids

IncA/C plasmids are low copy number, conjugative and self- transferable episomes with a size range of 40–230 Kb. According to the PBRT scheme the target gene is repA. IncA/C plasmids have a wide host range [396]. Two variants have been described within this group: A/C1 and A/C2; both IncA/C1 and IncA/C2 show 26 single nucleotide polymorphisms in the repA gene [397]. IncA/C plasmids have been found globally and are associated with MDR; they have been documented with ESBLs, AmpC beta lactamases, carbapenemases such as KPC-2, NDM-1, NDM-6, NDM-7 and VIM-4 [173, 389, 398, 399], and have been associated with other antibiotic resistance genes, *i.e*. tetracyclines (*tet(A)*), aminoglycosides (*aadA, aphA1, aacC, strA, strB*), chloramphenicol (*floR, catA1*) and trimethorprim (*drfA*) [400, 401].

1.8.2.3.3 InHI plasmids

IncHI plasmids have a wide host range, including *Enterobacteriacea* and other Gram negative bacteria [402]; they are a group of low copy number plasmids with a size ranging from 75 to 400Kb. The IncHI plasmid group has been distinguished into two groups as some members showed incompatibility with IncF plasmids. Therefore, IncHI1 group which have repFIA, are incompatible with IncF

plasmids but IncHI2 group is compatible with IncF plasmids [403].

IncHI plasmids have been reported to carry carbapenemase genes [319, 404, 405]; They are multidrug resistance plasmids, beyond genes encoding beta-lactamases including carbapenemases, they usually carry aminoglycoside, sulphonamide, streptomycin and tetracycline resistance genes [319, 404].

1.8.2.3.4 IncL/M plasmids

IncL/M are a broad host- range plasmids, their size range is 50-80Kb with a low copy number [406]; In the PBRT scheme repA, repB and repC are the target sites used.

A 60 Kb IncL/M plasmid is extensively reported worldwide associating with $bla_{\text{OXA-48}}$. *K. pneumoniae* bearing these IncL/M plasmids with $bla_{\text{OXA-48}}$ are considered a major cause of infection in hospitals [130, 319, 407].

One of the main causes of the successful spread of this plasmid is owed to the Tn*1999*, that harbor the $bla_{\text{OXA-48}}$, Tn*1999* inserts itself in the *tir* gene that encodes a transfer inhibition protein [218].

1.8.2.3.5 IncX plasmids

IncX plasmids were identified in *Klebsiella* and *Salmonella* isolates in the pre-antibiotic era [408]. IncX plasmids are narrow host range plasmids, reported extensively in *Enterobacteriaceae*, with a size range from 30 to ~50 Kb [368]. They have six subtypes: IncX1 to IncX6 [409, 410]. Molecular examination of IncX plasmids revealed genes, which are necessary for replication (*pir*), pilus synthesis (type IV fimbriae), assembly (mpf) and conjugative function (*taxAC*) enabling its

conjugation [411]. Moreover, these plasmids deliver accessory functions to their host bacteria, *e.g.* resistance to antibiotics and biofilm formation [412].

Members of this group have been found to carry a wide variety of resistance genes. For example, carbapenemases genes (mostly *bla*$_{KPC}$ and *bla*$_{NDM}$) have been described on IncX plasmids from different parts of the world [399, 410, 413]. IncX plasmids have been involved in the acquisition and spread of transferable AMR determinants, such as ESBLs (*bla*$_{TEM-52}$, *bla*$_{CTX-M-15}$ and *bla*$_{SHV-12}$), plasmid-mediated quinolone resistance genes (*qnrS1*, *oqxAB*) and colistin resistance genes (*mcr-1*). Furthermore, tetracycline and trimethoprim resistance determinants have been reported on IncX plasmids [230, 409, 410]. A special subgroup of the IncX plasmid, IncX3, has been described all over the world to carry various carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-181}$) in clinical isolates of *Enterobacteriaceae*, e.g. *E. coli*, *K. pneumonia*, *Citrobacter freundii*, *Enterobacter cloacae* and *Serratia marcescens* (illustrated in Table 2).

Table 2: Association of carbapenemase genes with IncX3 plasmid in clinical isolates of *Enterobacteriaceae*

Carbapenemase gene	**Year**	**Location**	**Organism**	**Size (Kb)**	**Reference**
bla$_{\text{NDM-1}}$	2012	China	*K. pneumoniae, E. coli Citrobacter freundii, Enterobacter aerogenes, E. cloacae*	54	[414]
bla$_{\text{KPC-2}}$	2013	China	*K. pneumoniae*	42	[415]
bla$_{\text{KPC-2}}$	2013	France	*K. pneumoniae*	53	[416]
bla$_{\text{NDM-1}}$, *bla*$_{\text{SHV-12}}$	2013	UAE	*E. coli, Citrobacter freundii, Enterobacter cloacae*	50	[417]
bla$_{\text{KPC-2}}$	2014	Brazil	*K. pneumoniae*	45	[256]
bla$_{\text{NDM-5}}$	2014	China	*E. coli*	47	[418]
bla$_{\text{NDM-5}}$	2015	India	*K. pneumoniae*	46	[419]
bla$_{\text{NDM-1}}$ *or* *bla*$_{\text{OXA-181}}$	2015	China	*E. coli*	51	[420]
bla$_{\text{NDM-7}}$	2013	Germany	*E. coli*	60	[421]
bla$_{\text{KPC-2}}$	2015	UAE	*K. pneumoniae*	45	[157]
bla$_{\text{NDM-4}}$	2015	Australia	*K. pneumoniae*	46	[422]
bla$_{\text{OXA-181}}$	2015	China	*E. coli*	51	[230]
bla$_{\text{NDM-7}}$	2015	Canada	*Serratia marcescens, E. coli, K. pneumoniae*	46	[423]
bla$_{\text{KPC-3}}$	2016	Italy	*K. pneumoniae*	63	[424]
bla$_{\text{OXA-181}}$	2016	West Africa	*E. coli*	54	[425]
bla$_{\text{KPC-2}}$	2017	Brazil	*K. pneumoniae*	45	[426]
bla$_{\text{NDM-7}}$	2017	Kuwait, Oman, UAE	*E. coli*	34-36	[318]
bla$_{\text{NDM-4}}$	2017	Myanmar	*E. coli*	46	[427]
bla$_{\text{NDM-7}}$	2017	Myanmar	*E. coli*	unknown	[427]
bla$_{\text{NDM-4}}$	2017	India	*E. coli*	45	[428]
bla$_{\text{KPC-3}}$	2017	Italy	*Serratia marcescens, E. coli, Citrobacter freundii*	53	[429, 430]
bla$_{\text{NDM-4}}$ or *bla*$_{\text{NDM-5}}$	2018	Czech Republic	*E. xiangfangensis, E. asburiae, K. intermedia, E. coli, Klebsiella oxytoca, Raoultella ornithinolytica*	45-55	[311]

1.9 Research hypothesis, aims and objectives

As shown in the previous sections antibiotic resistance, and specifically carbapenem resistance spreads both by dissemination of bacterial clones, as well as horizontal gene transfer. We hypothesized that both mechanisms contributed to the emergence of CRE in the UAE. Therefore, the aim of our study was to investigate the molecular epidemiology of carbapenem resistant *Enterobacteriaceae* in the UAE and its neighboring countries, with the following specific objectives:

- To study how the IncX3 plasmid-borne carbapenemases emerged in the UAE, and to what extent they contributed to the spread of CRE in the country.
- To assess the molecular epidemiology and antibiotic resistance of CRE collected systematically during a year-long period in five major hospitals of Dubai.
- To compare by whole-genome sequencing the core genome and the variety of plasmids carrying carbapenemase genes in carbapenem-resistant *Klebsiella pneumoniae* ST14, i.e. a CRE clone increasingly encountered in countries of the Arabian Peninsula.

Chapter 2: Materials and Methods

2.1 Bacterial isolates

To study the IncX3 plasmids carrying carbapenemases in the United Arab Emirates (UAE), 334 non-repeat carbapenem resistant *Enterobacteriaceae* (CRE) strains isolated between April 2009 and December 2014 in 12 hospitals of the UAE and submitted to the Department of Medical Microbiology and Immunology, UAE University (UAEU) for identification of the carbapenemases produced, were included. This collection included 90 isolates described earlier in [156, 157, 417, 431], and further 244 CRE isolated between May 2013 and December 2014 from six governmental hospitals of Abu Dhabi Emirate.

In order to assess to what extent *Klebsiella pneumoniae* clones contribute to the spread of CRE in Dubai, *Enterobacteriaceae* exhibiting decreased susceptibility to either ertapenem, imipenem or meropenem according to the criteria set by the Clinical and Laboratory Standards Institute (CLSI) [432] at the time of their isolation were collected, between June 2015 and June 2016, from hospitals having the broadest medical and surgical profile in Dubai. From the governmental health sector, the 625-bed Dubai Hospital and the 786-bed Rashid Hospital covering 70% of the city's governmental hospital capacity participated in the study. From the private health sector, the 254-bed American Hospital, the 280-bed Mediclinic City Hospital, and the 127-bed Welcare Hospital, altogether covering 35% of Dubai's private hospital capacity, were involved in the study. For CRE collection no further selection criteria were applied when submitting strains for the study to the Department of Medical Microbiology and Immunology, UAEU.

To investigate the genetic relatedness and variations in carbapenem-resistant *Klebsiella pneumoniae* ST14 (CRKP-ST14), *i.e.* a sequence type increasingly encountered in countries of the Arabian Peninsula, we tested 761 independent CRKP isolated between June 2011-June 2016 in Bahrain (n=20), Kuwait (n=66), Saudi Arabia (n=151), Oman (n=41) and the United Arab Emirates (n=483).

Until the investigations, all bacterial strains were stored at -80 °C in Tryptic Soy Broth (MAST, Merseyside, UK) containing 10% glycerol at the Department of Medical Microbiology and Immunology, UAEU.

2.2 Antibiotic susceptibility testing

For testing the antibiotic susceptibility, two approaches were used: broth microdilution and agar dilution. All tests were carried out and evaluated by the CLSI standards [432], except for tigecycline, colistin and fosfomycin for which no breakpoint values are available in this system. Thus, we used the EUCAST clinical breakpoints for interpretation of these latter antibiotics (www.eucast.org/clinical-breakpoints) (EUCAST 2018).

For the broth microdilution assay, antibiotics were serially diluted in 100 µl volume of Muller Hinton Broth (Oxoid, UK) in 96 well untreated microtiter plates (Sarstedt, Nümbrecht, Germany). Bacteria were inoculated using a Multipoint inoculator (MAST, UK) to give a final concentration of 10^5 CFU/ml. After 18 hours of incubation at 37 °C, the growth was visually assessed and the Minimum Inhibitory Concentration (MIC) of the drug was determined as the lowest concentration preventing visible growth of the organism tested. All tests were run in duplicates. The quantitative susceptibility test was established against: imipenem (IMP),

meropenem (MEM), ertapenem (ERT), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (AZT), ciprofloxacin (CIP), amikacin (AK), gentamicin (GN), trimethoprim-sulphamethoxazole (SXT), tetracycline (TET), chloramphenicol (CHL), and colistin (COL). For fosfomycin (FOS) and tigecycline (TGC), the agar dilution method was used to determine the MIC. Different concentrations of antibiotics were prepared in 1 ml and added to 24 ml molten Mueller Hinton Agar (MAST, UK). Plates with fosfomycin were supplemented with glucose-6-phosphate (Sigma, USA) at a concentration of 25 mg/L. One µl of 10^7 CFU/ml bacterial suspension was delivered by the multipoint inoculator to the surface of dry plates, *i.e.*, approximately 10^4 CFU/dot was inoculated.

For all sensitivity assays, *Escherichia coli* ATCC25922 was used as control. For testing colistin susceptibility ABC149, an mcr-1 producing *E. coli* ST131 was included as control with colistin MIC of 4 mg/L [433].

To group strains according to their level of resistance, the following categories were used: multi-drug resistant (MDR) if they were non-susceptible to ≥1 agent in >3 antimicrobial classes tested, extensively drug resistant (XDR) if they remained susceptible to agents in ≤2 classes, and pan-drug resistant (PDR) if non-susceptible to all antimicrobial agents tested.

2.3 Determination of carbapenemase production

Carbapenemase production was assessed using two phenotypic approaches, the CarbaNP test as described [434] and the modified Hodge test [435].

2.3.1 Modified Hodge Test (MHT)

Mueller-Hinton agar plates (Oxoid) were inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922. Meropenem disc was placed in the center of the plate. Whereas the test strain, a carbapenemase non-producing negative control strain (*E. coli* $J53_{RAZ}$), and a KPC producing strain GR-KPC-2 (positive control) were streaked starting from the disc towards the plate edges. After 16-24 hours incubation at 37 °C, the plate was inspected for a cloverleaf-type indentation at the intersection of the test organism and *E. coli* ATCC 25922.

2.3.2 CarbaNP test

Carba NP test was performed as described by Nordmann *et al.* [434]. A 10 µl loopful mass of an overnight culture of the test strain on Tryptic Soy Agar (TSA) was mixed in 100µl of B-PERII, Bacterial Protein Extraction Reagent (Thermo Scientific Pierce, Rockford, IL, USA), vortexed for 1 minute and incubated at room temperature for 30 minutes followed by centrifugation of this bacterial protein extraction at 10,000×g for 5 minutes at room temperature. In a 96 well plate, 30 µl of the supernatant was mixed with 100 µl of phenol red solution (containing 0.05% phenol red and 0.1 mmol/L $ZnSO_4$ adjusted to pH 7.8) supplemented with 3 mg/ml imipenem. As a negative control, the reaction was done in phenol red solution without the imipenem antibiotic. Plates were incubated at room temperature for a maximum of 2 hours. For carbapenemase producers, the color of the wells turned from red to orange or yellow. While, the color of the wells stayed red in case of no carbapenemase activity.

2.4 Crude extraction of bacterial genomic DNA

Three to five colonies, grown overnight on TSA with or without 0.5 mg/L ertapenem (according to the strain's susceptibility), were picked by sterile toothpick, and suspended in 200 µl of DNase free distilled water. The suspensions were kept in thermoblock (Eppendorf, Germany) at 99°C for 10 minutes, then centrifuged for 10 minutes at 14800 rpm. The supernatant collected in new sterile Eppendorf tubes was used as the DNA template in PCR reactions directly, or stored at 4°C.

2.5 Polymerase Chain Reaction (PCR)

PCR reactions for NDM, KPC, OXA, VIM and IMP were performed using primers and cycling conditions listed in Table 3. Negative controls used were the *E. coli* $J53_{RAZ}$ genomic DNA and ultra-pure distilled water. All PCR reactions were performed using the Applied Biosystems 2700 thermocycler and 2720 thermocyclers (Applied Biosystems, USA) using MicroAmp tubes (Applied Biosystems, USA).

Finally, amplicons were analyzed on 1% agarose gel (Promega, US) using ethidium bromide dye (Sigma). Gels were then visualized and photographed using Biometra gel documentation system (Biometra, Gottingen, Germany) at 302 nm.

To determine the alleles of $bla_{\text{OXA-48-like}}$ and bla_{NDM} genes the primers and conditions listed in Table 4 were used to generate amplicons.

DNA samples of isolates exhibiting resistance to colistin were tested for the presence of mobile colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3* and *mcr-4*) illustrated in Table 5.

Table 3: Primers and PCR reaction conditions for the detection of common carbapenemase genes

Gene	Primer	Sequence (5'-3')	Amplicon Size	Initial Denaturation	Cycle	Final Extension	Reference
bla$_{\text{OXA-48-like}}$	OXA-F	GCGTGGTTAAGGATGAACAC	438bp	10' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	5'at 72°C	[436]
	OXA-R	CATCAAGTTCAACCCAACCG					
bla$_{\text{KPC}}$	KPC-F	CGTCTAGTTCTGCTGTCTTG	798bp		40X (30"at 94°C, 40" at 52°C, 50" at 72°C)		
	KPC-R	CTTGTCATCCTTGTTAGGCG					
bla$_{\text{VIM}}$	VIM-F	GATGGTGTTTGGTCGCATA	390bp		40X (30"at 94°C, 40" at 52°C, 50" at 72°C)		
	VIM-R	CGAATGCGCAGCACCAG					
bla$_{\text{IMP}}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232bp		40X (30"at 94°C, 40" at 52°C, 50" at 72°C)		
	IMP-R	GGTTTAAYAAAACAACCACC					
bla$_{\text{NDM}}$	NDM1-Fo	TGCCGAGCGACTTGGCCTTG	379bp	5' at 94°C	30X (30"at 94°C, 30" at 60°C, 1' at 72°C)	7' at 72°C	[437]
	NDM1-Re	ACCGATGACCAGACCGCCCA					

Table 4: Primers and PCR conditions used for amplification of carbapenemase genes for allele determination

Gene targeted	**Primers**	**Sequences (5'- 3')**	**Amlicon size (bp)**	**Initial Denaturation**	**Cycle**	**Final extension**	**References**
bla$_{\text{OXA-48-like}}$	OXA-48 A	TTG GTG GCA TCG ATT ATC GG	744	5' at 94°C	40X (30" at 94°C, 30" at 60°C and 1' at 72°C)	10'at 72°C	[438]
	OXA-48 B	GAG CAC TTC TTT TGT GAT GGC					
bla$_{\text{NDM}}$	ASndm-1	GTC GCA AAG CCC AGC TTC GCA	945		40X (30" at 94°C, 30" at 58°C and 1' at 72°C)		[437]
	ASndm-2	GCC TCG CAT TTG CGG GGT TTT TA					

Table 5: Primers and PCR conditions used for amplification of *mcr-1*, *mcr-2*, *mcr-3* and *mcr-4*

Gene targeted	Primers	Sequences (5'- 3')	Amlicon size (bp)	Initial Denaturation	Cycle	Final extension	References
mcr-1	CLR-5F	CGGTCAGTCCGTTTGTTC	309	5' at 94°C	35X (30" at 94°C, 30" at 55°C and 1' at 72°C)	10'at 72°C	[84]
	CLR-5R	CTTGGTCGGTCTGTAGGG					
mcr-2	mcr2-IF	TGTTGCTTGTGCCGATTGGA	566	5' at 95°C	35X (30" at 95°C, 30" at 65°C and 1' at 72°C)	10'at 72°C	[439]
	mcr2-IR	AGATGGTATTGTTGGTTGCTG					
mcr-3	mcr3-F	TTGGCACTGTATTTTGCATTT	542	5' at 95°C	35X (30" at 95°C, 30" at 51°C and 1' at 72°C)	7'at 72°C	[440]
	mcr3-R	TTAACGAAATTGGCTGGAACA					
mcr-4	mcr4-F	ATTGGGATAGTCGCCTTTTT	487	5' at 95°C	35X (30" at 95°C, 30" at 51°C and 1' at 72°C)	7'at 72°C	[441]
	mcr4-R	TTACAGCCAGAATCATTATCA					

2.6 Molecular fingerprinting

Pulsed Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing, plasmid profile analysis, and core genome MLST (cgMLST) derived from bacterial whole genome sequences were used to establish the molecular types of the isolates.

2.6.1 Macrorestriction of the genome followed by Pulsed Field Gel Electrophoresis (PFGE)

The procedure of Gautom *et al.* was followed [442]. Bacterial strains grown on TSA plates overnight at 37^{o}C were suspended in 2ml of cell suspension buffer (100mM Tris: 100mM EDTA, pH8.0) and cell density was adjusted up to 2-3 McFarland unit to ensure an optimum amount of bacterial cells present in the agarose plug. Suspensions were kept on ice. Concurrently, plug agarose (Sigma, USA) containing 1% SDS was prepared in TE buffer (10mM Tris: 1mM EDTA, pH8.0).

In 1.5 ml Eppendorf tubes, 500 µl of bacterial suspensions were aliquoted and mixed with 25 µl of proteinase K (20 mg/ml) (Invitrogen) to inactivate the nucleases. The suspension was mixed with 525µl of 1% plug agarose and dispensed into 1 ml syringes and were left at room temperature for 15-30 minutes to allow the solidification of the agarose.

One mm thick slices of agarose plugs were directly cut into aliquots of 5 ml cell lysis buffer (50mM Tris: 50mM EDTA pH8.0, 1% Sarkosyl) supplemented with 25 µl proteinase K (20 mg/mL), then incubated at 50°C in a shaking water bath (200 rpm) for 2 hours. Subsequently, the plugs were washed twice with 10 ml of preheated sterile MilliQ water for 20 minutes in a 50°C shaking water bath. Afterward, they were washed four times for 20 minutes with 10 ml of preheated TE buffer. Finally, plugs were stored in 5 ml of fresh TE buffer at 4°C.

Genomic DNA within the plugs was digested overnight at 37°C in a 100 µl restriction mixture made of 10 µl of cut smart buffer (New England Biolabs, UK), 30 U (1.5 µl) of XbaI enzyme (New England Biolabs,UK) and 88.5 µl of sterile distilled water.

Following digestion, the restriction mixture was removed and the plugs were incubated in 250 µl of 0.5X TBE buffer for 30 minutes at room temperature. Subsequently, plugs were inserted into wells of 1.4% of agarose gel (Pulse Field Running Agarose A2929, Sigma, USA) prepared with 0.5X TBE buffer.

The two wells at the two sides of each gel and the middle well contained *Xba*I digested genome of *Salmonella* Braenderup H9812 as PFGE marker for standardization. Gels were run in CHEF Mapper (Biorad, USA) electrophoresis chamber containing 0.5X TBE buffer pre-chilled to 14°C. The running program consisted of 24 hours run at 6 V/cm with 120° angle and an initial switch time of 2.2 seconds and a final switch time of 54.2 seconds with linear ramp.

After electrophoresis, the gels were stained with ethidium bromide for 20 minutes, followed by de-staining in 400 ml of sterile MilliQ water for another 20 minutes. Using Biometra gel documentation system the genomic patterns were detected and photographed under UV light. Gel pictures were stored as .tif files for further analysis. GelCompare II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze and compare the banding patterns. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree graphically showing the level of relatedness between the isolates was created based on the Dice similarity coefficient (SD) (with a 1.5% position tolerance). Strains were considered to belong into the same PFGE pulsotype if they exhibited SD ≥ 80%. Sub-clusters were

defined by ≥90% pattern similarities.

2.6.2 Multi-locus Sequence Typing (MLST)

The seven housekeeping genes of *E. coli* (*adk, fumC, gyrB, icd, mdh, purA, recA), K. pneumoniae* (*gapA, infB, mdh, pgi, phoE, rpoB, tonB*) and *E. cloaceae* (*dnaA, fusA, gyrB, leuS, pyrG, rpIG, rpoB*) were amplified by the primers shown in Table 6. For *E. coli* the MLST protocol of Wirth *et al.* was used [443]. The isolate was then assigned to sequence types using the tools on the *E. coli* MLST webpage (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). For *E. cloaceae*, we used the MLST protocol of Miyoshi-Akiyama *et al.* [444]. Sequence types were established using *E. cloaceae* MLST webpage (http://pubmlst.org/ecloacae/). While for *K. pneumoniae* the protocol of Diancourt *et al*. [296] was applied and *K. pneumoniae* MLST webpage (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) was used to determine the sequence types.

Table 6: PCR programs and primers used for *E. coli*, *E. cloaceae* and *K. pneumoniae* MLSTs

Gene targeted	**Primers**	**Sequences (5'- 3')**	**PCR product size**	**Initial denaturation**	**Cycle**	**Final extension**
***E. coli* MLST(Wirth *et al.* 2006) [443]**						
adk	adk F	ATTCTGCTTGGCGCTCCGGG	583bp	5 minutes at 94°C	35X (60" at 94°C, 60" at 56°C and 60" at 72°C)	10'at 72°C
	adk R	CCGTCAACTTTCGCGTATTT				
fumC	fumC F	TCACAGGTCGCCAGCGCTTC	806 bp			
	fumC R	GTACGCAGCGAAAAAGATTC				
gyrB	gyrB F	TCGGCGACACGGATGACGGC	911 bp			
	gyrB R	ATCAGGCCTTCACGCGCATC				
icd	icd F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878 bp			
	icd R	GGACGCAGCAGGATCTGTT				
mdh	mdh F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932 bp			
	mdh R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT				
purA	purA F	CGCGCTGATGAAAGAGATGA	816 bp			
	purA R	CATACGGTAAGCCACGCAGA				
recA	recA F	CGCATTCGCTTTACCCTGACC	780 bp			
	recA R	TCGTCGAAATCTACGGACCGGA				

Table 6: PCR programs and primers used for *E. coli*, *E. cloaceae* and *K. pneumoniae* MLSTs (continued)

Gene targeted	Primers	Sequences (5'- 3')	PCR product size	Initial denaturation	Cycle	Final extension
***E.cloaceae* MLST (Miyoshi-Akiyama *et al.* . 2013) [444]**						
dnaA	dnaA-f2	AYAACCCGCTGTTCCTBTATGGCGGCAC	1151bp	2' at 95°C	35X (15" at 95°C, 10" at 50°C and 60" at 72°C)	7' at 72°C
	dnaA-r	KGCCAGCGCCATCGCCATCTGACGCGG				
fusA	fusA-f2	TCGCGTTCGTTAACAAAATGGACCGTAT	906bp			
	fusA-r2	TCGCCAGACGGCCCAGAGCCAGACCCAT				
gyrB	gyrB-F	TCGACGAAGCGCTCGCGGGTCACTGTAA	1153bp			
	gyrB-R	GCAGAACCGCCCGCGGAGTCCCCTTCCA				
leuS	leuS-f2	GATCARCTSCCGGTKATCCTGCCGGAAG	845bp			
	leuS-r	ATAGCCGCAATTGCGGTATTGAAGGTCT				
pyrG	pyrG-f	AYCCBGAYGTBATTGCRCAYMAGGCGAT	535bp			
	pyrG-r	GCRCGRATYTCVCCCTSHTCGTCCCAGC				
rplB	rplB-f	GTAAACCGACATCTCCGGGTCGTCGCCA	746bp			
	rplB-r	ACCTTTGGTCTGAACGCCCCACGGAGTT				
rpoB	rpoB-f	CCGAACCGTTCCGCGAACATCGCGCTGG	944bp			
	rpoB-r2	CCAGCAGATCCAGGCTCAGCTCCATGTT				

Table 6: PCR programs and primers used for *E. coli*, *E. cloaceae* and *K. pneumoniae* MLSTs (continued)

Gene targeted	**Primers**	**Sequences (5'- 3')**	**PCR product size**	**Initial denaturation**	**Cycle**	**Final extension**
***K. pneumoniae* MLST (Diancourt *et al.* 2005)[296]**						
rpoB	VIC3	GGC GAA ATG GCW GAG AAC CA	501bp	5' at 94°C	35X (60" at 94°C, 60" at 50°C and 60" at 72°C)	10' at 72°C
	VIC2	GAG TCT TCG AAG TTG TAA CC				
gapA	gapA173	TGA AAT ATG ACT CCA CTC ACG G	450bp			
	gapA181	CTT CAG AAG CGG CTT TGA TGG CTT				
mdh	mdh130	CCC AAC TCG CTT CAG GTT CAG	477bp			
	mdh867	CCG TTT TTC CCC AGC AGC AG				
pgi	pgi1F	GAG AAA AAC CTG CCT GTA CTG CTG GC	432bp			
	pgi1R	CGC GCC ACG CTT TAT AGC GGT TAA T				
phoE	phoE604.1	ACC TAC CGC AAC ACC GAC TTC TTC GG	420bp			
	phoE604.2	TGA TCA GAA CTG GTA GGT GAT				
infB	infB1F	CTC GCT GCT GGA CTA TAT TCG	318bp			
	infB1R	CGC TTT CAG CTC AAG AAC TTC				
tonB	tonB1F	CTT TAT ACC TCG GTA CAT CAG GTT	414bp			
	tonB2R	ATT CGC CGG CTG RGC RGA GAG				

2.6.3 Sequencing

PCR products were purified according to the manufacturer's instruction with Wizard PCR and gel purification kit (Promega, USA) before sequencing. The sequencing was done using the Big Dye Cycle Terminator V.3.1 (Applied Biosystems). Using the 3130X Genetic Analyzer (Applied Biosystems) samples were sequenced in both directions then analyzed using the MEGA7 (http://www.megasoftware.net/mega7/mega.html) and Clone Manager v9 (Scientific and educational software).

2.6.4 Plasmid profile analysis

For routine detection and isolation of large and small plasmids, the method of Kado and Liu [445] was used with slight adjustments. Bacterial cells were collected from an area of approximately 4 cm^2 of overnight confluent culture on TSA at 37°C. Cells were suspended gently into 250 µl of lysing solution (3% SDS, 50 mM Tris, pH 12.57), and mixed thoroughly by gentle agitation until having a homogenous and viscous suspension, which was incubated for 45 minutes at 60°C with gentle mix every 15 minutes. After the incubation period, 250 µl of 1:1 phenol- chloroform was added. Emulsification of the solution was achieved by gentle shaking. The emulsion was separated by centrifugation at 13,000 rpm for 15 minutes. The top aqueous layer was transferred to clean tubes without disrupting the precipitate at the interface. Electrophoresis of the samples for 3 hours at 120 Volt in 0.8% agarose gel was done. The gels was stained with ethidium bromide, destained with 400 ml sterile MilliQ water and was scanned using the Biometra gel documentation system.

2.6.5 Southern blot and hybridization

Plasmid gels prepared by the above method [445] were stained by ethidium bromide, photographed and scanned using the Biometra gel documentation system with rulers placed around the gel for further identification of bands.

Gels were depurinated in 0.25 M HCl, followed by denaturation in 0.5 M NaOH, 1 M NaCl and finally neutralized in 1 M Tris, 0.6 M NaCl. All treatment steps were 15 minutes long at room temperature with gentle shaking and were repeated twice. Between each step; gels were rinsed in sterile distilled water. The gels were capillary-transferred to Hybond N+ membranes (Roche) by soaking overnight in 20X SSC (Saline Sodium citrate). The following day the membranes were UV cross-linked at 70000 micro-joules.

Hybridization probes were made by PCR amplification of respective control strain for the following genes : $bla_{\text{NDM-1}}$[437], $bla_{\text{OXA-48}}$ [436], IncX3 [409] and *parA* gene of the IncL/M plasmid [215]; this was followed by DNA purification using Wizard SV Gel and PCR clean up system kit (Promega, USA) and quantification using the ND-1000 spectrophotometer (Nano Drop Technologies, USA). The DNA fragments were labeled using the DIG DNA labeling kit (Roche). Briefly, 200 ng of purified fragment were boiled for 10 minutes in a boiling water bath and rapidly chilled on ice. Then the denatured DNA was conjugated with digoxigenin according to the manufacturer's instructions. Membranes were pre-hybridized at optimal hybridization temperature, calculated based on the size of the probe and GC ratio using the formula suggested by the manufacturer ($T_{opt.}=T_m-20$ °C with $T_m=49.82+0.41(\%G+C)-600/L$ (L=length of the probe in base pairs)), in a pre-warmed hybridization buffer (5X SSC, 1% blocking solution, 0.1 % N-lauryl

sarcosine, 0.02% SDS) for 30 minutes followed by the addition of the hybridization buffer containing the probe. For post hybridization the blots were washed twice with 2X SSC / 0.1% SDS (Sigma) at room temperature for 5 minutes with gentle shaking followed by two successive washings in 0.1X SSC / 0.1 % SDS at 68°C for 15 minutes also with constant agitation. The membranes were briefly rinsed in washing buffer at room temperature, incubated in blocking solution for 30 minutes, and again incubated in antibody solution provided with the kit for 30 minutes. The blots were washed twice in washing buffer, equilibrated in detection buffer and finally incubated in the color substrate solution without shaking for various lengths of times in the dark till the desired spot or band intensity was visualized. The reaction was stopped using TE buffer (10mM Tris:1mM EDTA pH8 (Sigma)). The blots were digitized using the Biometra gel documentation system. In order to re-probe the membrane it was stripped with dimethyl formamide at 56°C followed by steps starting from the pre- hybridization.

2.7 Screening for the presence of IncX3 incompatibility type plasmids

Genomic DNA of isolates was subjected to PCR targeting the replicase of IncX3 incompatibility type plasmids as described [409], using primers IncX3-f – GTTTTCTCCACGCCCTTGTTCA and IncX3-r–CTTTGTGCTTGGCTATCATAA. The cycling conditions were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles 60 sec at 95°C, 30sec at 52°C and 60sec at 72°C, with a final extension of 5 minutes at 72°C.

2.8 PCR Based Replicon Typing (PBRT)

In order to identify plasmid incompatibility types, PCRs were performed using primers of the original PBRT scheme [371], and also others targetting IncHI1B and IncL/M-OXA-48 type plasmids [215, 319]. Primers and conditions used for these two latter types of plasmids are listed in Table 7.

DNA prepared of single plasmids containing derivatives was used as a template.

Table 7: Primers and PCR conditions used for targeting IncHI1B and IncL/M-OXA-48 type plasmids.

Primers	**DNA sequence (5'-3')**	**Amplicon size (bp)**	**Initial denaturation**	**Cycle**	**Final extension**
IncHI1B PCR (Dortet *et al.* 2012) [319]					
IncHI1B-Fw	CAA AAC AGA GAG TAT TCA ACC C	600	5' at 94°C	30 X (60" at 94°C, 30" at 52°C , 60" at 72°C)	5' at 72°C
IncHI1B-Rv	CTG ATT CTT TTC GAG ACA GGG				
Amplification of *repA*, *traU*, *parA* genes located on IncL/M plasmid (Poirel *et al.* 2012) [215]					
RepA-A	GACATTGAGTCAGTAGAAGG	925	5' at 94°C	35X (60" at 94°C, 60" at 51°C, 60" at 72°C)	10' at 72°C
RepA-B	CGTGCAGTTCGTCTTTCGGC				
TraU-A	ATCTCACGCAATCTTACGTC	577			
TraU-B	TCGCGTCATGCGTGATCTTC				
ParA-A	GCAGTGAAAACGTTGATCAG	532			
ParA-B	GATCGCAATGCGTCTTGGTG				

2.9 Plasmid transfer experiments

2.9.1 Conjugation

For conjugation experiments, *E. coli* $J53_{RAZ}$ (Na-azid resistant in-house derivative of the rifampin resistant J53 *E. coli* K-12) was used as recipient. Conjugation was attempted as follows: In a sterile 50 ml Falcon Tube (BD), log phase TSB cultures of the donor and recipient were combined in 1: 4 ratio in a 4 ml final volume. The mixture was incubated without shaking for an additional 4 hours, then it was centrifuged at 3500 rpm for 15 minutes. After resuspending the pellet in 200 µl TSB, 100 µl of suspension was added as a drop to the middle of a TSA plate without any antibiotics (two plates were prepared), incubated overnight at 37°C. On the following day, the growth was collected in 5 ml 1X PBS, centrifuged and washed once in 1X PBS, followed by resuspension of the pellet in 3 ml of PBS. This suspension was serially diluted and plated onto plates having 100 µg/ml Na-azide and either 8 mg/L ceftazidime for NDM-producer wild-type isolates, or 0.25 mg/L ertapenem for OXA-48-like-producer wild-type isolates and incubated overnight at 37°C. Next day single colonies were picked and subjected to plasmid electrophoresis and PCR.

2.9.2 Transformation

If we were unable to mobilize a specific plasmid into an *E. coli* $J53_{RAZ}$ by conjugation; transformation of the plasmid was attempted. For these experiments competent cells of *E. coli* DH5α, or *E. coli* GM2163 were used as a recipient.

2.9.2.1 Competent cells preparation

Cells were made competent by the following method: *E. coli* DH5α, or *E. coli* GM2163 was cultured overnight at 37°C in 10 ml of TSB. This culture was used to

inoculate 500 ml Luria-Bertani broth and incubated at 37°C in shaken culture until OD_{600} reached 0.5-0.7 (2-4 hours). The culture was centrifuged at 5000 rpm for 10 min at 4°C. Pellet was resuspended in 50 ml ice-cold 50mM $CaCl_2$ on ice and centrifuged at 5000 rpm for 5 min at 4°C. The pellet was then resuspnded in 50 ml ice-cold 50mM $CaCl_2$ and left on ice for 20 minutes. The suspension was centrifuged at 5000 rpm for 10 min at 4°C. The pellet was resuspended in 25 ml of freezing solution of (50mM $CaCl_2$ containing 20% glycerol), aliquoted in 150µl and snap freezed in liquid Nitrogen. Aliquots were stored at -80°C.

2.9.2.2 Heat-shock transformation

One µg of plasmid DNA purified from the wild type strain using Qiagen plasmid Maxi prep kit was used for transformation. The DNA was mixed with one aliquot (150µl) of competent cells, and the mixture was kept on ice for 20 minutes, followed by a heat shock at 42°C for 5 minutes in a thermoblock, with a final incubation on ice for 2 minutes. The transformation mixture was incubated for 1 hour at 37°C with shaking, and spread on plates containing either 8 mg/L ceftazidime for NDM-producer wild-type isolates or 0.25 mg/L ertapenem for OXA-48-like-producer wild-type isolates and incubated overnight at 37°C. Next day single colonies were picked and subjected to plasmid electrophoresis and PCR.

2.9.3 Plasmid purification

Plasmids were extracted and purified using Qiagen plasmid Maxi kit (Qiagen, Germany). Briefly, 100 mL of Luria Bertani broth was inoculated and incubated for 12-16 hour at 37°C with vigorous shaking. Cells were harvested by centrifugation at 4°C at 6000 x *g* for 15 min. The pellet was re-suspended in 10 ml of Buffer P1 that contained RNase A and 10 ml of P2 Buffer was added. The suspension was mixed

immediately by vigorously inverting the tube 4–6 times then incubated at room temperature for 5 min. After adding 10 ml of chilled Buffer P3 and mixing vigorously, suspensions were incubated on ice for 20 min, followed by centrifugation at ≥20,000 x g for 30 min at 4°C. Meanwhile, the Qiagen column was equilibrated with 10 ml of Buffer QBT and allowed to empty by gravity flow. Supernatant containing plasmid DNA was added into the Qiagen column Qiagen-tip 100, allowed to enter the resin by gravity flow. The Qiagen column was washed twice with 30 ml Buffer QC, and the DNA was eluted with 15 ml Buffer QF. In order to precipitate DNA, 10.5 ml room-temperature isopropanol was added to the eluted DNA, mixed and centrifuged immediately at 4000 x g for 75 min at 4°C. 5 ml room-temperature 70% ethanol was added to the DNA pellet, followed by centrifugation at 4000 x g for 30 minutes. The pellet was air-dried. The DNA was dissolved in nuclease free water. DNA concentration and quality were assessed using the ND-1000 spectrophotometer (Nano Drop Technologies, USA), and were also analyzed by agarose gel electrophoresis.

2.9.4 Establishing the complete plasmid sequences

Plasmid DNA purified of single plasmid containing transconjugants or transformants (TF) were sent for commercial next generation sequencing on the Illumina MiSeq platform (performed at the CCIB DNA Core Facility in Massachusetts General Hospital (Cambridge, MA)). The gaps between contigs assembled were closed by PCR and by direct sequencing of the amplicons. The complete plasmid sequences were assembled with Clone Manager v9.0 (Sci-Ed Software, Cary, NC, US), annotated using Geneious R11.0.4 (Biomatters Ltd.,

Auckland, New Zealand) and Sequin (http://www.ncbi.nlm.nih.gov/Sequin), and submitted to GenBank.

2.9.5 Phylogenetic analysis of the conserved regions of plasmids

Regions of plasmid backbone sequences present in each plasmid were aligned using ClustalW. Phylogenetic (Neighbor-Joining) tree was constructed using the Jukes-Cantor genetic distance model with 500 bootstrap replicates. Missing data were ignored. For this analysis Geneious R11.0.4 software (Biomatters Ltd., Auckland, New Zealand) was used.

2.10 Whole genome sequencing

2.10.1 Genomic DNA extraction for whole genome sequencing by Illumina MiSeq

Genomic DNA was isolated using Wizard genomic DNA Purification kit (Promega, USA) as per the manufacturer's instructions. Briefly: 1.5 mL of overnight culture in TSB was centrifuged at 14800 rpm for 3 minutes, the pellet was resuspended gently with 600µl of Nuclei Lysis Solution, and the mixture was incubated at 80°C for 5 minutes to lyse the cells; then cooled to room temperature. 3µl of RNase Solution was added to the cell lysate, mixed and incubated at 37°C for 45 minutes. 200µl of Protein Precipitation Solution was added to the sample after cooling to room temperature, vortexed, and incubated on ice for 5 minutes. Sample was then centrifuged for 3 minutes at 14800 rpm, the supernatant containing the DNA was transferred to a clean Eppendorf tube containing 600µl of room temperature isopropanol, mixed by inverting, and centrifuged for 3 minutes at 14800 rpm. The pellet was washed with 600µl of room temperature 70% ethanol and centrifuged at 14800 rpm for 3 minutes. After removing the ethanol, the pellet was

air-dried. The DNA was dissolved in nuclease free water. DNA concentration and quality was assessed using the ND-1000 spectrophotometer (Nano Drop Technologies, USA), and was also analyzed by agarose gel electrophoresis.

2.10.2 Nextera XT DNA library preparation

Qubit dsDNA BR Assay system was used for quantitation of the input bacterial genomic DNA (gDNA) as follows: A mixture of 2 µl of each DNA sample + 198 µl of the Qubit working solution was prepared for sample quantification. UV absorbance was then used to assess the DNA Quality.

Bacterial DNA libraries were prepared using the Illumina Nextera®XT DNA Library Preparation Kit (Illumina, USA). An accurate quantified amount of 0.2 ng/µl input bacterial gDNA of the samples were used.

First tagmentation of input bacteria gDNA was performed *i.e*. gDNA was fragmented and tagged with adaptor sequences to the ends using the Nextera XT transposome. Briefly, this was achieved by addition of 10 µl Tagment DNA buffer (TD) to each well of the 96-well Nextera XT Tagment Amplicon Plate (NTA) then 5 µl gDNA of each sample were added (calculated at 0.2 ng/µl '1 ng total') to their assigned well in the NTA plate. This was followed by additing 5 µl of Amplicon Tagment Mix to each well of the NTA plate. Sealing and centrifuging the NTA plate at 280 x g for 1 minute at 20°C was done, and then it was placed in the thermocycler with the following program: "55°C for 5 minutes - Hold at 10°C". Five µl NT Buffer was then added to each well and the NTA plate was sealed and centrifuged again at 280 x g for 1 minute at 20 °C.

Second, PCR amplification of the tagmented DNA while the addition of index 1 (i7) and index 2 (i5) for cluster formation. This was achieved as follows; 15 µl Nextera PCR Master Mix were added to each well of the NTA plate and the arrangement of the index primers (i5 and i7) in the TruSeq Index Plate Fixture was done according to the manufacturer recommendations. Using a multi-channel pipette, 5 µl index 2 primers were added to each column in the NTA plate while 5 µl index 1 primers were added to each row of the NTA plate. Finally the NTA plate was sealed and centrifuged at 280 x g for 1 minute at 20°C.

PCR using the following program was implemented: 72°C for 3 minutes, 95°C for 30 seconds; 12 cycles of : 95°C for 10 seconds / 55°C for 30 seconds / 72°C for 30 seconds / 72°C for 5 minutes; Hold at 10°C.

Third, the PCR cleanup step was performed to purify the library DNA using AMPure XP beads. Using a multi-channel pipette, PCR products from the NTA plate were transferred to a new labelled Clean Amplified plate (CAA). This was followed by the addition of 30 µl AMPure XP beads to each well of the CAA, then the plate was incubated at room temperature for 5 minutes, after that the CAA plate was transferred to the magnetic stand and was left for two minutes (until the supernatant has been cleared); the supernatant was then removed and discarded. The beads were then washed twice with freshly prepared 80% ethanol at which 200 µl 80% ethanol were added to each sample, followed by incubating the plate on the magnetic stand for 30 seconds and finally removing and discarding the supernatant.

After removal of the CAA plate from the magnetic stand, 52.5 µl resuspension buffer were added to each well and then the plate was left at room temperature for two minutes. CAA plate was again transferred to the magnetic stand

for two minutes (until the supernatant has been cleared). Then 50 µl of the supernatant from the CAA plate were transferred to a new labelled NTA plate.

Library normalization step was performed to ensure an equal representation of the library in the pooled samples. 20 µl of the supernatant taken earlier were transferred to a new labelled Library Normalization Plate (LNP). A mixture was prepared using Library Normalization Additives 1 (LNA1) together with Library Normalization Beads (1LNB1) as recommended by the supplier then 45 µl combined LNA1/LNB1 were added to each well of the LNP containing libraries then the plate was placed on microplate shaker set at 1800 rpm for 30 minutes. LNP was then transferred to a magnetic stand and left for two minutes until the supernatant was cleared, supernatant was then removed and discarded. Washing each sample well twice, using 45 µl LN Wash1. The plate was then transferred to a magnetic stand and left for two minutes until the supernatant was cleared, supernatant was then removed and discarded in an appropriate hazardous waste container. For elution, a fresh stock solution of 30 µl 0.1 N NaOH were added to each well, the plate was then placed on shaker for 5 minutes at 1800 rpm. The LNP was then placed on the magnetic stand for two minutes until the liquid was cleared. 30 µl from the supernatant were transferred from the LNP plate to a new labelled storage plate (SGP) where 30 µl LN Storage buffer 1 were added to each well. Sealing and centrifugation of the SGP for 1 minute at 1000 x g was done.

Library Pooling and MiSeq Sample Loading where equal volumes (5 µl) of the normalized library were combined into a fresh Eppendorf tube then diluted by hybridization buffer; this was followed by heat denaturing (at 96°C for 2 minutes) before sequencing, finally the diluted amplicon library was loaded into the Miseq

reagent cartridge into the Load Samples reservoir. Sequence of the library was performed as indicated in the MiSeq System User Guide.

Illumina sequencing was performed to result in paired end 250 bp reads in the sequencing facility of New York University, Abu Dhabi.

2.10.3 Analysis of bacterial whole genome sequences.

Illumina paired end 250 bp reads in fastq format were assembled using Velvet 1.1.04. MLST and cgMLST of strains was established and the minimum spanning tree was build using Ridom^{TM} SeqSphere+. The assembled reads were uploaded to the pathogen watch web based service (https://pathogen.watch) for the analysis by Kleborate for the virulence gene and capsule locus related information [55, 446].

Similarly, for the detection of acquired resistance genes the assembled contigs were uploaded to the ResFinder website (https://cge.cbs.dtu.dk//services/ResFinder/) [447].

In case the Velvet assembly lacked an expected carbapenemase gene, the respective reads were uploaded to the Center of Genomic Epidemiology website for assembly using SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/) and the contigs produced were re-analyzed in the ResFinder.

2.11 Statistical analysis

Two-tailed Fisher's exact test was used to compare categorical variables of different groups of isolates, while in the case of continuous variables the unpaired t-test was employed using the GraphPad Prism software.

The normal distribution of age was confirmed by calculating the Skewness and Kurtosis values for each data sets. p values ≤0.05 were considered statistically significant.

2.12 Ethical approval

For samples collected from Dubai hospitals ethical approval was obtained from the Dubai Scientific Research Ethics Committee of Dubai Health Authority with the reference code DSREC-11.

Studying the role of IncX3 plasmids and the *K. pneumoniae* ST4 clone in the emergence of CRE in the UAE involved previously established collections of strains stored without using any patient identifiers; therefore, ethical approval was not necessary.

Chapter 3: Results

3.1 Carbapenemase carrying IncX3 plasmids in the United Arab Emirates

3.1.1 Characteristics of carbapenemase producing *Enterobacteriaceae* harboring IncX3 type plasmids

Of the 334 non-repeat carbapenem resistant *Enterobacteriaceae* (CRE) isolated between April 2009 and December 2014 from 12 hospitals of the UAE (Figure 4) screening for the IncX3 type by PCR identified 32 strains carrying IncX3 plasmids, *i.e.* 13 *Escherichia coli*, 15 *Klebsiella pneumoniae*, 2 *Enterobacter cloacae* and one *Citrobacter freundii* and one *Morganella morganii*, respectively. The distribution of IncX3 plasmids among strains with various carbapenem resistance mechanisms is shown in Table 8. Two *K. pneumoniae* carrying IncX3 plasmids did not produce any carbapenemase.

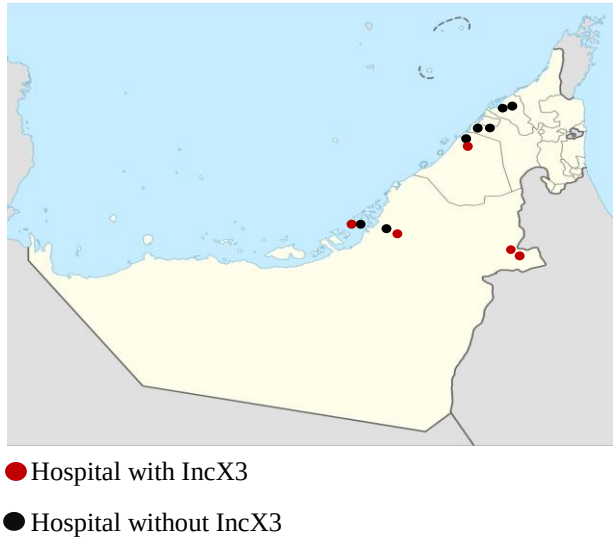

Figure 4: Geographical location of the 12 hospitals where 334 CRE were collected

Table 8: Distribution of IncX3 plasmid carrying isolates among strains expressing different carbapenem resistance mechanisms

***Enterobacteriaceae* species**	**IncX3 plasmid**		**Total**
	Detected	Not detected	
Non carbapenemase-producer [n, (%)]	2 (5.1)	37 (94.9)	39
Carbapenemase-producer [n, (%)]	30 (10.2)	265 (89.8)	295
KPC	2	-	
NDM	18	71	
OXA-48-like	6	120	
NDM and OXA-48-like	4	71	
VIM	0	3	
Total	32	302	334

In all the 30 carbapenemase producer *Enterobacteriaceae* (CPE) strains, the respective carbapenemase genes, in case of the double carbapenemase producers the *bla*$_{\text{NDM}}$ gene, were located on the IncX3 plasmid. Altogether 22 IncX3 plasmids carried *bla*$_{\text{NDM}}$ (13 NDM-1, 1 NDM-4, 6 NDM-5 and 2 NDM-7), 6 contained *bla*$_{\text{OXA-181}}$ and 2 harbored *bla*$_{\text{KPC-2}}$. The characteristics of these strains and their respective IncX3 plasmids are shown in Table 9.

Some clonal clustering among the strains was encountered. The 13 *E. coli* strains represented 8 sequence types, of which only two contained more than 2 strains. All members of one of these clusters (ST410) produced OXA-181, while single members of the ST167 cluster produced OXA-181, NDM-7 or NDM-5.

The 13 *K. pneumoniae* strains were divided into 6 sequence types, of which a cluster of 5 *K. pneumoniae* ST11 strains produced NDM-1 and were isolated over a three years period of time from the same hospital. Also, a triplet of ST1318 coproducing NDM-1 and OXA-48 was identified (Table 9).

All IncX3 positive carbapenemase producer isolates were non-susceptible to at least one carbapenem. Resistance to 3rd generation cephalosporins, aztreonam, aminoglycosides, ciprofloxacin, co-trimoxasole, tetracycline, tigecycline, colistin and fosfomycin was variable (Table 10).

Table 9: Characteristics of carbapenemase producing *Enterobacteriaceae* harboring IncX3 type plasmids with carbapenemase genes

Isolate							Plasmid			
Name	**Date of isolation**	**Hospital**	**Specimen**	**Species**	**Carbapenemase produced**	**MLST**	**Name**	**Size (bp)**	**Resistance gene(s)**	**GenBank Acc. No**
ABC133	12/14/2012	TH	sputum	*E.coli*	NDM-7	ST4108	pABC133-NDM	37070	bla_{NDM-7}	KX214671*
ABC239	8/15/2013	RH	urine	*E.coli*	OXA-181	ST410	pABC239-OXA-181	51479	$bla_{OXA-181}$ + *qnrS1*	MK412916
ABC264	6/9/2014	TH	unknown	*E.coli*	OXA-181	ST410	pABC264-OXA-181	51479	$bla_{OXA-181}$ + *qnrS1*	MK412917
ABC356	8/8/2014	MH	urine	*E.coli*	OXA-181	ST410	pABC356-OXA-181	51479	$bla_{OXA-181}$ + *qnrS1*	MK412918
ABC381	11/4/2014	AAH	rectal swab	*E.coli*	OXA-181	ST167	pABC381-OXA-181	51479	$bla_{OXA-181}$ + *qnrS1*	MK412919
ABC218	12/25/2012	RH	wound	*E.coli*	NDM-7	ST167	pABC218-NDM	34403	bla_{NDM-7}	KX214670*
ABC233	7/21/2013	RH	urine	*E.coli*	NDM-5	ST167	pABC233-NDM-5	46161	bla_{NDM-5}	MK372390
ABC384	11/5/2014	AAH	urine	*E.coli*	NDM-5	ST1284	pABC384-NDM-5	46161	bla_{NDM-5}	MK372389
ABC54	1/2/2011	TH	urine	*E.coli*	NDM-1	ST2206	pABC54-NDM-1	53023	bla_{NDM-1} + bla_{SHV-12}	MK372382
BC-13-836	9/24/2013	TH	blood	*E.coli*	NDM-1	ST446	pBC836-NDM-1	52565	bla_{NDM-1} + bla_{SHV-11}	MK372387
ABC280	7/15/2014	TH	urine	*E.coli*	NDM-5	ST448	pABC280-NDM5	35502	bla_{NDM-5}	MK372392
ABC286	8/15/2014	TH	blood	*E.coli*	NDM-5	ST448	NT	NT	NT	NT
ABC268	6/11/2014	AAH	urine	*E.coli*	NDM-5	ST2083	pABC268-NDM-5	45232	bla_{NDM-5}	MK372391
ABC40	10/27/2009	TH	wound	*E. cloacae*	NDM-1	ST417	pABC40-NDM-1	54035	bla_{NDM-1} + bla_{SHV-12}	MK372380
ABC302	2/26/2014	MH	urine	*E. cloacae*	NDM-4	ST200	ABC302-NDM-4	49402	bla_{NDM-4}	MK372388
BC-13-947	7/11/2013	TH	blood	*K. pneumoniae*	OXA-181	ST2095	pBC947-OXA-181	51479	$bla_{OXA-181}$ + *qnrS1*	MK412920
ABC260	3/31/2014	TH	rectal swab	*K. pneumoniae*	OXA-181	ST3545	pABC260-OXA-181	51480	$bla_{OXA-181}$ + *qnrS1*	MK412915
ABC369	9/23/2014	TH	abdominal fluid	*K. pneumoniae*	NDM-5 + OXA-162	ST307	pABC369-NDM-5	45252	bla_{NDM-5}	MK372393
ABC137	1/14/2013	MH	wound	*K. pneumoniae*	NDM-1 + OXA-48	ST1318	pABC137-NDM-1	53022	bla_{NDM-1} + bla_{SHV-12}	MK372384
ABC141	4/20/2013	MH	unknown	*K. pneumoniae*	NDM-1 + OXA-48	ST1318	NT	NT	NT	NT
ABC155	6/5/2013	SKMC	blood	*K. pneumoniae*	NDM-1 + OXA-48	ST1318	NT	NT	NT	NT
ABC220	10/5/2012	RH	wound	*K. pneumoniae*	KPC-2	ST14	pABC220-KPC-2	46900	bla_{KPC-2}	MK412914
ABC224	3/17/2013	RH	sputum	*K. pneumoniae*	KPC-2	ST14	NT	NT	NT	NT
ABC52	9/19/2010	TH	sputum	*K. pneumoniae*	NDM-1	ST11	pABC52-NDM-1	52565	bla_{NDM-1} + bla_{SHV-12}	MK372381
ABC53	9/19/2010	TH	sputum	*K. pneumoniae*	NDM-1	ST11	NT	NT	NT	NT
BC680	7/18/2012	TH	blood	*K. pneumoniae*	NDM-1	ST11	NT	NT	NT	NT
BC700	7/24/2012	TH	blood	*K. pneumoniae*	NDM-1	ST11	pBC700-NDM-1	52565	bla_{NDM-1} + bla_{SHV-11}	MK372386
BC-13-817	9/17/2013	TH	blood	*K. pneumoniae*	NDM-1	ST11	NT	NT	NT	NT
ABC80	5/8/2011	TH	urine	*Citrobacter freundii*	NDM-1	NA	pABC80-NDM-1	53023	bla_{NDM-1} + bla_{SHV-12}	MK372383
ABC140	3/25/2013	MH	perianal swab	*Morganella morganii*	NDM-1	NA	pABC140-NDM-1	52591	bla_{NDM-1}	MK372385

MLST Multi-Locus Sequence Typing, ST sequence type, NA not applicable, NT not tested, * from [431]

Table 10: Minimum inhibitory concentration of different antibiotics against the clinical isolates of carbapenemase producing *Enterobacteriaceae* harboring IncX3 type plasmids with carbapenemase genes and their derivatives

Strain	Carbapenemase produced	Wild type/ transconjugant/ transformant	Ertapenem	Imimpenem	Meropenem	Ceftazidime	Cefotaxime	Aztreonem	Ciprofloxacin	Gentamicin	Amikacin	Co-trimoxazole	Tetracycline	Colistin	Tigecycline	Fosfomycin
ABC220	KPC-2	WT	256	64	128	>128	>128	>128	64	256	16	>256	4	32	0.5	256
GM3163(pABC220-KPC-2)	KPC-2	TF	4	4	2	16	4	>128	≤0.125	1	1	≤0.5	≤0.5	≤0.5	≤0.125	16
ABC224	KPC-2	WT	256	64	128	>128	>128	>128	32	256	32	>256	4	32	0.5	128
ABC140	NDM-1	WT	2	32	8	128	32	4	8	2	4	≤0.5	16	>256	4	>512
DH5α(pABC140-NDM-1)	NDM-1	TF	0.5	2	≤0.25	>128	64	32	≤0.125	≤0.5	2	≤0.6	≤0.5	≤0.5	≤0.125	≤0.25
ABC40	NDM-1	WT	32	16	16	>128	>128	>128	>64	128	4	≤0.7	256	2	1	8
J53RAZ(pABC40-NDM-1)	NDM-1	TC	0.25	4	4	>128	64	32	≤0.125	≤0.5	≤0.5	≤0.8	≤0.5	≤0.5	≤0.125	0.5
ABC52	NDM-1	WT	64	32	64	>128	>128	>128	>64	>256	>256	>256	4	≤0.5	1	4
J53RAZ(pABC52-NDM-1)	NDM-1	TC	1	4	8	>128	64	64	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	1
ABC53	NDM-1	WT	64	64	64	>128	>128	>128	>64	>256	>256	>256	4	≤0.5	2	16
ABC54	NDM-1	WT	8	16	16	>128	128	>128	0.25	1	4	>256	128	≤0.5	0.25	0.5
DH5α(pABC54-NDM-1)	NDM-1	TF	0.25	4	4	>128	64	32	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	0.5
ABC80	NDM-1	WT	8	8	4	>128	128	>128	4	32	1	128	≤0.5	≤0.5	0.25	0.5
J53RAZ(pABC80-NDM-1)	NDM-1	TC	0.5	8	4	>128	64	32	≤0.125	1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	0.5
BC680	NDM-1	WT	16	32	64	>128	>128	>128	>64	4	16	>256	2	≤0.5	2	4
BC700	NDM-1	WT	16	64	32	>128	>128	>128	>64	2	16	>256	2	≤0.5	2	4
J53RAZ(pBC700-NDM-1)	NDM-1	TC	2	4	4	>128	64	≤0.25	≤0.125	1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	0.5
BC-13-817	NDM-1	WT	16	32	32	>128	>128	>128	>64	4	16	>256	2	≤0.5	2	4
BC-13-836	NDM-1	WT	4	16	16	>128	>128	≤0.25	≤0.125	2	4	≤0.5	128	≤0.5	≤0.125	0.5
DH5α(pBC836-NDM-1)	NDM-1	TF	0.25	2	≤0.25	>128	64	≤0.25	≤0.125	≤0.5	1	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC141	NDM-1 + OXA-48	WT	16	64	32	>128	>128	>128	>64	128	8	>256	>256	4	16	8
ABC155	NDM-1 + OXA-48	WT	>256	>128	>128	>128	>128	>128	4	128	4	>256	>256	≤0.5	2	128
ABC137	NDM-1+ OXA-48	WT	16	64	32	>128	>128	>128	4	128	4	>256	>256	≤0.5	2	8
J53RAZ(pABC137-NDM-1)	NDM-1	TC	0.25	4	4	>128	64	32	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	0.5
ABC302	NDM-4	WT	128	64	128	>128	>128	>128	64	>256	>256	>256	8	≤0.5	2	16
DH5α(pABC302-NDM-4)	NDM-4	TF	0.25	2	≤0.25	>128	64	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC233	NDM-5	WT	32	16	32	>128	>128	>128	>64	>256	>256	128	1	≤0.5	≤0.125	0.5
DH5α(pABC233-NDM-5)	NDM-5	TF	0.5	2	≤0.25	>128	64	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC268	NDM-5	WT	32	16	32	>128	>128	32	>64	64	4	256	1	≤0.5	0.25	1
DH5α(pABC268-NDM-5)	NDM-5	TF	0.25	2	≤0.25	>128	64	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25

WT wild type, TC transconjugant, TF transformant, R recipient

Table 10: Minimum inhibitory concentration of different antibiotics against the clinical isolates of carbapenemase producing *Enterobacteriaceae* harboring IncX3 type plasmids with carbapenemase genes and their derivatives (continued)

Strain	Carbapenemase produced	Wild type/ transconjugant/ transformant	Ertapenem	Imimpenem	Meropenem	Ceftazidime	Cefotaxime	Aztreonem	Ciprofloxacin	Gentamicin	Amikacin	Co-trimoxazole	Tetracycline	Colistin	Tigecycline	Fosfomycin
ABC280	NDM-5	WT	32	16	16	>128	>128	>128	>64	64	8	>256	1	≤0.5	0.25	1
J53RAZ(pABC280-NDM-5)	NDM-5	TC	0.5	4	8	>128	128	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	1
ABC286	NDM-5	WT	64	8	32	>128	>128	>128	>64	32	4	256	1	≤0.5	0.25	1
ABC384	NDM-5	WT	64	128	32	>128	>128	>128	>64	64	8	256	>256	≤0.5	≤0.125	0.5
J53RAZ(pABC384-NDM-5)	NDM-5	TC	0.25	4	4	>128	64	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	1
ABC369	NDM-5 + OXA-162	WT	4	8	8	>128	>128	>128	>64	256	>256	>256	4	≤0.5	2	4
DH5α(pABC369-NDM-5)	NDM-5	TF	0.25	2	≤0.25	>128	64	≤0.25	≤0.125	≤0.5	1	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC133ETP	NDM-7	WT	128	128	128	>128	>128	64	>64	64	8	≤0.5	>256	≤0.5	0.25	0.5
DH5α(pABC133-NDM)	NDM-7	TF	1	4	2	>128	64	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC218	NDM-7	WT	64	16	32	>128	>128	>128	>64	256	8	256	1	≤0.5	≤0.125	0.5
J53RAZ(pABC218-NDM)	NDM-7	TF	2	8	8	>128	128	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	1
ABC239	OXA-181	WT	2	0.5	≤0.25	>128	>128	>128	>64	128	8	>256	>256	≤0.5	≤0.125	0.5
DH5α(pABC239-OXA-181)	OXA-181	TF	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC260	OXA-181	WT	128	64	32	2	2	0.5	16	1	1	>256	4	16	2	>512
DH5α(pABC260-OXA-181)	OXA-181	TF	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC264	OXA-181	WT	2	1	≤0.25	>128	>128	>128	>64	4	4	256	>256	≤0.5	0.25	1
DH5α(pABC264-OXA-181)	OXA-181	TF	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC356	OXA-181	WT	2	1	0.5	>128	>128	>128	>64	128	8	>256	>256	≤0.5	0.25	0.5
DH5α(pABC356-OXA-181)	OXA-181	TF	0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
ABC381	OXA-181	WT	16	8	8	>128	128	8	64	2	2	256	256	≤0.5	0.25	≤0.25
DH5α(pABC381-OXA-181)	OXA-181	TF	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
BC-13-947	OXA-181	WT	≤0.125	2	0.5	16	2	64	1	2	2	>256	1	8	0.25	16
DH5α(pBC947-OXA-181)	OXA-181	TF	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
DH5α	none	R	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	1	1	≤0.5	≤0.5	≤0.5	≤0.125	≤0.25
GM2163	none	R	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	2	4	≤0.5	≤0.5	≤0.5	≤0.125	16
J53Raz	none	R	≤0.125	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.125	1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.125	0.5

WT wild type, TC transconjugant, TF transformant, R recipient

3.1.2 Analysis of the complete sequence of IncX3 plasmids

Based on the type of carbapenemase produced, the MLST and plasmid profile similarities of the host strains (shown in the Appendix); 21 IncX3 plasmids were selected for further analysis.

First, the complete sequences of these plasmids (Table 9) were compared to the sequences of IncX3 plasmids previously described from the UAE (pABC133-NDM and pABC218-NDM) carrying $bla_{\text{NDM-7}}$ [431] (Figure 5A, 5B, 5C).

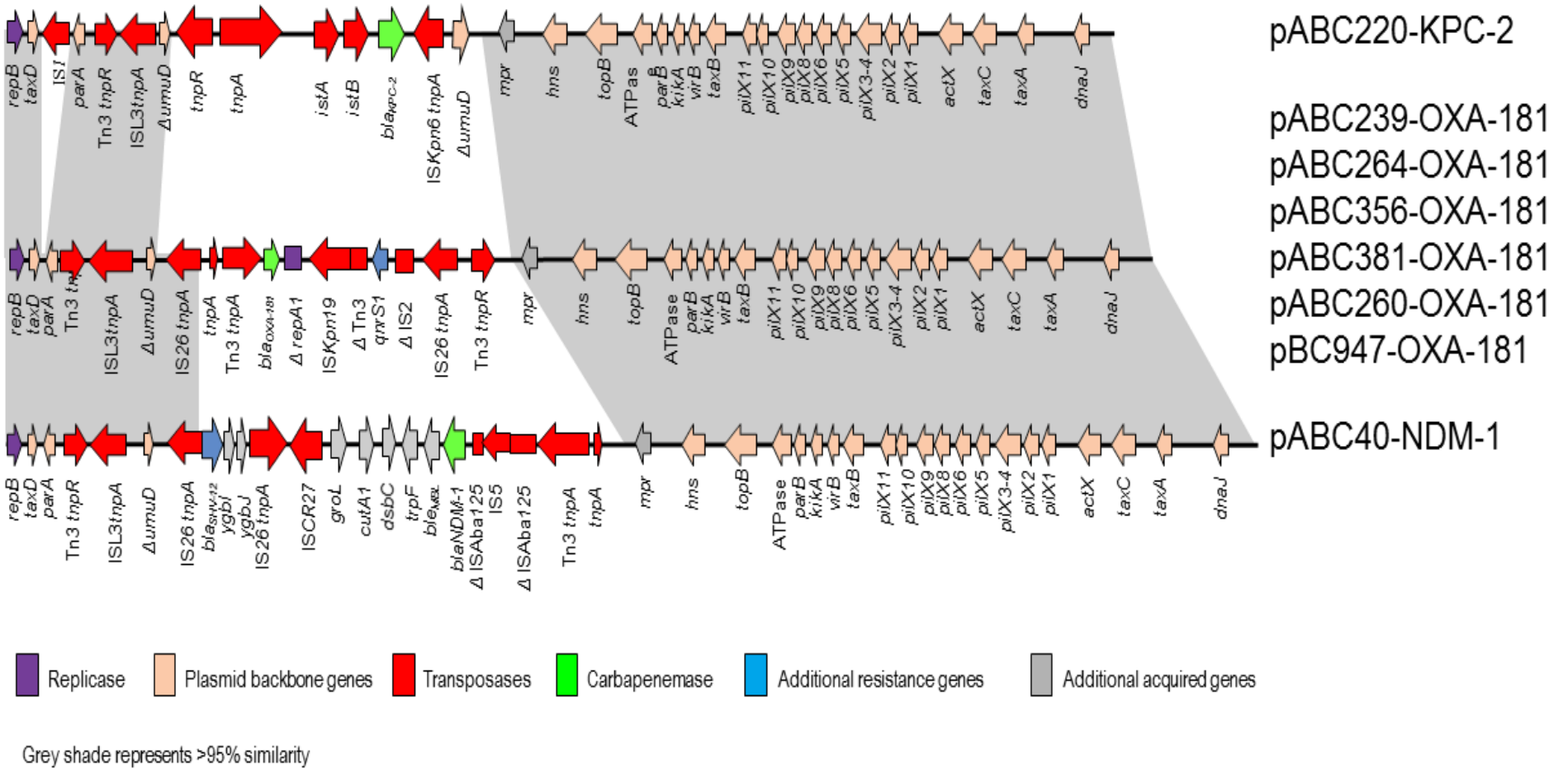

Figure 5A: Schematic representation and comparison of linearized carbapenemase carrying IncX3 plasmids from the UAE

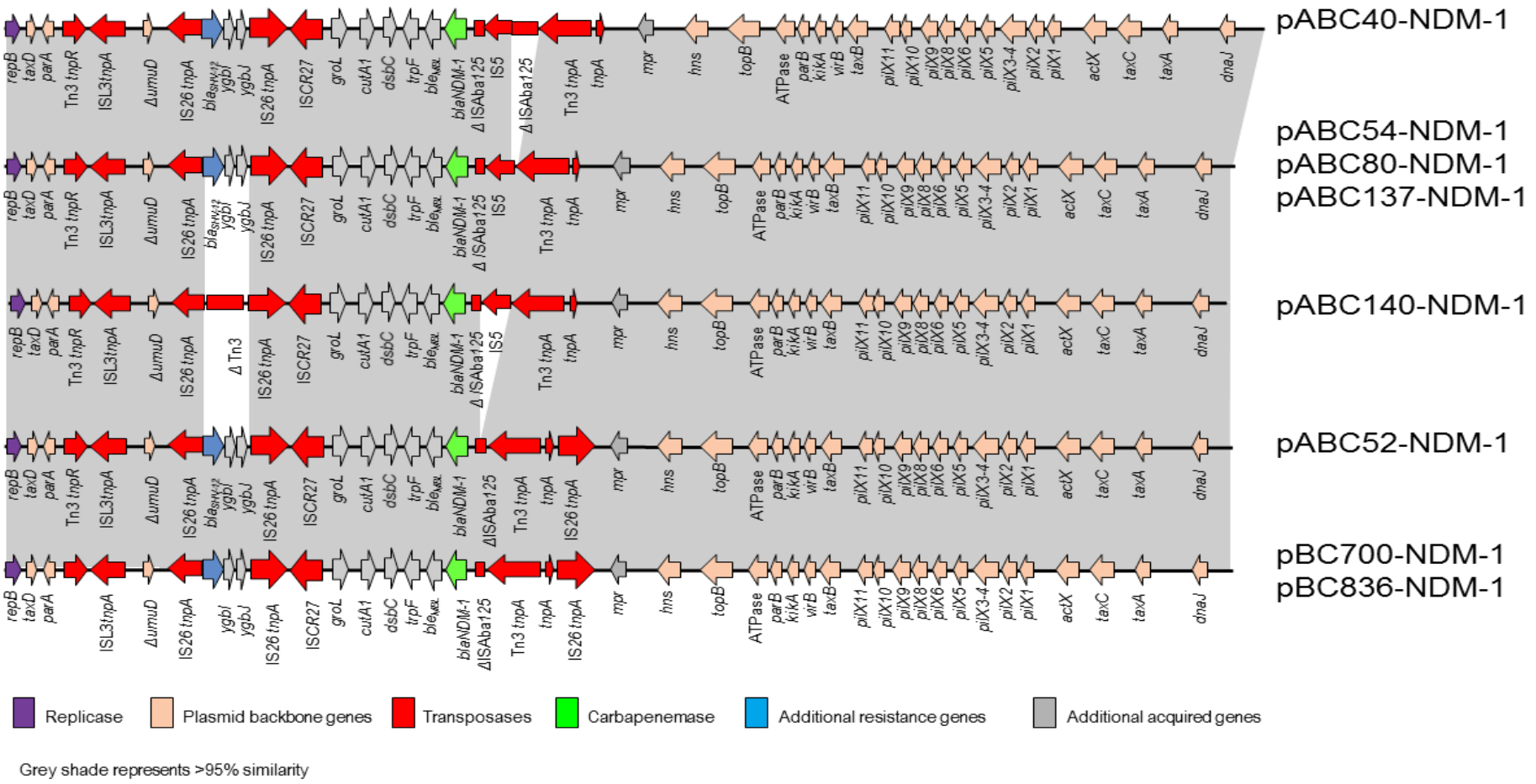

Figure 5B: Schematic representation and comparison of linearized carbapenemase carrying IncX3 plasmids from the UAE (continued)

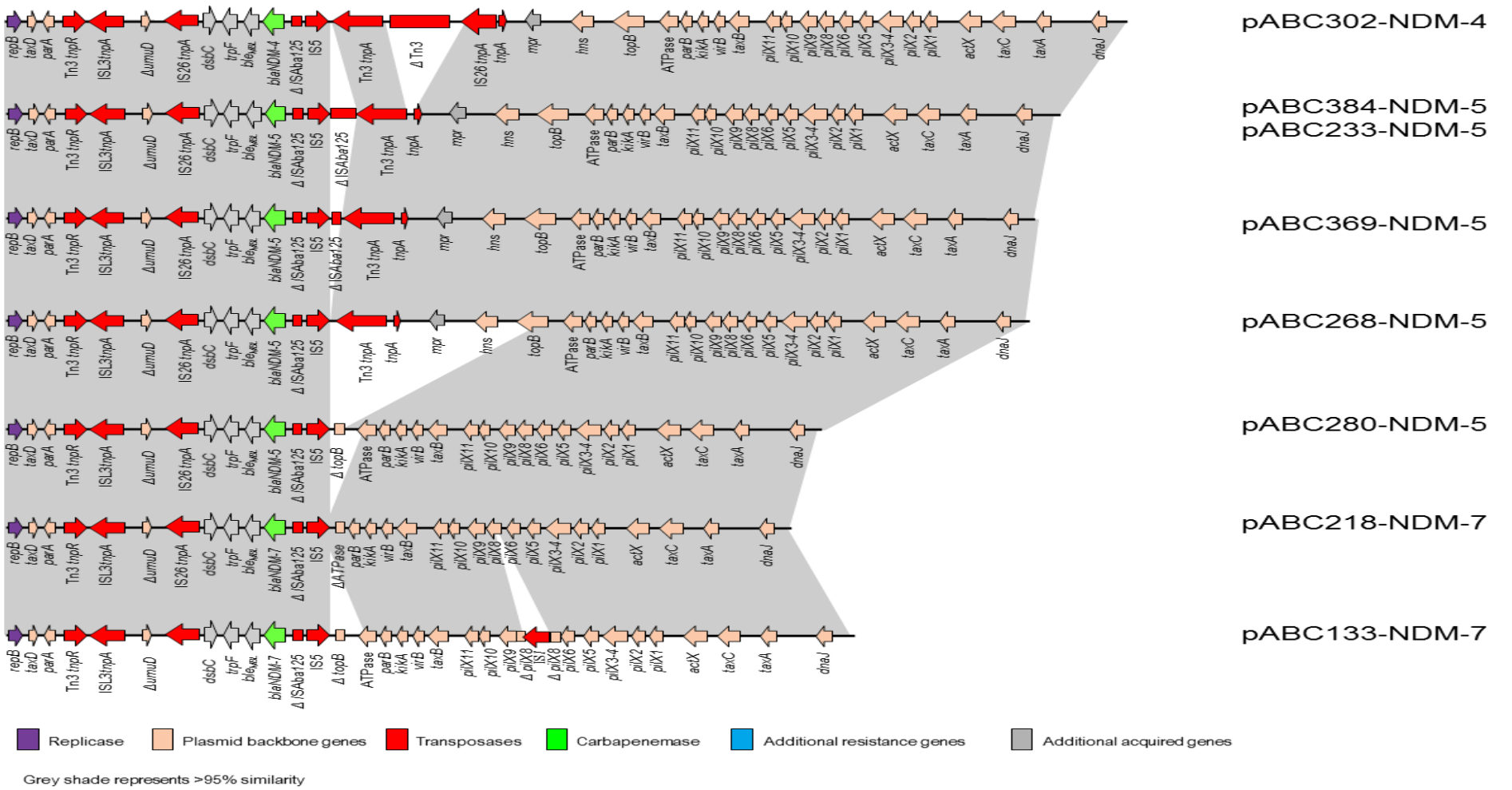

Figure 5C: Schematic representation and comparison of linearized carbapenemase carrying IncX3 plasmids from the UAE (continued)

All the six *bla*$_{\text{OXA-181}}$ carrying plasmids had identical genetic load region containing the carbapenemase gene and a *qnrS1* quinolone resistance gene as well.

Seven of the eight *bla*$_{\text{NDM-1}}$ carrying plasmids also carried a *bla*$_{\text{SHV}}$ ESBL gene in the genetic load region: two the *bla*$_{\text{SHV-11}}$, and five the *bla*$_{\text{SHV-12}}$ allele, respectively. Both *bla*$_{\text{SHV}}$ alleles were present in an IS*26* bracketed composite transposon. In pABC140-NDM-1, this structure contained a truncated Tn*3* transposase instead of the ESBL gene.

IncX3 plasmids carrying NDM alleles other than *bla*$_{\text{NDM-1}}$ did not harbor any other resistance genes. The genetic surroundings of *bla*$_{\text{NDM-4,}}$ *bla*$_{\text{NDM-5,}}$ and *bla*$_{\text{NDM-7}}$ between IS*26* and IS*5* were identical.

In pABC220-KPC-2 the *bla*$_{\text{KPC-2}}$ gene was located on a Tn*4401b* transposon, and no further resistance gene was carried by this plasmid.

Regarding the conserved regions of the plasmids, they were very similar to each other, with the variable presence of the region between the *hns* and *parA* genes described earlier in IncX3 plasmids as part of the basic core structure [409]. Notably, this region was partially or completely missing in pABC280-NDM-5, pABC218-NDM and pABC133-NDM. Therefore, when comparing the plasmid backbones of IncX3 plasmid sequences, we used a 24905 bp-long region from *parB* gene to *parA* gene (from position 1286 to 26190 in pABC218-NDM, Acc.No. KX214670). This region coded for plasmid replication, partitioning and mobilization.

In order to assess the similarities of locally encountered IncX3 plasmids to those identified elsewhere, the above described backbone sequences of 23 UAE IncX3 plasmids were compared to the respective backbone regions of 35 IncX3

plasmids from different geographical regions deposited in the GenBank (listed in Table 11).

Table 11: IncX3 plasmids (retrieved from GenBank in January 2019) from different geographical regions with unique backbone sequences and beta-lactamase genes carried

Resistance genes	**Country**	**Name**	**GenBank Accession No**
bla$_{KPC-2}$	Hong Kong	pKPC-NY79	JX104759
bla$_{NDM-17}$	China	pAD-19R	KX833071
bla$_{NDM-4}$	Myanmar	pM216_X3	AP018146
bla$_{NDM-4}$	Australia	pJEG027	KM400601
bla$_{NDM-4}$	Czech Republic	pEncl-922cz	MG252892
bla$_{NDM-5}$	Czech Republic	pEsco-5256cz	MG252891
bla$_{NDM-5}$	India	pNDM-MGR194	KF220657
bla$_{NDM-5}$	Hong Kong	pNDM-HK2998	MH234508
bla$_{NDM-5}$	Hong Kong	pNDM-HK2967	MH234509
bla$_{NDM-5}$	South Korea	pCREC-591_4	CP024825
bla$_{NDM-7}$	South Korea	pCREC-532_3	CP024833
bla$_{NDM-7}$	Oman	pOM26-NDM	KP776609
bla$_{NDM-7}$	Kuwait	pKW53T-NDM	KX214669
bla$_{NDM-7}$	Canada	pKpN01-NDM-7	CP012990
bla$_{NDM-7}$	Myanmar	pM110-X3	AP018141
bla$_{NDM-7}$	China	pEC50-NDM-7	KX470735
bla$_{OXA-181}$	Italy	pKP_BO_OXA-181	MG228426
bla$_{OXA-181}$, *qnr*S1	China	pOXA-181	KP400525
bla$_{OXA-181}$, *qnr*S1	Switzerland	pKS22	KT005457
bla$_{OXA-181}$, *qnr*S1	Germany	pOXA-181-IHIT35346	KX894452
bla$_{OXA-181}$, *qnr*S1	South Korea	pD6-OXA_1_1	MG702491
bla$_{OXA-181}$, *qnr*S1	Myanmar	pM206-OXA181	AP018831
bla$_{OXA-181}$, *qnr*S1	Czech Republic	pOXA181_29144	KX523903
bla$_{OXA-181}$, *qnr*S1	Lebanon	pSTIB_IncX3_OXA_181	MG570092
bla$_{OXA-181}$, *qnr*S1	Denmark	pAMA1167-OXA-181	CP024806
bla$_{SHV-11}$	Italy	pIncX-SHV	JN247852
bla$_{SHV-11}$, *bla*$_{KPC-3}$	Italy	p45-IncX3	KT362706
bla$_{SHV-12}$	Netherlands	pEC-393	KX618697
bla$_{SHV-12}$	Netherlands	pEC-125	KX618703
bla$_{SHV-12}$, *aac(6')-Ib*	USA	pKPN-819	CP008799
bla$_{SHV-12}$, *bla*$_{KPC-2}$	France	pKpS90	JX461340
bla$_{NDM-1}$ + *bla*$_{SHV-12}$	China	pNDM-HN380	JX104760
bla$_{NDM-1}$ + *bla*$_{SHV-12}$	Hong Kong	pNDM-HK3694	MH234505
bla$_{SHV-12}$, *bla*$_{TEM-1}$, *qnr*S1	Netherlands	pEC-NRS18	KX618696
None	USA	pUCLAOXA232-2	CP012563

The Neighbor-Joining tree constructed using the Jukes-Cantor genetic distance model with 500 bootstrap replicates of the 58 IncX3 plasmids' backbone sequences aligned using ClustalW, depicting the relationship between these plasmid backbones and the evolutionary history inferred is presented in Figure 6.

The comparison shows three distinct clades: one with $bla_{\text{NDM-1,}}$ $bla_{\text{NDM-4}}$ and $bla_{\text{NDM-7}}$ carrying plasmids from the UAE and with plasmids carrying similar carbapenemase genes not only from different countries of the Middle-East and Asia, but also with $bla_{\text{NDM-7}}$ carrying plasmid from Canada and $bla_{\text{SHV-12}}$ carrying plasmid from the Netherlands. $bla_{\text{NDM-5}}$ carrying plasmids formed a distinct clade. $bla_{\text{OXA-181}}$ carrying IncX3 plasmids originating from various parts of the world formed another distinct clade with a single outlier (MG228426) from Italy, only. Plasmids coding for KPC were more heterogenous.

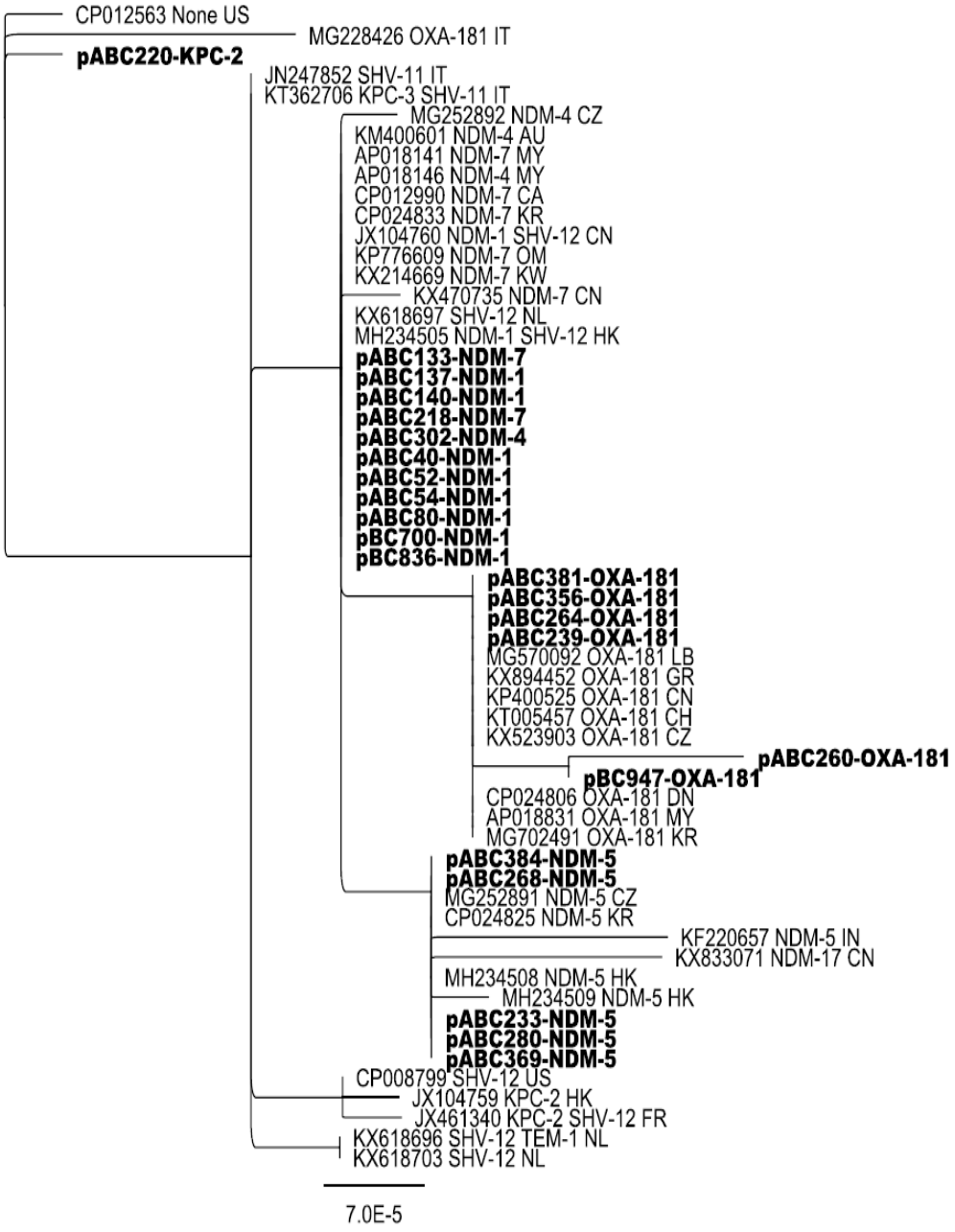

Figure 6: Phylogenetic tree of IncX3 plasmid backbones

Plasmid names printed in bold represent IncX3 plasmids from the UAE, for plasmids retrieved from GenBank the accession number, the beta-lactamase gene carried, and the country of isolation is shown.
(AU Australia, US United States of America, IT Italy, MY Myanmar, CA Canada, KR South Korea, CN China, OM Oman, KW Kuwait, NL The Netherlands, HK Hong Kong, LB Lebanon, CH Switzerland, CZ Czech Republic, GR Germany, DN Denmark, IN India, FR France)

3.2 Characterization of carbapenem resistant *Enterobacteriaceae* isolated in 2015-2016 in Dubai, United Arab Emirates

3.2.1 The collection

During the study period, *i.e.* between June 2015 and June 2016, of the 13025 *Enterobacteriacea* isolates encountered in the participating hospitals 4.6% was identified as CRE, a feature being the most common (16.2%) among *K. pneumoniae* (Table 12).

Table 12: Rate of CRE isolated and included in the study

Species	**Total number isolated**	**CRE encountered N (%)**	**CRE studied N (%)**
Klebsiella pneumoniae	2900	470 (16.2)	70 (14.9)
Escherichia coli	8112	52 (0.6)	13 (25.0)
Other *Enterobacteriaceae*	2013	81 (4.0)	6 (7.4)
All	13025	603 (4.6)	89 (14.8)

Of these, altogether 107 strains were submitted for the current study. Two strains were excluded for being carbapenem susceptible by the time of the confirmatory test and further 16 were omitted as they represented multiple isolates from same patients. The remaining 89 CRE strains (14.8% of all CRE isolated during the study period), i.e. 70 *Klebsiella pneumoniae*, 13 *Escherichia coli*, 3 *Enterobacter cloacae*, 2 *Citrobacter* sp. and one *Serratia marcescens* were subjected to further investigations. Details of the collection are summarized in Table 13.

Table 13: General characteristics and distribution of various carbapenemases in different species of CRE studied

Characteristics	***Enterobacteriaceae* infections** n (%)				
	All	**Carbapenamase-producer**			**Non carbapenemase-producer ***
		NDM	**OXA-48-like**	**NDM and OXA-48-like**	
Age					
<65 years	31 (34.8%)	9	14	4	4
≥65	58 (65.2%)	10	27	13	8
Gender					
Female	33 (37%)	10	12	4	7
Male	56 (63%)	9	29	13	5
Ethnicity					
UAE	31(34.8%)	5	14	7	5
Non-UAE national	44 (49.4%)	11	20	8	5
Unknown	14(15.7%)	3	7	2	2
Hospital					
Private	25 (28.1%)	8	10	3	4
Public	64 (72.9%)	11	31	14	8
Sample source					
Blood	7 (7.9%)	2	5	0	0
Respiratory	20 (22.5%)	1	12	6	1
Skin and soft tissue	17 (19%)	3	7	1	6
Urine	45 (50.6%)	13	17	10	5
Organism					
Escherichia coli	13 (14.6%)	2	7	0	4
Klebsiella pneumoniae	70 (78.7%)	16	31	17	6
Enterobacter cloacae	3 (3.4%)	1	0	0	2
Citrobacter species	2 (2.2%)	0	2	0	0
Serratia marcescens	1 (1.1%)	0	1	0	0

* confirmed with the carbaNP test

The *mcr-1, -2, -3* or *-4* genes were not detected among the colistin resistant members of the pool. Eighty seven percent of the isolates produced carbapenemase, while 13% of them tested negative with the carbaNP test. bla_{IMP}, bla_{VIM}, and bla_{KPC} genes were not identified in the 77 carbapenemase producer strains, whereas in 53% of them $bla_{\text{OXA-48-like}}$, in 25% bla_{NDM}, and in 24% both $bla_{\text{OXA-48-like}}$ and bla_{NDM} were

detected, respectively. The distribution of the carbapenemase genes detected in various species is shown in Table 13.

3.2.2 Antibiotic susceptibility and the presence of antibiotic resistance genes

The antibiotic susceptibilities, the MIC_{90} and MIC_{50} figures for *K. pneumoniae* and *E. coli* isolates, *i.e.* species for which these calculations were possible, are summarized in Table 14.

Table 14: Antibiotic susceptibility of the CRE strains isolated in Dubai

Organism (Nº tested) /antibiotic	**MIC (mg/L)**		**% Susceptible**
	50%	90%	
All isolates (89)			
ampicillin	>32	>32	0
amoxicillin/clavulanate	>32/16	>32/16	0
piperacillin/ tazobactam	>128/4	>128/4	0
cefotaxime	>64	>64	4.5
ceftazidime	>64	>64	9.0
cefepime	>64	>64	16.7
aztreonam	>128	>128	12.4
ertapenem	>64	>64	0
imipenem	>16	>16	21.3
meropenem	>16	>16	16.9
amikacin	32	>64	46.1
gentamicin	8	>16	43.8
ciprofloxacin	>4	>4	4.5
nitrofurantoin	128	256	10.1
trimethoprim/sulfamethoxazole	>16/304	>16/304	18.0
tigecycline	≤1	>8	61.8
chloramphenicol	32	>256	29.2
colistin	≤0.5	32	73
XDR (%)	32.6		
PDR (%)	4.5		

Table 14: Antibiotic susceptibility of the CRE strains isolated in Dubai (continued)

Organism (Nº tested) /antibiotic	**MIC (mg/L)**		**% Susceptible**
	50%	**90%**	
***Klebsiella pneumoniae* (70)**			
ampicillin	NA	NA	NA
amoxicillin/clavulanate	>32/16	>32/16	0
piperacillin/ tazobactam	>128/4	>128/4	0
cefotaxime	>64	>64	1.4
ceftazidime	>64	>64	8.6
cefepime	>64	>64	14.3
aztreonam	>128	>128	11.4
ertapenem	>64	>64	0
imipenem	>16	>16	15.7
meropenem	>16	>16	7.1
amikacin	64	>64	35.7
gentamicin	8	>16	45.7
ciprofloxacin	>4	>4	2.9
nitrofurantoin	256	256	1.4
trimethoprim/sulfamethoxazole	>16/304	>16/304	12.9
tigecycline	≤1	>8	57.1
chloramphenicol	64	>256	20
colistin	≤0.5	32	68.6
XDR (%)	40		
PDR (%)	5.7		
***Escherichia coli* (13)**			
ampicillin	>32	>32	0
amoxicillin/clavulanate	>32/16	>32/16	0
piperacillin/ tazobactam	>128/4	>128/4	0
cefotaxime	>64	>64	15.4
ceftazidime	>64	>64	7.7
cefepime	>64	>64	30.8
aztreonam	>128	>128	15.4
ertapenem	>64	>64	0
imipenem	2	>16	30.8
meropenem	1	>16	53.8
amikacin	4	>64	84.6
gentamicin	>16	>16	38.5
ciprofloxacin	>4	>4	0
nitrofurantoin	64	256	53.8
trimethoprim/sulfamethoxazole	>16/304	>16/304	23.1
tigecycline	≤1	≤1	10
chloramphenicol	8	256	76.9
colistin	≤0.5	≤0.5	100
XDR (%)	0		
PDR (%)	0		

All strains were multi-drug resistant, of these 29 (32.6%) also qualified as extremely-drug resistant (XDR). All *E. coli* strains remained susceptible to tigecycline and colistin, and none of them were XDR. On the other hand, in *K. pneumoniae* the rate of XDR was 40%, the non-susceptibility to tigecycline was 42.9%, and to colistin was as high as 31.4%, respectively. Four *K. pneumoniae* isolates did not show susceptibility to any of the antibiotics tested. The distribution of XDR and PDR isolates among various species is shown in Table 15.

Table 15: Distribution of XDR and PDR isolates among various species

	K. pneumoniae	***Serratia marcescens***	***E. coli***	***E. cloacae***	***Citrobacter spp.***	**All**
XDR (n)	24	1	0	0	0	25
PDR (n)	4	0	0	0	0	4

3.2.3 Molecular typing

Pulsed field gel electrophoresis confirmed the diversity of the *E. coli*, *E. cloacae* and *Citrobacter* sp. isolates (Figure 7-9).

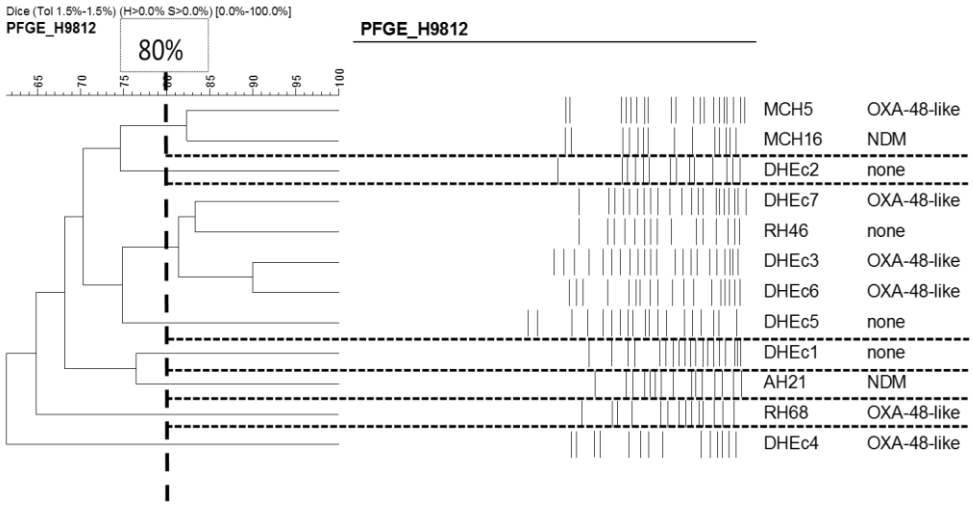

Figure 7: PFGE comparison of carbapenem resistant *E. coli*

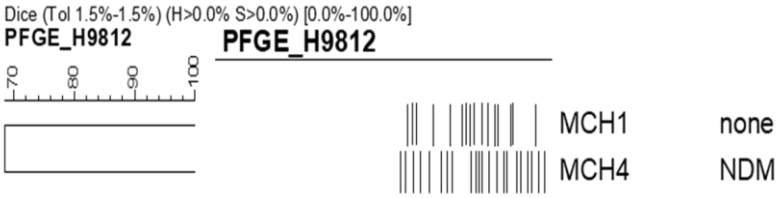

Figure 8: PFGE comparison of carbapenem resistant *E. cloacae*

Figure 9: PFGE comparison of carbapenem resistant *Citrobacter sp.*

This was in sharp contrast with the clustering of *K. pneumoniae* isolates. In this species 35 strains (51.4% of the typable strains) exhibited PFGE patterns with >80% similarity (cluster PF-KP5). The remaining 33 strains grouped into 16 PFGE patterns with two 6-membered clusters, one triplet, five duplets and 8 unique patterns (Figure 10). Two *K. pneumoniae* were untypable by PFGE.

Within cluster PF-KP5 six sub-clusters of >90% similarity, were identified. In each of them at least one strain of every unique carbapenemase gene patterns (i.e. carrying exclusively bla_{NDM} or $bla_{\text{OXA48-like}}$ or both) were subjected to MLST. All 19 strains selected belonged to *K. pneumoniae* ST14 (Figure 10). The sequence type of the four pan-drug resistant *K. pneumoniae* was also determined. Of those two, DHKp8 and DHKp26, *i.e.* strains from the PF-KP5 group, also belonged to ST14, while the two unrelated pan-drug resistant isolates, DHKp9 and DHKp34 exhibited sequence types ST15 and ST231, respectively. Furthermore, selected isolates having unique carbapenemases in the two 6-membered clusters were also MLST-ed. With the exception of the NDM and OXA-48-like co-producer *K. pneumoniae* ST1513 isolate, the other members of these clusters belonged to *K. pneumoniae* ST147 (Figure 10).

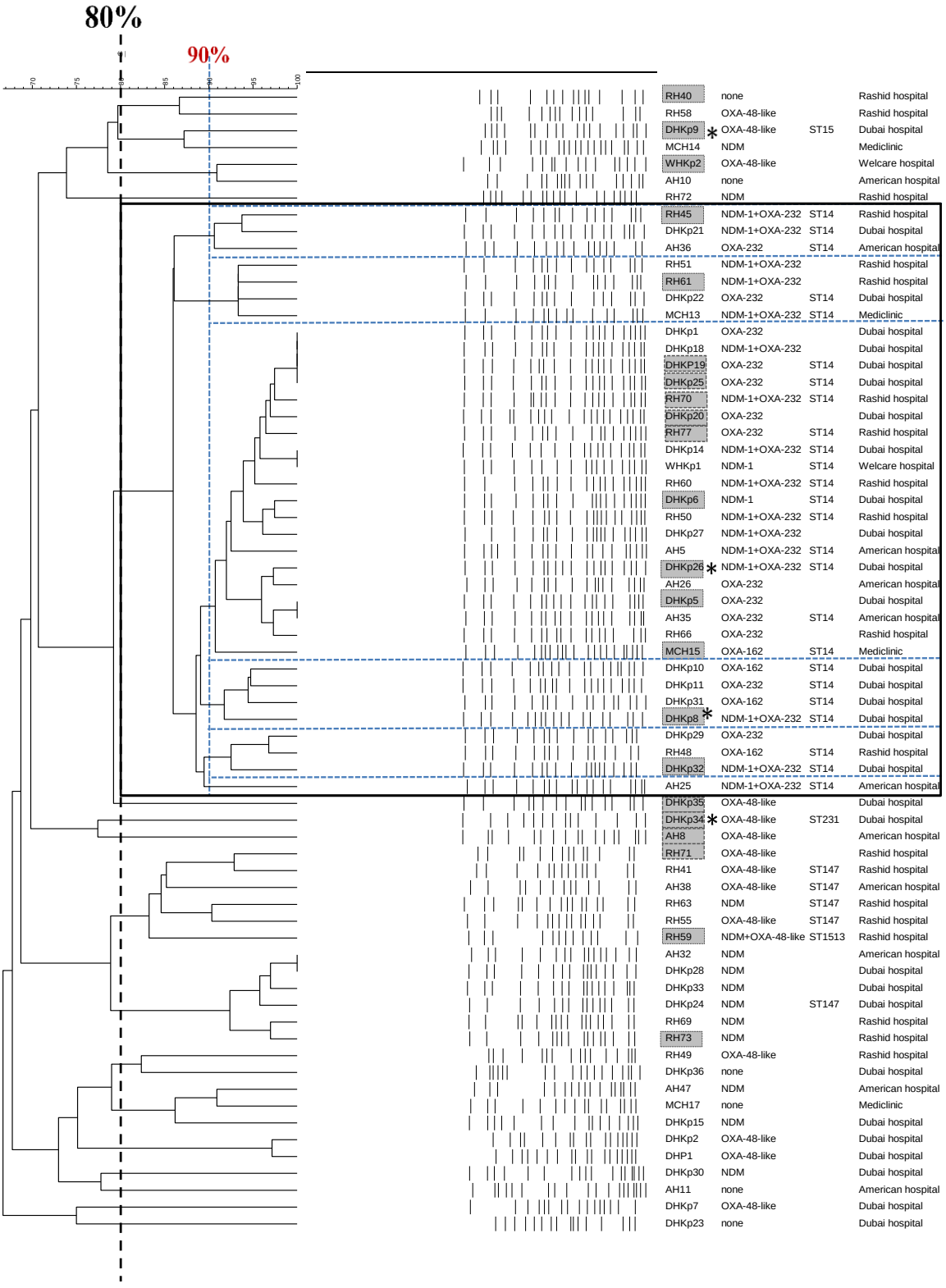

Figure 10: PFGE comparison of carbapenem resistant *Klebsiella pneumoniae*

Grey boxes indicate colistin resistant strains, * marks pan-drug resistant strains, bold rectangular box represent the ST-14 looking like isolates (PF-KP5)

3.2.3.1 Features associated with the *K. pneumoniae* ST14 PF-KP5 group

Strains belonging to this cluster were distributed among all participating hospitals. Analyzing their characteristics revealed that members of this ST14 clone were significantly more likely to be isolated from Emirati patients, to be amikacin non-susceptible, extremely drug resistant (XDR), and to co-produce OXA-48-like and NDM carbapenemases than isolates outside of this cluster (Table 16).

Table 16: Association of various factors with *K. pneumoniae* PF-KP5

	Members of the PF-KP5 pattern (N=35)	**Other *K. pneumoniae* (N=35)**	**P value**
Specimen type			
Blood	1	3	0.6139
Urine	19	18	1.0000
Respiratory	12	5	0.0928
Skin and soft tissue	3	9	0.1103
Patients' characteristics			
Mean age in years ±SD	70.9±12.8	66.5±15.1	0.2198*
Male:female ratio	25:10	22:13	0.6113
Emirati**	15	3	0.0015
Carbapenemase carried			
Carbapenemase non-producer	0	6	0.0248
NDM and OXA-48-like co-producer	16	1	0.0001
Single OXA-48-like producer	17	14	0.6307
Single NDM producer	2	14	0.0012
Antibiotic susceptibility			
Gentamicin non-susceptible	20	18	0.8106
Amikacin non-susceptible	31	14	0.0001
Tigecycline non-susceptible	15	15	1.0000
Colistin resistant	13	9	0.4436
XDR and PDR	19	9	0.0273

*student t test

**Calculated for 59 patients with known nationality

All but one of the 17 NDM-OXA dual carbapenemease producers (94.4%), belonged to *K. pneumoniae* PF-KP5. Although colistin resistance was not significantly associated with this clone, it was more common within the cluster (37.1%) than in *K. pneumoniae* having different pulsotypes (27.3%). Furthermore, all colistin resistant *K. pneumoniae* outside of the PF-KP5 group exhibited distinct PFGE patterns (Figure 10).

3.3 Characterization of *K. pneumoniae* ST14 strains isolated in the Arabian Peninsula

Among 761 non-repeat carbapenem resistant *Klebsiella pneumoniae* (CRKP) isolated in June 2011- June 2016 from five cities in the UAE (Dubai, Abu Dhabi, Sharjah, Um Al Qwain, Al Ain) and from four other countries of the Arabian Peninsula (Kingdom of Saudi Arabia (KSA), Kuwait, Bahrain and Oman), we identified 172 *K. pneumoniae* exhibiting PFGE profiles with ≥80% similarity to known CRKP-ST14 (Figure 11). The number of strains with PFGE patterns similar to CRKP-ST14 in each country is shown in Table 17.

Table 17: Proportion of strains with PFGE patterns similar to CRKP-ST14 in different countries

	UAE	**KSA**	**Kuwait**	**Bahrain**	**Oman**	**All**
N	483	151	66	20	41	761
ST14-like	162 (33.5%)	9 (6%)	0	1 (5%)	0	172 (22.6)

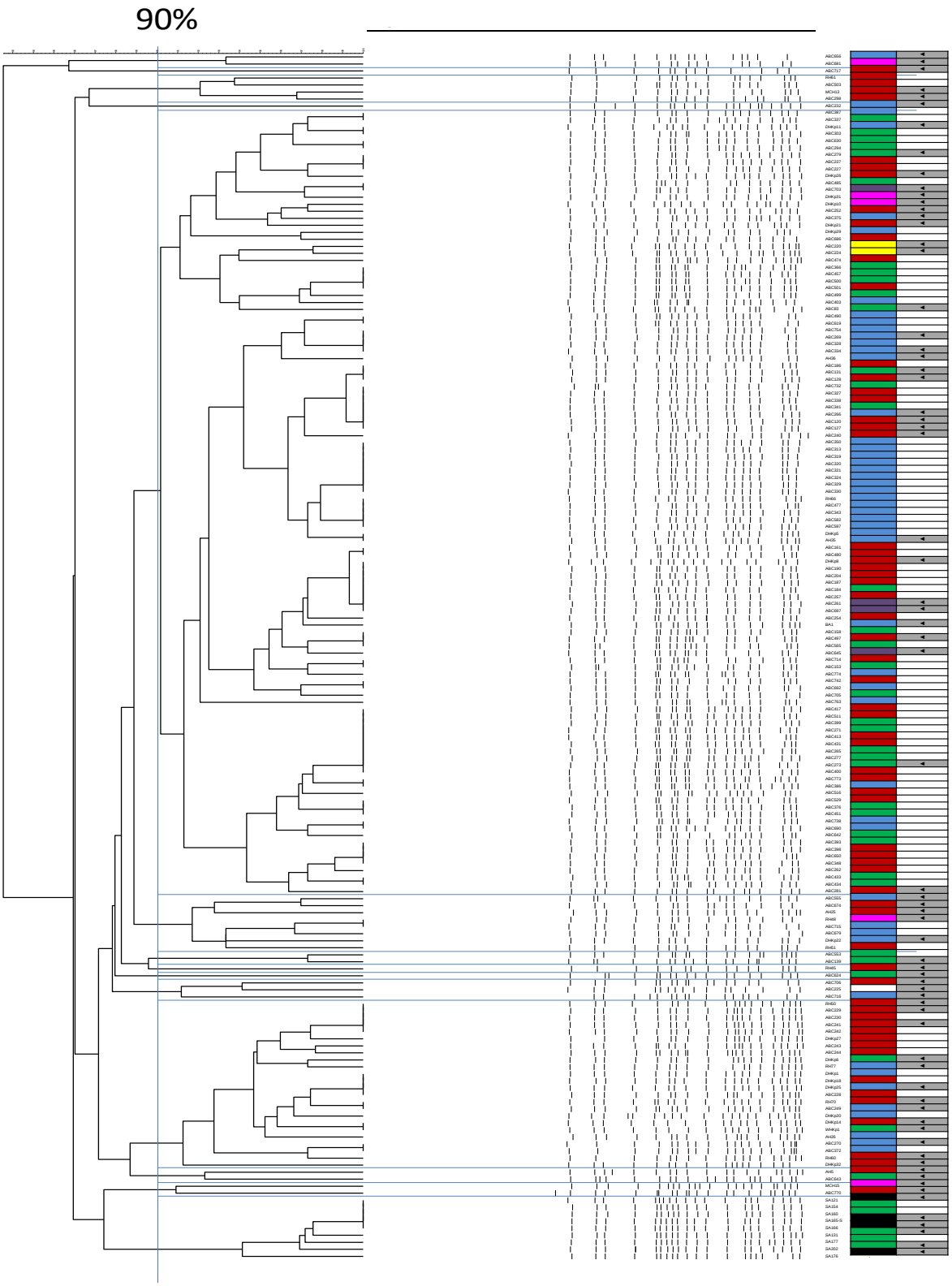

Figure 11: PFGE comparison of 172 carbapenem-resistant *Klebsiella pneumoniae* exhibiting profiles with ≥80% similarity to known CRKP-ST14

The results of KPC-, NDM-, OXA-48-like, VIM-, IMP-carbapenemase alleles identified in the 172 *K. pneumoniae* are shown in Table 18.

Table 18: Carbapenemase genes identified in the 172 CRKP exhibiting ST14 like PFGE patterns

Carbapenemase allele	**N**
bla$_{\text{NDM-1}}$	41
bla$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-232}}$	65
bla$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-162}}$	4
bla$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$	4
bla$_{\text{OXA-162}}$	5
bla$_{\text{OXA-232}}$	50
bla$_{\text{KPC-2}}$	2
No carbapenemase	1

Within the 172 CRKP ST14-like isolates, 14 subgroups exhibiting ≥90% PFGE pattern similarity were formed. At least one isolate from each subgroup, or if they harboured different carbapenemases, one of each with non-identical carbapenemases were subjected to MLST (n=70), which proved that they indeed belonged to ST14 (Figure 11).

To localize the carbapenemase genes in the CRKP-ST14 isolates, plasmid gels were hybridized with the respective probes. (All gel pictures and hybridization membranes are shown in the Appendix). The comparison of plasmid profiles with the schematic presentation of the plasmid hybridizing with the respective carbapenemase probes is shown in Figure 12. However, we were unable to localize the *bla*$_{\text{OXA-232}}$ in ABC241 and in ABC334, and the *bla*$_{\text{NDM-1}}$ in ABC643.

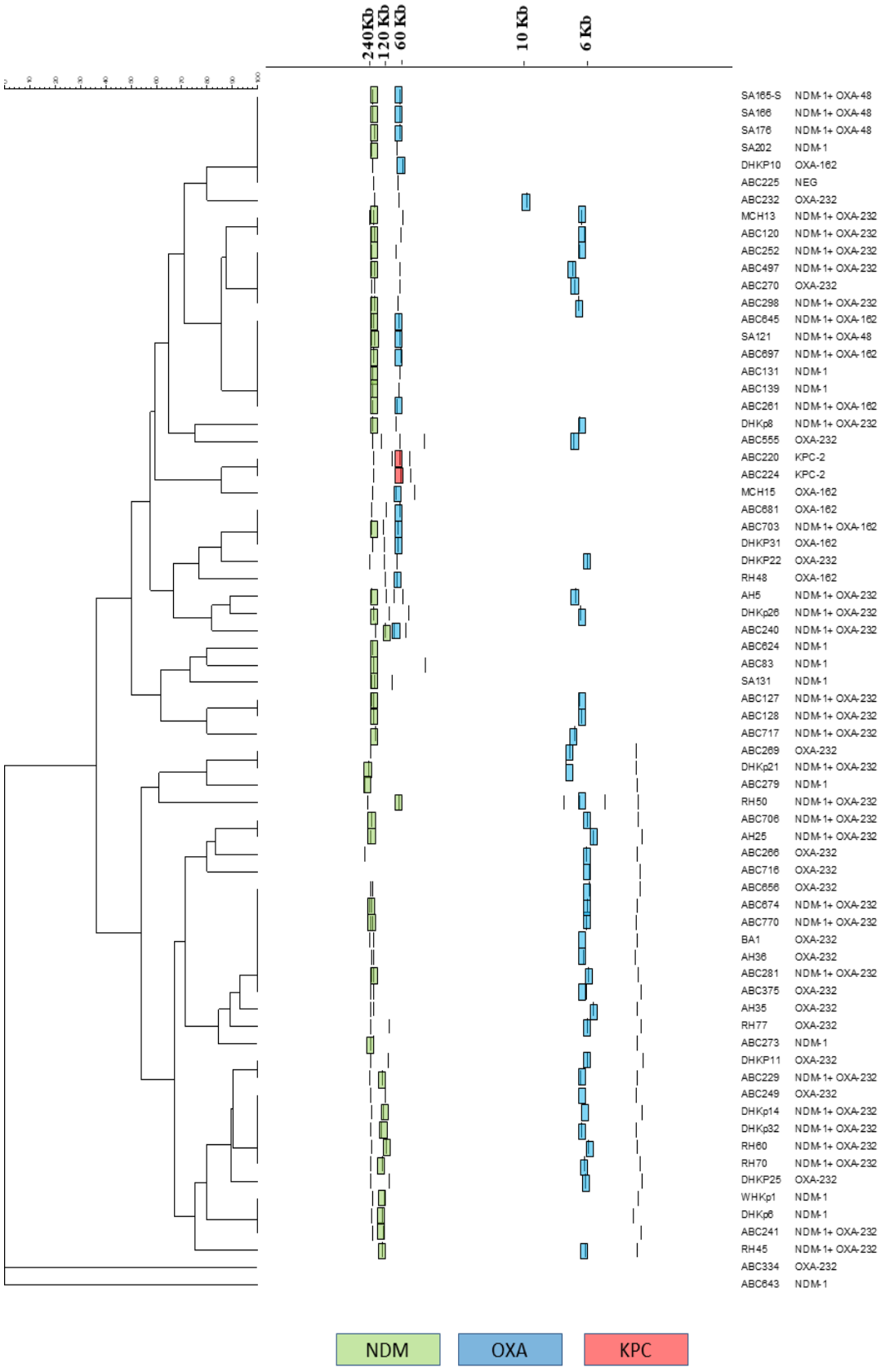

Figure 12: Localization of carbapenemase genes in the 70 CRKP-ST14

Based on the alleles of the carbapenemase genes and the plasmid localizations of these genes, we formed 15 subgroups (SGs) of the 70 CRKP-ST14 as listed in Table 19.

Table 19: Subgroups of CRKP-ST14 with various alleles of carbapenemases located on different size plasmids

Subgroup	N	NDM allele	NDM plasmid size	OXA allele	OXA plasmid size
A	**17**	NDM-1	≥240Kb	OXA-232	≈6Kb
B	**4**	NDM-1	≥240Kb	OXA-48	≈60Kb
C	**4**	NDM-1	≥240Kb	OXA-162	≈60Kb
D	**8**	NDM-1	≥240Kb	none	NA
E	**1**	NDM-1	≈120Kb	OXA-232	≈60Kb
F	**7**	NDM-1	≈120Kb	OXA-232	≈6Kb
G	**2**	NDM-1	≈120Kb	none	NA
H	**1**	NDM-1	≈120Kb	OXA-232	impossible to localize by hybridization
I	**5**	none	NA	OXA-162	≈60Kb
J	**1**	none	NA	OXA-232	≈10Kb
K	**15**	none	NA	OXA-232	≈6Kb
L	**1**	none	NA	OXA-232	impossible to localize by hybridization
M	**1**	NDM-1	impossible to localize by hybridization	none	NA
N	**2**	**KPC-2**			
O	**1**	**Carbapenemase non-producer**			

Of these isolates we selected minimum 4 representatives of each subgroups (if present) for characterization by whole genome sequencing (Table 20).

Table 20: Characteristics of strains subjected to whole genome sequencing

Subgroup	Strain	Year of isolation	Specimen	Hospital	PFGE subgroup
A	ABC127	2012	Blood	AQH, Sharjah	5
A	ABC252	2014	Unknown	ZMH, Abu Dhabi	5
A	ABC497	2015	Urine	TH, Al Ain	5
A	DHKp21	2016	Urine	DH, Dubai	5
B	SA121	2015	Unknown	KFSH, Riyadh, KSA	14
B	SA165/S	2015	Unknown	KFSH, Riyadh, KSA	14
B	SA166	2015	Unknown	KFSH, Riyadh, KSA	14
B	SA176	2015	Unknown	KFSH, Riyadh, KSA	14
C	ABC261	2014	Perianal swab	KH-Um Al Qwain	5
C	ABC645	2015	Urine	SKMC, Abu Dhabi	5
C	ABC697	2014	Perianal swab	KH-Um Al Qwain	5
C	ABC703	2015	Wound	KH-Um Al Qwain	5
D	ABC83	2011	Nasal screen	MH, Abu Dhabi	5
D	ABC131	2012	Rectal swab	AQH, Sharjah	5
D	ABC139	2013	Sacral skin	MH, Abu Dhabi	7
D	ABC273	2014	Sputum	AAH, Al Ain	5
D	SA131	2015	Unknown	KFSH, Riyadh, KSA	14
E	ABC240	2013	Blood	RH, Dubai	5
F	ABC229	2013	Urine	RH, Dubai	11
F	RH60	2016	Urine	RH, Dubai	11
F	RH45	2016	Sputum	RH, Dubai	8
F	DHKp14	2015	Sputum	DH, Dubai	11
G	DHKp6	2015	Urine	DH, Dubai	11
G	WHKp1	2014	Urine	WC, Dubai	11
H	ABC241	2013	Sputum	RH, Dubai	11
I	ABC681	2015	Urine	SKMC, Abu Dhabi	1
I	MCH15	2016	Urine	MCH, Dubai	13
I	DHKp10	2015	Sputum	DH, Dubai	5
I	RH48	2016	Urine	RH, Dubai	6
I	DHKp31	2016	Urine	DH, Dubai	5
J	ABC232	2013	Wound	RH, Dubai	4
K	ABC270	2014	Urine	AAH, Al Ain	11
K	ABC716	2015	Blood	ZMH, Abu Dhabi	10
K	BA1	2015	Blood	BDF, Riffa, Bahrain	5
K	ABC375	2014	Rectal swab	TH, Al Ain	5
L	ABC334	2014	Blood	SKMC, Abu Dhabi	5
M	ABC643	2015	Urine	SKMC, Abu Dhabi	12
N	ABC220	2012	Wound	RH, Dubai	5
O	ABC225	2013	Urine	RH, Dubai	10

The result of the Velvet assembly of the Illumina reads is summarized in Table 21.

Table 21: Assembly report of strains subjected to whole genome sequencing

Genome	Genome Length (bp)	No. Contigs	Smallest Contig	Largest Contig	Average Contig Length	N50	cgMLST good target (%)
ABC127	5809195	125	265	849538	46473	191826	99.5
ABC131	5961443	232	221	524733	25695	173434	99.6
ABC139	5889327	250	200	288472	23557	83557	98.4
ABC220	5699694	171	225	324953	33331	158617	99.3
ABC225	5642359	286	206	130827	19728	49230	98.9
ABC229	5761817	563	209	307021	10234	60604	98.7
ABC232	5860727	393	200	205328	14912	39841	98.6
ABC240	5786071	213	200	393110	27164	97639	99.2
ABC241	5693070	258	206	174151	22066	58851	98.6
ABC252	5846567	156	209	512109	37477	191768	99.4
ABC261	5956594	228	209	696470	26125	140697	99.3
ABC270	5914488	194	204	382134	30487	157499	99.3
ABC273	5815262	230	204	222781	25283	79005	98.2
ABC334	5791551	244	202	173939	23735	64126	99.0
ABC375	5851467	159	213	411735	36801	163126	99.3
ABC497	5734851	159	253	387861	36068	130040	99.4
ABC643	5787685	235	200	329878	24628	65791	98.4
ABC645	5884874	357	206	162653	16484	40201	98.7
ABC681	5789489	812	205	301084	7129	66565	98.9
ABC697	5838890	308	200	249991	18957	49675	99.2
ABC703	5922381	1128	201	164112	5250	38915	98.6
ABC716	5647419	297	200	150240	19014	51873	98.4
ABC83	5861041	162	262	849851	36179	231631	99.5
BA1	5810838	326	202	162719	17824	38062	98.6
DHKp10	5962056	698	201	165672	8541	39392	98.6
DHKp14	5915588	1681	205	75275	3579	20264	96.1
DHKp21	5665306	279	201	238917	20305	53745	98.9
DHKp31	5734296	313	200	230361	18320	50506	98.8
DHKp6	5702039	190	206	393204	30010	97105	98.1
MCH15	5859798	269	200	221252	21783	64226	98.6
RH45	5504435	219	202	213230	25134	75850	99.3
RH48	5573400	457	201	150330	12195	39548	98.4
RH60	5770882	445	200	120659	12968	28971	97.2
SA121	6193334	1940	205	162670	3192	50257	98.4
SA131	5765041	882	200	65483	6536	14475	94.7
SA165-S	5762375	414	202	203167	13918	34431	98.3
SA166	5890440	413	201	101901	14262	36261	98.6
SA176	6273034	2278	205	114849	2753	26574	97.6
WHKp1	5727737	244	201	300112	23474	56026	98.7

Core genome MLST of the 39 isolates performed using Ridom ™ SeqSphere+ software revealed three clusters that differed by up to 14 out of 2358 alleles, and seven singletons. The minimum spanning tree (MST) based on the cgMLST of isolates is shown in Figure 13.

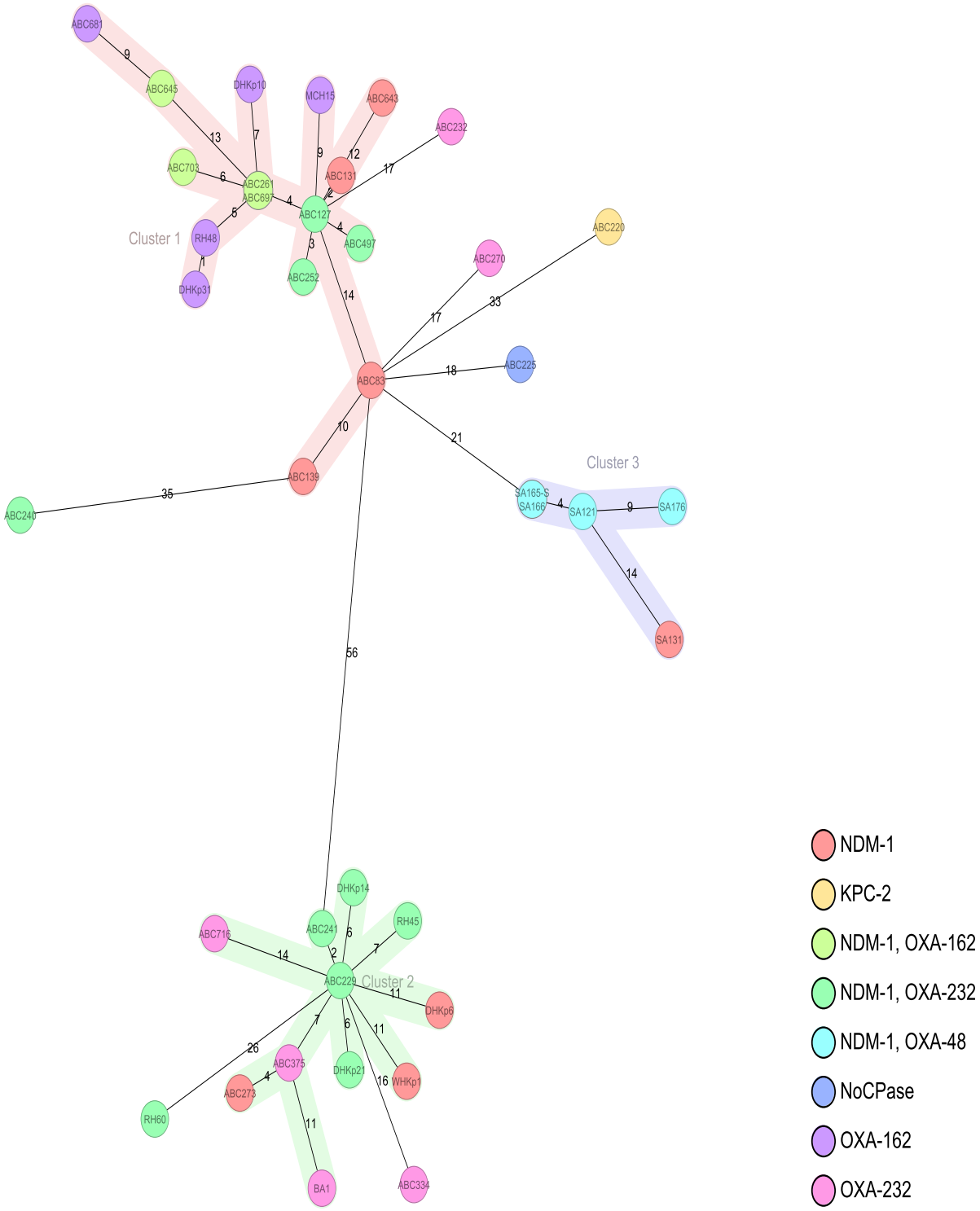

Figure 13: Minimum spanning tree based on cgMLST of CRKP-ST14 (color coded based on the carbapenemase gene harbored)

As it is shown in Figure 13 each cluster was comprised of strains having various carbapenemases.

In Figure 14 the isolates in the MST were color coded based on the city of isolation, which demonstrates that isolates of Cluster 1 and Cluster 2 were recovered in multiple cities of the UAE and in Bahrain, and Cluster 3 contained the five isolates of Riyadh, Saudi Arabia.

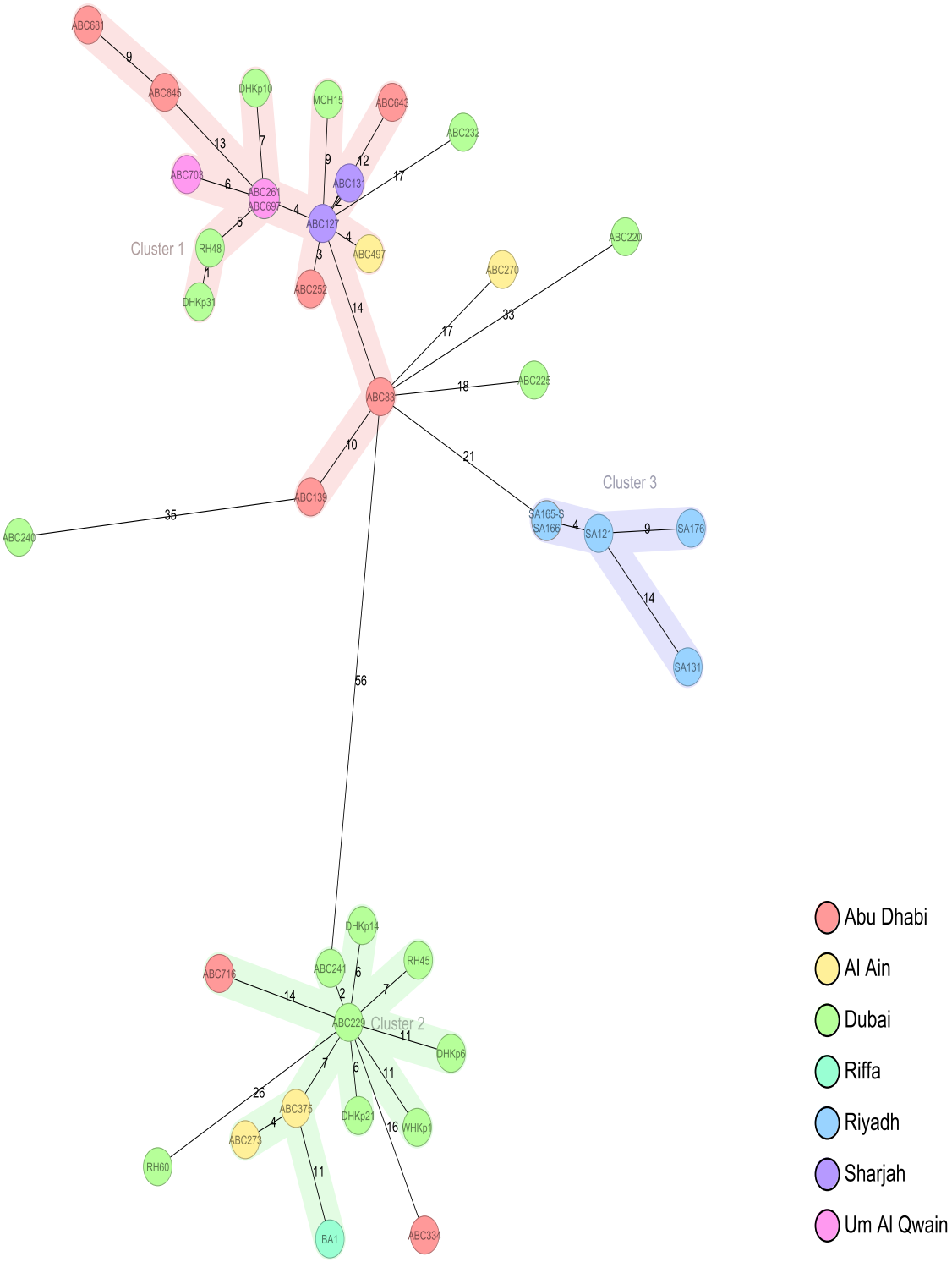

Figure 14: Minimum spanning tree based on cgMLST of CRKP-ST14 (color coded based on the city of isolation)

Analysis of the assembled contigs using Kleborate (on https://pathogen.watch/) demonstrated that all but one CRKP-14 isolate harbored yersiniabactin on ICE*Kp5*, and the sequence type of yersiniabactin was YbST140 or its single, double or triple locus variants with the exception of SA131 harboring double locus variants of YbST138 (Table 22). Other virulence genes (colibactin, aerobactin, salmochelin, *rmpA* and *rmpA2*) were not present in any of the isolates WGS-ed. The capsular locus variants were also tested by Kleborate, confirming the presence of KL2 in 26 isolates, and interestingly KL64 in 13 isolates tested. Comparing the capsular locus variations with the cgMLST clustering revealed that Cluster 2 and two singletons related closely to Cluster 2 harbored the KL64 variant (Figure 15 and Table 22).

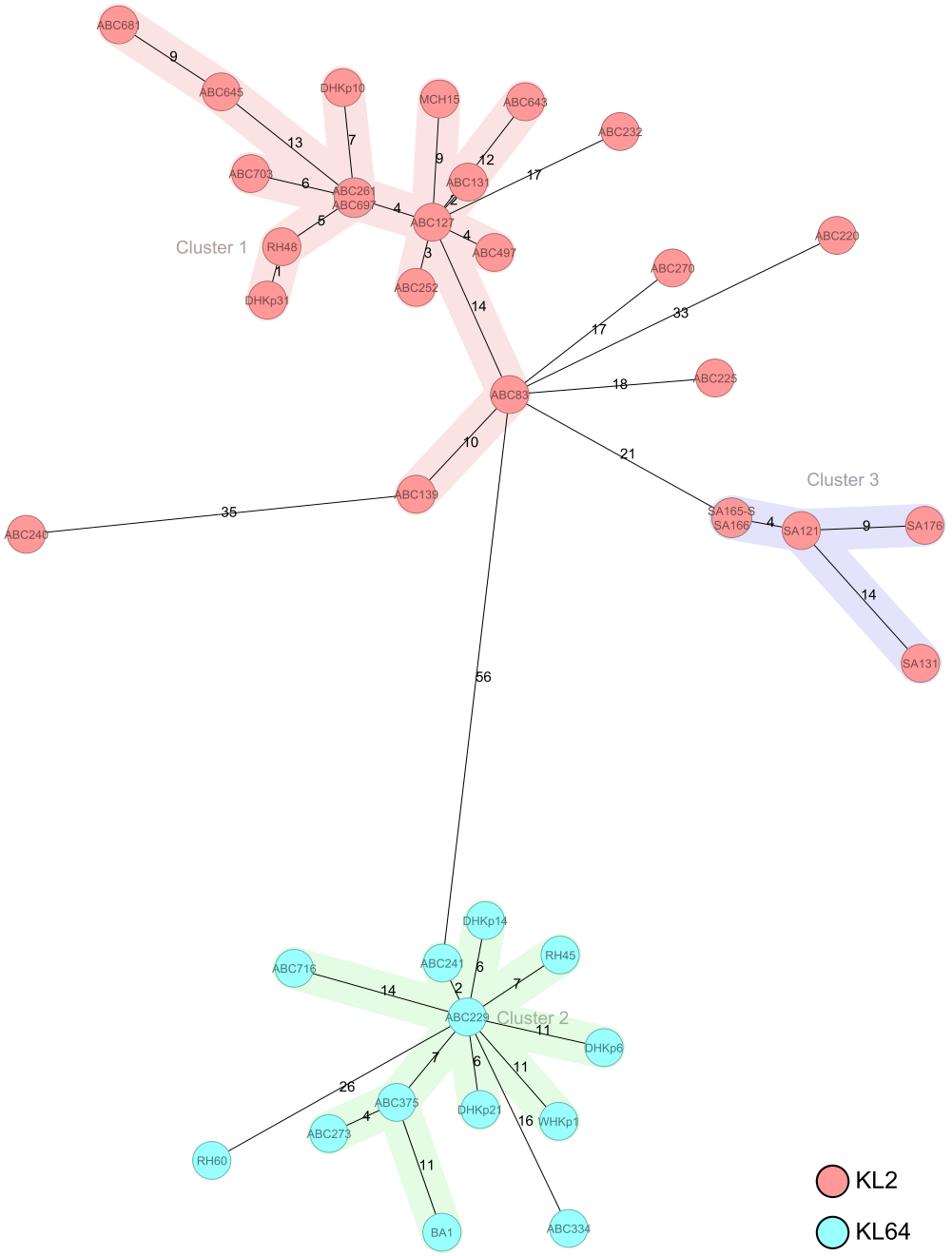

Figure 15: Minimum spanning tree based on cgMLST of CRKP-ST14 (color coded based on the capsular loci)

Table 22: cgMLST clustering, yersiniabactin and capsular types of the CRKP-14 isolates

Strain	SG	PFGE SG	cgMLST cluster	Yersinia-bactin	Yersinia-bactin encoding mobile element	YbST	wzi allele	K locus
ABC127	A	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC252	A	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC497	A	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
DHKp21	A	5	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
SA121	B	14	C3	ybt 14	ICEKp5	140-1LV	wzi2	KL2
SA165/S	B	14	C3	ybt 14	ICEKp5	140-1LV	wzi2	KL2
SA166	B	14	C3	ybt 14	ICEKp5	140-1LV	wzi2	KL2
SA176	B	14	C3	ybt 14	ICEKp5	140-1LV	wzi2	KL2
ABC261	C	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC645	C	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC697	C	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC703	C	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC131	D	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC139	D	7	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC273	D	5	C2	ybt 14	ICEKp5	140-1LV	wzi64	KL64
ABC83	D	5	C1	ybt 14	ICEKp5	140	wzi2	KL2
SA131	D	14	C3	ybt 14	ICEKp5	138-2LV	wzi2	KL2
ABC240	E	5	S5	ybt 14	ICEKp5	140	wzi2	KL2
ABC229	F	11	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
DHKp14	F	11	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
RH45	F	8	C2	ybt 14	ICEKp5	140-3LV	wzi64	KL64
RH60	F	11	S6	ybt 14	ICEKp5	140-2LV	wzi64	KL64
DHKp6	G	11	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
WHKp1	G	11	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
ABC241	H	11	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
ABC681	I	1	C1	-	-	-	wzi2	KL2
DHKp10	I	5	C1	ybt 14	ICEKp5	140-1LV	wzi2	KL2
DHKp31	I	5	C1	ybt 14	ICEKp5	140-1LV	wzi2	KL2
MCH15	I	13	C1	ybt 14	ICEKp5	140	wzi2	KL2
RH48	I	6	C1	ybt 14	ICEKp5	140-1LV	wzi2	KL2
ABC232	J	4	S1	ybt 14	ICEKp5	140	wzi2	KL2
ABC270	K	11	S2	ybt 14	ICEKp5	140	wzi2	KL2
ABC375	K	5	C2	ybt 14	ICEKp5	140-1LV	wzi64	KL64
ABC716	K	10	C2	ybt 14	ICEKp5	140-2LV	wzi64	KL64
BA1	K	5	C2	ybt 14	ICEKp5	140-1LV	wzi64	KL64
ABC334	L	5	S7	ybt 14	ICEKp5	140-1LV	wzi64	KL64
ABC643	M	12	C1	ybt 14	ICEKp5	140	wzi2	KL2
ABC220	N	5	S4	ybt 14	ICEKp5	140	wzi2	KL2
ABC225	O	10	S3	ybt 14	ICEKp5	140	wzi2	KL2

As shown in Table 22, the only group of strains in which the cgMLST and PFGE grouping overlapped was Cluster 3 with the isolates from Riyadh. Members of other PFGE subgroups were exhibiting various cgMLST types.

The contigs assembled with Velvet were also analyzed for the presence of acquired antibiotic resistance genes using ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/) (Table 23). In three isolates (RH45, DHKp14 and ABC241), the *bla*$_{\text{OXA-232}}$ gene previously identified by PCR was not present in the Velvet assembly. Therefore, reads of these isolates were re-assembled using SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/). The SPAdes assembly confirmed the presence of *bla*$_{\text{OXA-232}}$ gene in both RH45 and DHKp14. However, in ABC241, previously positive by PCR for *bla*$_{\text{OXA-232}}$ but negative by hybridization, the WGS data assembled by either Velvet or SPAdes could not confirm the presence of this gene.

The array of acquired antibiotic resistance genes was remarkable: *bla*$_{\text{CTX-M-15}}$ ESBL gene was present in all but two isolates; and beyond a wide variety of genes coding for aminoglycoside modifying enzymes, the ribosomal methylase *armA* was possessed by more than half of the isolates tested.

Table 23: Acquired antibiotic resistance genes of CRKP-14 isolates detected by ResFinder

cgMLST cluster	SG	Strain ID	Carbapenemases					Other beta-lactamases							Aminoglycoside resistance genes												PMQR			Macrolide					Phenicol		Co-trimoxazole					Rif	Tetra-cycline	
			blaNDM-1	*blaOXA-48*	*blaOXA-162*	*blaOXA-232*	*blaKPC-2*	*blaTEM-1A*	*blaTEM-1B*	*blaOXA-1*	*blaOXA-9*	*blaCTX-M-15*	*blaSHV-13*	*blaSHV-28*	*armA*	*aadA1*	*aadA2*	*aac(3)-Iia*	*aac(3)-IId*	*aac(6')Ib*	*aph(3'')-Ib*	*aph(6')-Id*	*aph(3')-VIa*	*aac(6')-Ib3*	*aph(3')-Ia*	*aac(6')Ib-cr*	*QnrB1*	*QnrS1*	*msr(E)*	*mph(A)*	*mph(E)*	*erm(B)*	*ere(A)*	*cmlA1*	*catA1*	*sul1*	*sul2*	*dfrA1*	*dfrA12*	*dfrA14*	*ARR-2*	*tet(A)*	*tet(D)*
C1	C	ABC645																																								
C1	C	ABC697																																								
C1	C	ABC703																																								
C1	C	ABC261																																								
C1	A	ABC252																																								
C1	A	ABC497																																								
C1	A	ABC127																																								
C1	D	ABC83																																								
C1	D	ABC131																																								
C1	D	ABC139																																								
C1	M	ABC643																																								
C1	I	MCH15																																								
C1	I	DHKP10																																								
C1	I	DHKP31																																								
C1	I	ABC681																																								
C1	I	RH48																																								
C2	A	DHKP21																																								
C2	H	ABC241																																								
C2	D	ABC273																																								
C2	G	DHKP6																																								
C2	F	ABC229																																								
C2	F	DHKP14																																								
C2	F	RH45																																								
C2	G	WHKP1																																								
C2	K	ABC375																																								
C2	K	ABC716																																								
C2	K	BA1																																								
C3	B	SA121																																								
C3	D	SA131																																								
C3	B	SA165S																																								
C3	B	SA166																																								
C3	B	SA176																																								
S1	J	ABC232																																								
S2	K	ABC270																																								
S3	O	ABC225																																								
S4	N	ABC220																																								
S5	E	ABC240																																								
S6	F	RH60																																								
S7	L	ABC334																																								

Colored boxes demonstrate the presence of a gene, SG: subgroup classified in Table 19

Genes coding resistance to: Beta-lactam, Aminoglycoside, Quinolone, Macrolide, Chloramphenicol, Co-trimoxazole, Rifampicin, Tetracycline

Table 24: Antibiotic susceptibility of CRKP-14 isolates tested by WGS

cgMLST cluster	SG	Strain	MIC (mg/L)														
			Ertapenem	Imipenem	Meropenem	Ceftazidime	Cefotaxime	Aztreonam	Ciprofloxacin	Gentamicin	Amikacin	Co-trimoxazole	Tetracycline	Chloramphenicol	Colistin	Tigecycline	Fosfomycin
C1	**A**	**ABC127**	128	64	64	>128	>128	2	>64	256	>256	>256	8	>256	≤0.5	2	64
C1	**A**	**ABC252**	128	128	64	>128	>128	>128	>64	>256	>256	>256	16	>256	≤0.5	8	32
C1	**A**	**ABC497**	64	128	64	>128	>128	>128	>64	>256	>256	>256	4	>256	16	1	16
C2	**A**	**DHKP21**	128	128	64	>128	>128	>128	>64	256	>256	>256	8	32	≤0.5	2	32
C3	**B**	**SA121**	128	128	128	>128	>128	>128	>64	>256	>256	>256	8	>256	64	1	32
C3	**B**	**SA165S**	64	64	64	>128	>128	>128	>64	>256	>256	>256	8	>256	64	1	16
C3	**B**	**SA166**	64	64	64	>128	>128	>128	>64	>256	>256	>256	8	>256	64	1	16
C3	**B**	**SA176**	128	64	128	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	2	64
C1	**C**	**ABC261**	128	64	64	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	2	64
C1	**C**	**ABC645**	128	128	64	>128	>128	>128	>64	>256	>256	>256	8	256	32	4	32
C1	**C**	**ABC697**	128	64	128	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	2	64
C1	**C**	**ABC703**	128	64	64	>128	>128	>128	>64	>256	>256	>256	8	256	≤0.5	2	64
C1	**D**	**ABC131**	64	64	64	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	2	64
C1	**D**	**ABC139**	128	64	64	>128	>128	>128	>64	>256	>256	>256	>256	>256	≤0.5	4	64
C2	**D**	**ABC273**	64	32	64	>128	>128	>128	>64	256	>256	>256	8	>256	≤0.5	4	16
C1	**D**	**ABC83**	256	64	64	>128	>128	>128	>64	>256	>256	>256	>256	>256	≤0.5	4	32
C3	**D**	**SA131**	64	64	64	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	2	64
S5	**E**	**ABC240**	128	128	128	>128	>128	>128	>64	256	16	>256	8	>256	≤0.5	1	128
C2	**F**	**ABC229**	128	128	128	>128	>128	>128	>64	256	>256	>256	4	32	4	0.5	32
C2	**F**	**DHKP14**	128	128	128	>128	>128	>128	>64	256	>256	>256	8	64	≤0.5	1	16
C2	**F**	**RH45**	128	64	64	>128	>128	>128	>64	4	32	4	8	16	16	1	128
S6	**F**	**RH60**	128	64	32	>128	>128	>128	>64	>256	>256	>256	8	32	≤0.5	2	32
C2	**G**	**DHKP6**	64	128	32	>128	>128	>128	64	>256	>256	>256	1	2	16	0.25	16
C2	**G**	**WHKP1**	128	128	128	>128	>128	>128	>64	256	>256	>256	16	64	≤0.5	2	16
C2	**H**	**ABC241**	128	128	64	>128	>128	>128	>64	256	>256	>256	32	32	≤0.5	8	32
C1	**I**	**ABC681**	16	4	4	64	>128	>128	>64	256	>256	>256	4	64	16	2	32
C1	**I**	**DHKP10**	16	16	8	>128	>128	>128	>64	2	4	>256	2	>256	≤0.5	1	64
C1	**I**	**DHKP31**	4	8	2	>128	>128	>128	32	>256	>256	>256	1	32	≤0.5	0.25	32
C1	**I**	**MCH15**	128	32	32	>128	>128	>128	>64	256	>256	>256	8	>256	4	2	64
C1	**I**	**RH48**	64	16	8	>128	>128	>128	>64	128	16	4	8	256	≤0.5	2	64
S1	**J**	**ABC232**	64	8	16	>128	>128	>128	>64	>256	>256	>256	8	>256	≤0.5	1	64
S2	**K**	**ABC270**	128	16	16	>128	>128	>128	>64	128	16	>256	8	>256	≤0.5	2	64
C2	**K**	**ABC375**	64	8	16	32	>128	128	>64	>256	>256	>256	4	>256	>256	1	32
C2	**K**	**ABC716**	32	8	16	>128	>128	>128	>64	>256	>256	>256	>256	16	64	2	8
C2	**K**	**BA1**	64	8	16	128	>128	>128	>64	>256	>256	>256	>256	>256	≤0.5	1	16
S7	**L**	**ABC334**	64	8	32	128	>128	>128	64	256	>256	>256	>256	>256	≤0.5	0.25	32
C1	**M**	**ABC643**	64	128	128	>128	>128	>128	>64	>256	>256	>256	4	256	≤0.5	2	64
S4	**N**	**ABC220**	256	64	128	>128	>128	>128	64	256	16	>256	4	256	32	0.5	256
S3	**O**	**ABC225**	16	1	2	>128	>128	>128	>64	256	32	>256	8	>256	≤0.5	1	64

The array of acquired antibiotic resistance genes matched the susceptibilities of the isolates, *e.g.* high-level tetracycline resistance was observed with isolates possessing the *tet(D)* gene. Similarly, the few isolates susceptible to chloramphenicol were the ones lacking any phenicol resistance genes. However, the lack of acquired phenicol resistance genes did not translate always to susceptibility to chloramphenicol, suggesting alternative resistance mechanisms. The colistin resistance observed in one third of the isolates was not due to the presence of mobile colistin resistance genes and did not correlate with certain clusters. Nevertheless, detection of chromosomal mutations leading to colistin, to tigecycline or to ciprofloxacin resistance was beyond the scope of our current investigations.

In order to investigate the carbapenemase carrying plasmids one representative of each subgroup defined in Table 19 was chosen for plasmid transfer experiments.

All NDM-plasmids were conjugative into *E. coli* $J53_{RAZ.}$ Conjugal transfer of bla_{NDM-1} from ABC643, a strain in which the hybridization did not localize the gene on a plasmid, was successful, resulting in transconjugant harboring a plasmid of approximately 240kb size. Among the OXA-plasmids, those carrying bla_{OXA-48} could be mobilized into *E. coli* $J53_{RAZ}$. One of the $bla_{OXA-162}$ carrying plasmid was also conjugative. Those harboring $bla_{OXA-232}$ were non-conjugative. These latter ones were transferred into *E. coli* DH5α by $CaCl_2$ transformation. The plasmid incompatibility types determined by PBRT of the single plasmid containing derivatives are shown in Table 25.

Table 25: Basic characteristics of carbapenemase carrying plasmids of CRKP-14 isolates

SG	Strain	NDM-1 plasmid			OXA plasmid			
		Size	Inc type	C	OXA allele	size	Inc type	C
A	**ABC252**	≥240Kb	IncHI1B	C	OXA-232	≈6Kb	IncColKP3	NC
B	**SA121**	≥240Kb	IncHI1B	C	OXA-48	≈60Kb	IncL/M	C
C	**ABC261**	≥240Kb	IncHI1B	C	OXA-162	≈60Kb	IncL/M	C
D	**ABC131**	≥240Kb	IncHI1B	C	none	NA	NA	NA
E	**ABC240**	≈120Kb	IncFII	C	OXA-232	≈60Kb	IncColKP3, IncR	NC
F	**RH60**	≈120Kb	IncFII	C	OXA-232	≈6Kb	IncColKP3	NC
G	**DHKp6**	≈120Kb	IncFII	C	none	NA	NA	NA
H	**ABC241**	≈120Kb	IncFII	C	OXA-232	NL	NA	NA
I	**MCH15**	NA	NA	NA	OXA-162	≈60Kb	IncL/M	NC
J	**ABC232**	NA	NA	NA	OXA-232	≈10Kb	IncColKP3	NC
K	**ABC270**	NA	NA	NA	OXA-232	≈6Kb	IncColKP3	NC
M	**ABC643**	≥240Kb	IncHI1B	C	none	NA	NA	NA

C conjugative, NA not applicable, NL not localized on plasmid, NC non-conjugative

To compare the CRKP-ST14 isolates of this study to international members of this clone we performed the cgMLST on our strains along with complete genomes of *K. pneumoniae* ST14 retrieved from GenBank: PittNDM01, KP617, and NUHL24835 (Figure 16). The main characteristics of these reference sequences are listed in Table 26.

Table 26: Characteristics of *K. pneumoniae* ST14 complete genome reference sequences

Name	**Acc. No**	**Country/ year of isolation**	**Sample**	**Carbapenemase genes carried**	**Capsular type**
PittNDM01	CP006798 - CP006802	USA / 2013	Urine	$bla_{\text{OXA-232}}$ and $bla_{\text{NDM-1}}$	K2
KP617	CP012753 - CP012755	South Korea/ 2013	unknown	$bla_{\text{OXA-232}}$ and $bla_{\text{NDM-1}}$	K2
NUHL24835	CP014004 - CP014006	China / 2014	Urine	$bla_{\text{NDM-5}}$	K2

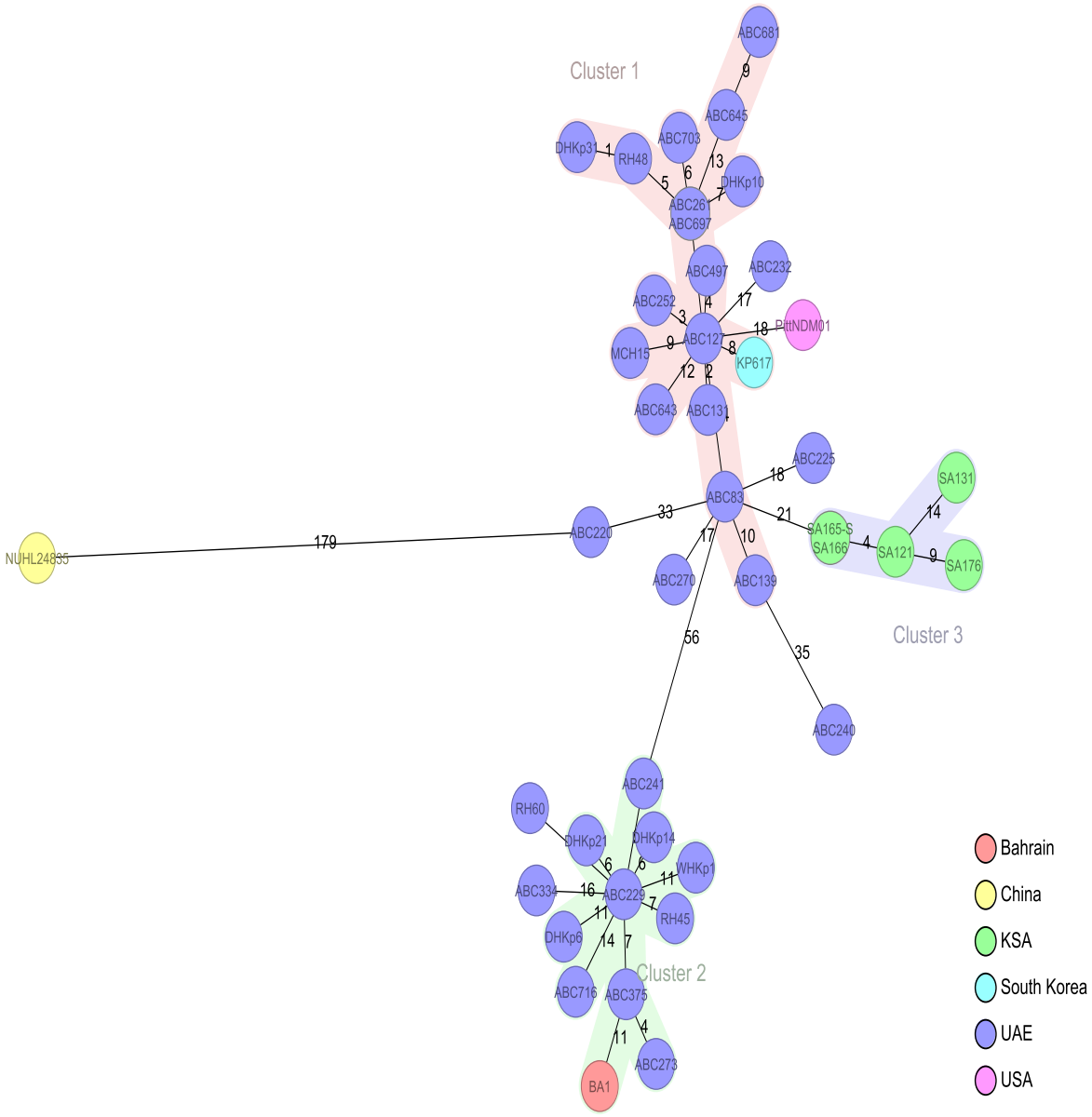

Figure 16: Minimum spanning tree based on cgMLST of CRKP-ST14 (color coded based on the country of isolation)

As it is shown in Figure 16, the reference genomes of CRKP-ST14 harboring *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-232}}$ were highly similar to isolates in cluster C1 in our study.

Chapter 4: Discussion

Although the presence of CRE has been reported repeatedly from countries of the Arabian Peninsula, only few studies used detailed molecular investigations to reveal the driving force behind the emergence of this critical type of human pathogen [156, 172, 194, 195, 314, 317-319, 326]. These studies documented limited clonal expansion as well as plasmid mediated outbreak behind the emergence of certain carbapenemase type producing isolates. Clonal expansion was mainly seen with *K. pneumoniae* producing NDM-1 or OXA-48-like carbapenemases [156, 317], whereas plasmid mediated spread explained a temporary increase of VIM-4 carbapenemase producing isolates in Kuwait [172]. The CRE encountered in this region produced mainly OXA-48 or NDM-1carbapenemases [195, 314, 317], with sporadic occurrence of VIM-4 [173, 326], OXA-181[319], OXA-162, OXA-232 [329], NDM-5 [314], NDM-7 [318] and KPC-2 [157] producing isolates. Infrequently, double carbapenemase producing isolates were also reported [314, 317, 329]. In the early stage of the emergence of NDM-producing *Enterobacteriaceae* in the UAE, the phenomenon was partially attributed to the spread of a specific type of conjugative plasmid, IncX3 harboring the $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ beta-lactamases [417]. The same plasmid type carried $bla_{\text{NDM-7}}$ in all isolates identified in the Arabian Peninsula [318], and $bla_{\text{KPC-2}}$ in the first reported KPC-producing *K. pneumoniae* strains from the region [157]. These all suggested the prominent role of this plasmid to acquire and disseminate carbapenemase genes in this geographic area.

To investigate this hypothesis, we systematically screened CRE isolated in the UAE, and found that the carbapenemase producer *Enterobacteriaceae* (CPE) isolates harbored IncX3 type plasmids twice as frequently as non-carbapenemase

producer CRE, which was largely due to the high proportion of IncX3 plasmid carrying isolates in the NDM-producing group. Transfer and complete sequencing of these plasmids from CPE proved that indeed all of them harbored one of the following carbapenemase genes: *bla*$_{\text{NDM-1}}$, *bla*$_{\text{NDM-4}}$, *bla*$_{\text{NDM-5,}}$ *bla*$_{\text{NDM-7}}$, *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{KPC-2}}$.

However, this observation from the UAE was not unique, as all of these carbapenemases have been reported to be carried on IncX3 plasmids in various regions of the world as shown in Table 2.

In order to study whether the IncX3 plasmids occur in the UAE as a result of a local evolution, or rather as a consequence of international transfer, we compared the conserved regions of plasmids of the UAE to the ones reported earlier from various countries (Table 11) [230, 311, 318, 395, 415, 416, 427, 428, 430, 448, 449]. The analysis identified clades based on the carbapenemase genes carried, *i.e.* close phylogenetic relationship of IncX3 plasmids harboring *bla*$_{\text{NDM-1,}}$ *bla*$_{\text{NDM-4}}$ and *bla*$_{\text{NDM-7}}$ from the UAE and from different countries of the Middle-East, Asia, Europe and North-America was observed. On the other hand, *bla*$_{\text{NDM-5}}$ carrying plasmids from the UAE, Czech Republic, China, Hong Kong, India and South Korea formed a distinct clade. *bla*$_{\text{OXA-181}}$ carrying IncX3 plasmids originating from the UAE, as well as from Lebanon, Germany, Denmark, Czech Republic, Switzerland, China, South Korea and Myanmar formed another distinct clade with a single outlier (MG228426) from Italy, only. The KPC-IncX3 plasmids were phylogenetically heterogeneous: while two *bla*$_{\text{KPC-2}}$ harbouring plasmids from Hong Kong and from France mapped relatively close (JX104759 and JX461340), the backbone of the plasmid coding for the same allele from of the UAE (pABC220-KPC-2) and that of an Italian plasmid

carrying *bla*$_{KPC-3}$ (KT362706) were distant. These data suggest that the various carbapenemase gene carrying IncX3 plasmids recovered in the UAE are rather the result of international transfer of this successful plasmid type, and not the consequence of local evolution.

The sequence types of the isolates harboring IncX3 type plasmids partially support this notion, as OXA-181 producing *E. coli* ST410 harboring the carbapenemase on IncX3 plasmids were reported from countries of three continents: China, Italy, Poland and Canada [230, 450-452]. Recently it was established that this sequence type of *E. coli* is to be considered an emerging high-risk clone, too [453]. Similarly, *E. coli* ST167 harboring *bla*$_{NDM-5}$ was present in the Czech Republic [311] and China [454], and the same clone was also reported to harbor *bla*$_{NDM-7}$ on IncX3 plasmid from France [455] and India [456]. However, *E. coli* ST167 with *bla*$_{OXA-181}$ carrying IncX3 plasmid has not been encountered yet, although a single locus variant of ST167 was reported to carry this carbapenemase gene from São Tomé and Príncipe [457].

On the other hand, the clonal spread of *K. pneumoniae* ST1318, and especially the five *K. pneumoniae* ST11 harboring *bla*$_{NDM-1}$ demonstrates that the international transfer is not the only mechanism behind the emergence of this plasmid. Combination of the IncX3 plasmid and the *K. pneumoniae* ST11 clone, both considered to have epidemic potential [238], is especially worrisome.

Apart from *K. pneumoniae* ST11, member of another high-risk *K. pneumoniae* clone was encountered, a single *K. pneumoniae* ST307 carrying *bla*$_{NDM-5}$ on an IncX3 plasmid. This finding is novel, as members of this clone have been described to carry various carbapenemases, *e.g.* *bla*$_{NDM-1,}$ *bla*$_{NDM-6,}$ *bla*$_{OXA-48,}$ *bla*$_{KPC-2,}$

bla$_{KPC-3}$ and *bla*$_{VIM-1}$, in Spain, Italy, Pakistan, South Korea [310, 449, 453, 458] and even in the UAE [329], but production of NDM-5 by this clone has not been reported yet. Since the *K. pneumoniae* ST307 reported earlier from the UAE and the one described in this study were isolated in the same hospital, and both carried *bla*$_{OXA-162}$ as well [329], the possibility of local acquisition of *bla*$_{NDM-5}$ carrying IncX3 plasmid cannot be excluded.

Beyond the role of epidemic plasmids, earlier observations suggested that clonal dissemination of *K. pneumoniae* ST14 has also played a role in the emergence of CRE in the UAE [156, 329]. The systematic study conducted in 2015 June-2016 June in Dubai has confirmed these early observations. The data of this study unveiled a large, multi-hospital dissemination of the clone, in a scenario where 39% of all CRE cases investigated were attributed to *K. pneumoniae* ST14. Although the first reported NDM-producing *K. pneumoniae* belonged to this clone, *i.e.* ST14 [174], and it has been also described from India, the United Kingdom and Sweden to carry *bla*$_{NDM}$ [299] or from the USA and South Korea to co-produce OXA-48-like and NDM carbapenemases [404, 459], to the best of our knowledge, our findings were the first report on the spread of carbapenemase producing *K. pneumoniae* ST14 clone on a multi-hospital scale. KPC-producing isolates were not identified in this collection, although the first KPC producing *K. pneumoniae* strains from the Arabian Peninsula were reported from Dubai as *K. pneumoniae* ST14 carrying *bla*$_{KPC-2}$ on IncX3 plasmid [157]. The most common mechanism of carbapenem resistance in CRE of Dubai were the production of OXA-48-like and NDM carbapenemases, like it is common in the Arabian Peninsula [156, 194, 317]. The identification of the alleles and genetic support of the NDM or OXA-48-like genes was beyond the scope

of this study; however the whole genome sequencing project discussed later shed light to the complexity of carbapenemases and plasmids.

K. pneumoniae ST14 high risk clone was reported to cause neonatal outbreaks: in Italy *K. pneumoniae* ST14 producing FOX-7 AmpC beta-lactamase [460], in Tanzania *K. pneumoniae* ST14 producing CTX-M-15 ESBL occurred in clusters of neonatal infections, respectively [461]. Furthermore, a retrospective study conducted in Shanghai showed a clonal dissemination of NDM-producing *K. pneumoniae* ST14 isolates at different times in pediatric patients [462]. We have not observed this association with a certain age group; however, the clone was significantly more likely to be isolated from Emirati patients (Table 16).

It was also notable that almost 20% of the isolates from Dubai co-produced NDM and OXA-48-like carbapenemases, and this feature was significantly associated with the *K. pneumoniae* ST14 clone, and only one double carbapenemase producing isolate belonged to a different sequence type, *K. pneumoniae* ST1513.

K. pneumoniae ST14 was not only associated with double carbapenemase production, but also with extreme drug resistance (Table 16). Furthermore, two of the four pan-drug resistant isolates also belonged to this clone, with the other to being ST15 and ST231, respectively.

Beyond the spread of *K. pneumoniae* ST14 clone, smaller clusters of *K. pneumoniae* ST147 producing either NDM (n=6) or OXA-48-like (n=4) carbapenemase were also encountered. This high risk clone has been reported from the region from Oman [156] and from the UAE, where a pan-drug resistant members

of this clone occurred in multiple hospitals [314]. In Dubai, the pan-drug resistant isolates belonged to different STs, as discussed above.

This study also revealed an overall high rate of colistin resistance (31.4%) in carbapenem resistant *K. pneumoniae* (CRKP). This rate of resistance is particularly alarming, as higher rate of colistin resistance in CRKP was only reported from Italy (43%), where the majority of colistin resistance was due to clonal spread of KPC-producer *K. pneumoniae* CC258 [463]. In Dubai the picture seemed to be more complex: as only 59% of colistin resistant CRKP belonged to the locally prevalent ST14 clone, a large part (41%) was due to sporadic isolates. No plasmid-coded colistin resistance gene was detected, although such mechanism has already been reported from human isolates in the region, even among carbapenem resistance strains [433]. Further investigating the chromosomal mutations leading to colistin resistance was beyond the scope of the study.

A limitation of the study was that the collection did not encompass all non-repeat isolates of the period, as we faced considerable logistic constrains in the submission of strains. Nevertheless, this was a problem occurring randomly, and no specific selection criteria were applied by the participating hospitals. The fact that the proportion of *K. pneumoniae* in the collection studied (78.6%) was practically identical to those among all CRE encountered during the study period (77.9%) suggests that the random submission of the strains had limited, if any, impact on the validity of our findings.

The other drawback was the almost complete lack of epidemiological data, although, the fact that *K. pneumoniae* ST14 strains occurred in all hospitals highlights the need for such data. The presence of a highly resistant clone clearly

seems to affect a large proportion of local hospitals both in the state, as well as in the private sector. Furthermore, such data would be needed to verify and explain the current unexpected observation regarding the significant association of the ST14 clone with Emirati patients, for which, currently, without access to detailed clinical information on the patients, we do not have a specific explanation.

For a deeper understanding of the dynamics of CRKP-ST14 we selected strains of our Arabian Peninsula-wide collection, and screened them first by PFGE. If the PFGE pattern was ≥80% similar to known *K. pneumoniae* ST14, we considered them ST14-like. With this pre-selection, CRKP-ST14-like isolates were identified from Bahrain, Saudi Arabia and the UAE. As CRKP from various countries were not collected systematically, but for smaller scale studies only, the fact that the clone was not identified by us in Kuwait or Oman, does not exclude the possibility of its presence in those countries as well. Furthermore, the selection based on PFGE pattern similarity might have excluded potential CRKP-ST14 isolates with different PF patterns to known *K. pneumoniae* ST14, but for practical reasons we used this approach as MLST of over 700 isolates would have been very laborious. Even with this pre-selection criteria we found a remarkable variety in the carbapenemase genes, e.g. ST14-like CRKP carried $bla_{\text{NDM-1}}$, $bla_{\text{OXA-162}}$, $bla_{\text{OXA-232}}$, $bla_{\text{KPC-2}}$ or combinations of either $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-232}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-162}}$, or $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$. Multi-locus sequence typing of ST14-like CRKP isolates with variable carbapenemase genes and PFGE patterns with <90% similarity proved that all 70 CRKP selected for MLST indeed belonged to ST14. This variety of carbapenemase gene content further confirms the earlier observations describing this high-risk clone as one, which is particularly fit to acquire resistance plasmids and spread the resistance traits [235]. This clone has been reported to produce various

ESBLs (mostly CTX-M-15, SHV-2), AmpC cephalosporinases (DHA, FOX-7 and CMY-2) and carbapenemases (KPC-2, KPC-3, OXA-48, OXA-181, OXA-232, NDM-1, NDM-5) [238, 404, 458, 459], but to the best of our knowledge our study is the first to detect $bla_{\text{OXA-162}}$ in *K. pneumoniae* ST14.

We have selected 39 CRKP-ST14 isolates for whole genome sequencing based on the alleles of the carbapenemase genes and the plasmid localizations of these genes from the 15 subgroups listed in Table 19. All of this 39 isolates were multi-drug or extremely drug resistant (Table 24), and harbored a remarkable array of acquired resistance genes coding for beta-lactam, aminoglycoside, sulfonamide, trimethoprim, phenicol and macrolide non-susceptibility. Tetracycline, rifampicin and plasmid mediated quinolone resistance genes were less uniformly harbored by the isolates sequenced (Table 23). The non-carbapenemase genes did not show a particular association with the subgroups defined (Table 19 and Table 23).

cgMLST of the selected CRKP-ST14 isolates grouped them into three clusters that differed by up to 14 out of 2358 alleles, and seven singletons. These clusters only showed association with the city and hospital of isolation for the isolates of Saudi Arabia. Clusters were not associated with particular type of carbapenemase or plasmid size/type either (Figure 13, Figure 14, Table 19 and Table 22).

Nevertheless, a clear association of clustering and harbouring KL2 or KL64 capsular locus was demonstrated (Figure 15 and Table 22). *K. pneumoniae* ST14 clone have been extensively reported and associated with serotype K2, which is one of the most prevalent *K. pneumoniae* serotype linked to hypervirulence and invasive diseases [297-300]. To the best of our knowledge this is the first report of *K.*

pneumoniae ST14 harboring KL64. This capsular serotype was reported to be associated with numerous STs, including ST11, ST20, ST859, ST247, ST1711 and overall it was the most prevalent in Taiwan [464]. Capsular type K64 has recently been associated with a fatal bacteremia caused by a hypermucoviscous $bla_{\text{KPC-2}}$ producing ST11 *K. pneumoniae* [464] and another case of $bla_{\text{NDM-1}}$ producing hypermucoviscous and hypervirulent ST1764 *K. pneumoniae* in China [465]. Furthermore, it has been described in hypervirulent *K. pneumoniae* ST147 in the United Kingdom [316]. Further studies are needed to investigate whether this capsular switch in CRKP-ST14 from the well-known K2 type to K64 increases or changes virulence, or simply helps the pathogen to avoid immune recognition, and helps the dissemination of this clone, as was hypothesized in case of ST11 and CC258 with capsular variations [238].

As we pre-selected the strains for whole genome sequencing, statistical analysis of association with capsular types and the type of infections would have been skewed, nevertheless in the pool of strains subjected to whole genome sequencing the proportion of bloodstream and respiratory isolates was higher in the group of strains harboring KL64 capsular locus.

Irrespective of a potential change in virulence caused by the capsular switch, the phenomenon certainly has an implication if the phage derived capsule-targeting depolymerase therapy will be considered as an alternative or additional therapy to antibiotics [466].

It was interesting to note that CRKP-ST14-K2 and CRKP-ST14-K64 isolates overlapped both in time and in terms of the hospitals they were isolated from. Actually, this latter observation was true for referral hospitals: the two major

governmental hospitals of Dubai (RH and DH), a tertiary care hospital in Al Ain (TH) and two other tertiary care hospitals in Abu Dhabi (SKMC, ZMH), as well (Tables 20 and 22).

Although we tested isolates from 5 different locations from Saudi Arabia (data not shown), we have encountered strains with PFGE patterns ≥80% similarity to known *K. pneumoniae* ST14 isolated in a single Saudi hospital only, which formed a distinct group and were far more similar to each other, than the CRKP-ST14 of the UAE. This suggests that the clone has only recently been arisen in that country, supported also by the fact that all these isolates were from 2015.

Comparing our isolates to the internationally encountered CRKP-ST14 (Table 26) revealed that isolates in Cluster 1 (CRKP-ST14 from the UAE) were highly related to both of the NDM-1 and OXA-232 producing *K. pneumoniae* ST14 reference genome, to the extent that the Korean [459] even fitted into the cluster with 8 locus differences only. The CRKP-ST14 from the USA, originally considered to be imported from India [404], was also very close (18 locus different of the 2358) by cgMLST to *K pneumoniae* ABC127 isolated in 2011 in Sharjah (Figure 16). The NDM-5 producing ST14 isolate from China [467] exhibited more distant cgMLST profile, with being closest (179 allele difference) to KPC-2 producing isolate ABC220 (Figure 16). Interestingly, the carbapenemase genes; $bla_{\text{NDM-5}}$ in NUHL24835 [467] and $bla_{\text{KPC-2}}$ in ABC220 [157] were both located on IncX3 plasmids. All three reference genomes were of CRKP-ST14 with capsular type K2, suggesting that the capsular switch encountered in the UAE was a recent evolutionary event.

Our investigations have several limitations. Unfortunately, our data could not be aligned with epidemiological observations, indeed, lack of certain correlates such as travel information, dietary habits, clinical presentations and their associated outcomes have impeded our understanding of the dissemination of CREs in the study setting. The various sub-clones of *K. pneumoniae* ST14 strains occurring in 12 hospitals of the UAE highlight the need for such data.

A further shortcoming of our studies on CRKP-ST14 was that we have not tested the level of susceptibility to disinfectants, which may explain the sustained presence of this clone in the hospitals [243]. Nevertheless, the lack of generally accepted breakpoints for biocide resistance makes the interpretation of such tests difficult.

Furthermore, assessing the molecular mechanisms other than the presence of acquired resistance genes (*e.g.* chromosomal mutations, porin-loss, and efflux pump hyper-expression) causing resistance to colistin, tigecycline, chloramphenicol and ciprofloxacin was beyond the scope of our study.

Chapter 5: Conclusion

Systematic screening of 334 carbapenem resistant *Enterobacteriaceae* for IncX3 incompatibility type plasmids, *i.e.* for a type of plasmid implicated in the early emergence of CRE in the United Arab Emirates, revealed 30 carbapenemase carrying IncX3 plasmids, i.e. 9.6% of CRE carried carbapenemase on this specific type of plasmid.

The CRE strains carrying carbapenemase genes on IncX3 plasmids were diverse; they belonged to 16 different sequence types of five species of *Enterobacteriaceae*. Some of these strains belonged to high-risk epidemic clones of *Enterobacteriaceae*, *e.g. K. pneumoniae* ST11, *K. pneumoniae* ST14, and *E. coli* ST410.

The carbapenamase genes carried on IncX3 plasmids were also diverse: $bla_{\text{NDM-1}}$, $bla_{\text{NDM-4}}$, $bla_{\text{NDM-5}}$, $bla_{\text{NDM-7}}$, $bla_{\text{OXA-181}}$ and $bla_{\text{KPC-2}}$ were encountered.

Establishing the complete sequence of 21 IncX3 plasmids of the UAE harboring various carbapenemase genes showed high level of similarity in regions coding for plasmid replication, partitioning and mobilization, suggestive of a potential local evolution of these plasmid acquiring various genetic loads, *i.e.* resistance genes.

Nonetheless, phylogenetic analysis of the conserved regions of local IncX3 plasmids and of those described globally clustered them according to the carbapenemase genes carried, suggesting that they do not evolve locally, rather, were the result of international transfer.

Furthermore, investigating the molecular epidemiology of carbapenem-resistant *Enterobacteriaceae* (CRE) isolated over a year-long period in major hospitals of Dubai revealed an alarmingly high rate (33%) of extremely drug resistant, or colistin resistant (27%) isolates.

Thirty-nine percent of this collection belonged to a *K. pneumoniae* ST14 clone, members of which were identified in all participating hospitals.

This clone showed significant association with double carbapenemase production, with extreme drug resistance, and with being isolated from Emirati patients.

To gain a deeper insight into the molecular features of this clone, 39 CRKP-ST14 selected from five cities of the UAE, from Bahrain and from Saudi Arabia were subjected to whole genome sequencing (WGS), and their resistome, virulome and core genome MLST was assessed.

One of the isolates selected for WGS did not produce a carbapenemase, but 38 of them carried either single carbapenemases: *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-162}}$, *bla*$_{\text{OXA-232}}$, *bla*$_{\text{KPC-2,}}$ or a combinations of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-232}}$, *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-162}}$, or *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$. Although *K. pneumoniae* ST14 has been known to carry a variety of carbapenemases, our study demonstrated the first time the production of OXA-162 by *K. pneumoniae* ST14.

bla$_{\text{NDM-1}}$ was located on conjugative an IncHI1b or on an IncFII type plasmids, *bla*$_{\text{OXA-162}}$ and *bla*$_{\text{OXA-48}}$ on IncL/M type plasmid some of which were self-transmissible, whereas *bla*$_{\text{OXA-232}}$ was located on non-conjugative plasmids of IncColKP3 type or in one isolate on a composite IncColKP3-IncR type plasmid.

cgMLST revealed three clusters of 16 isolates from five UAE cities (C1), 11 isolates from three UAE cities and Bahrain (C2) and 5 isolates from Saudi Arabia (C3), respectively, and seven singletons. Resistance gene profile and carbapenemase carrying plasmid types were variable in both C1 and C2 clusters, while C3 clustering the isolates from Saudi Arabia was more uniform.

Although, *K. pneumoniae* ST14 is generally believed to be associated with capsular serotype K2, we found that isolates of cgMLST cluster C2, and two closely related singletons, carried genes of capsular locus KL64, a capsular type, which, to the best of our knowledge, has never been described earlier in this clone.

The capsular switch within the clone not only further emphasises its genetic flexibility, but may directly promote its transmission by evading immune recognition.

Altogether, our data showed that the emergence of CRE in the United Arab Emirates is a complex phenomenon of likely international transfer of successful plasmids and clones, combined with countrywide clonal dissemination of a genetically flexible high-risk *Klebsiella pneumoniae* clone.

Chapter 6: Recommendation

Our data demonstrating a nationwide spread of a high-risk *Klebsiella pneumoniae* clone highlights the necessity of surveillance and epidemiological investigations to monitor the antibiotic resistance situation countrywide in the United Arab Emirates.

The continuous surveillance should be supported by the establishment of a national antimicrobial reference laboratory able to conduct confirmatory tests and molecular epidemiological investigations.

To control the clonal spread occurring locally, as well as, to monitor the internationally transferred CRE, heightened infection control measures including active screening for CRE is needed in all hospitals of the UAE.

Furthermore, as the non-judicious use of antibiotics facilitates the spread of drug-resistant organisms by increasing the selection pressure, antibiotic stewardship programs in the inpatient care should be extended to the outpatient healthcare settings, along with enforced reduction of antibiotics consumption in the agriculture, in this region too.

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List of Publications

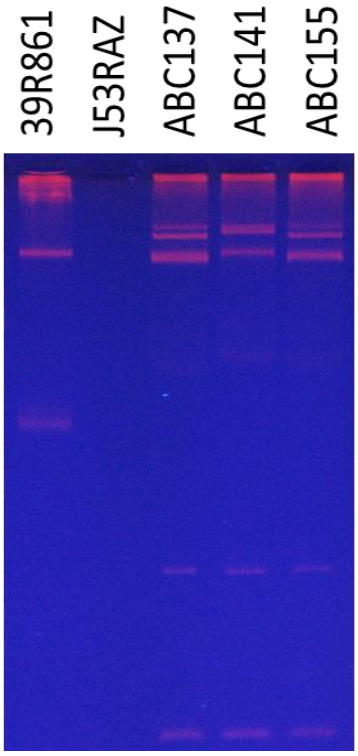
Moubareck CA[#], Mouftah SF[#], Pál T, Ghazawi A, Halat DH, Nabi A, AlSharhan MA, AlDeesi ZO, Peters CC, Celiloglu H, Sannegowda M, Sarkis D, Sonnevend A. Clonal emergence of Klebsiella pneumoniae ST14 co-producing OXA-48-type and NDM carbapenemases with high rate of colistin resistance in Dubai, United Arab Emirates. *International Journal of Antimicrobial Agents* 2018, 52(1):90-95.
(# equally contributing authors).

Appendix

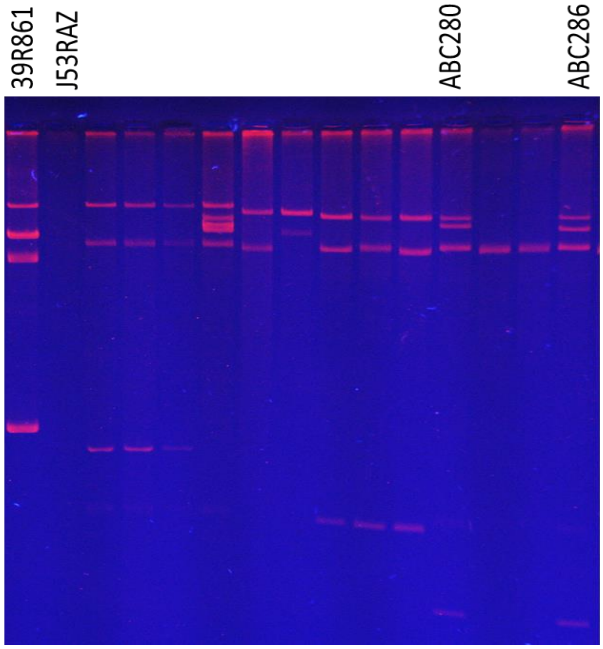
Part 1 Carbapenemase carrying IncX3 plasmids in the United Arab Emirates

Illustrating the plasmid profile similarities between host strains

I.Plasmid profile analysis of ABC137, ABC141 and ABC155



II.Plasmid profile analysis of ABC280 and ABC286



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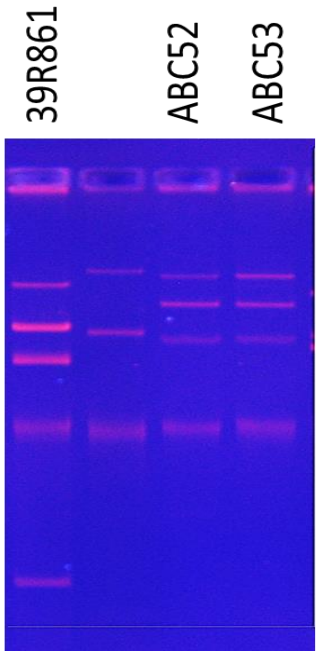
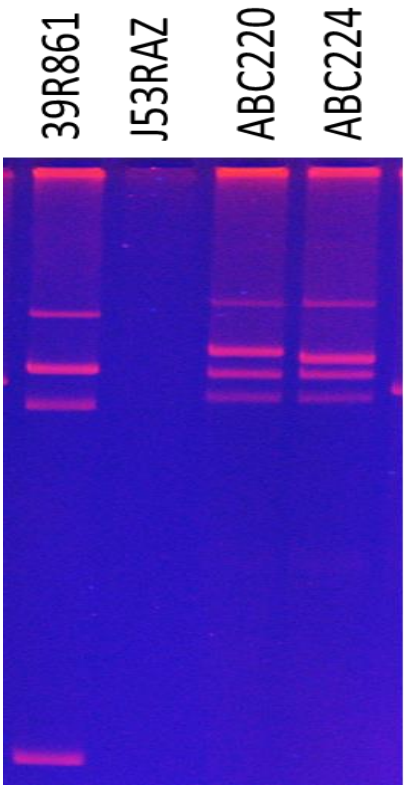
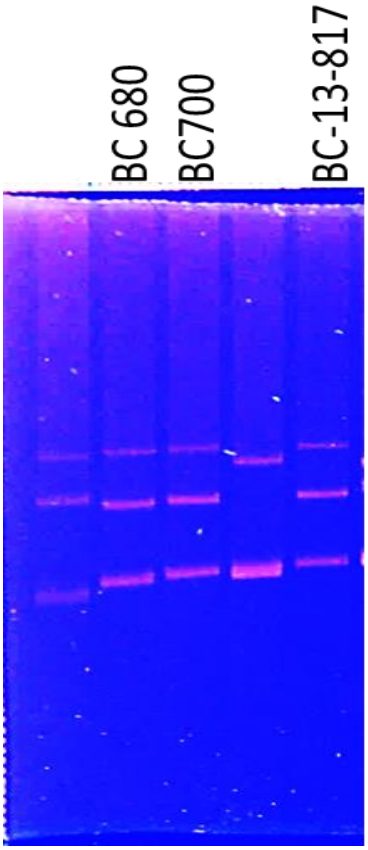


IV.Plasmid profile analysis of ABC220 and ABC224

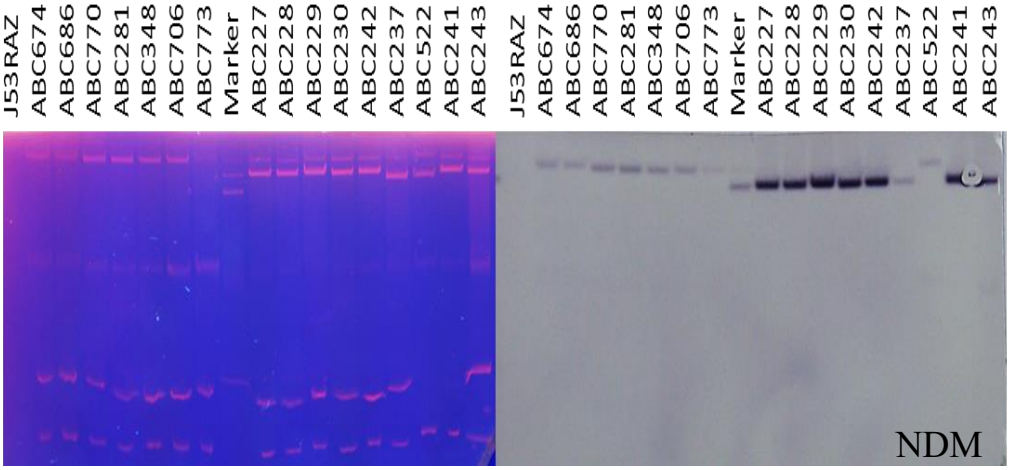


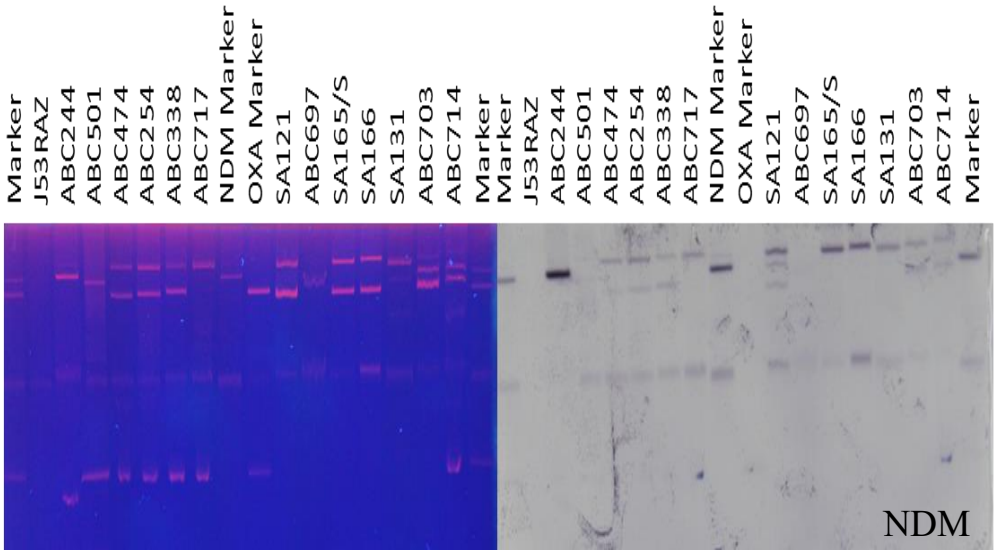
V.Plasmid profile analysis of BC-680, BC-700 and BC-13-817

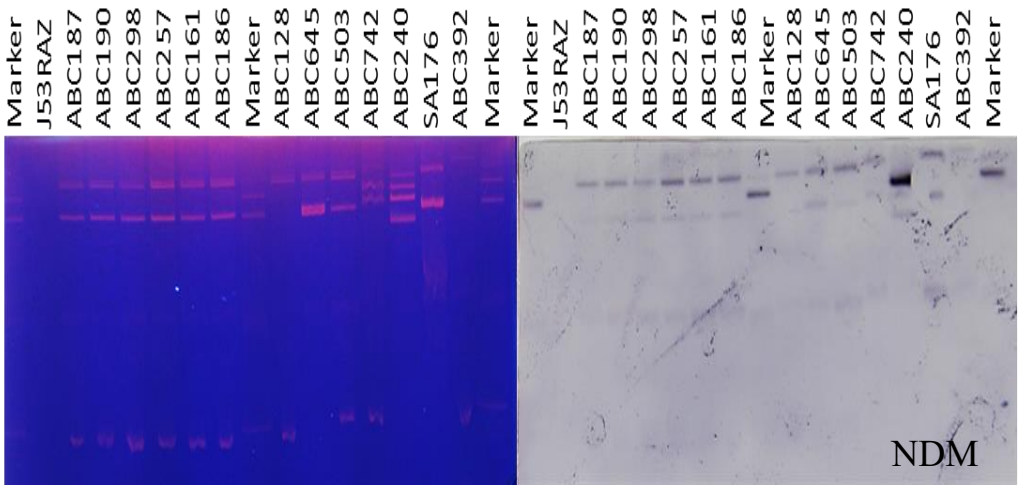


Part 2 In order to localize the carbapenemase genes; Hybridization with NDM-probe, OXA-48-like probe were performed for the 170 ST14-looking like isolates as shown in the following figures.

I. NDM hybridization



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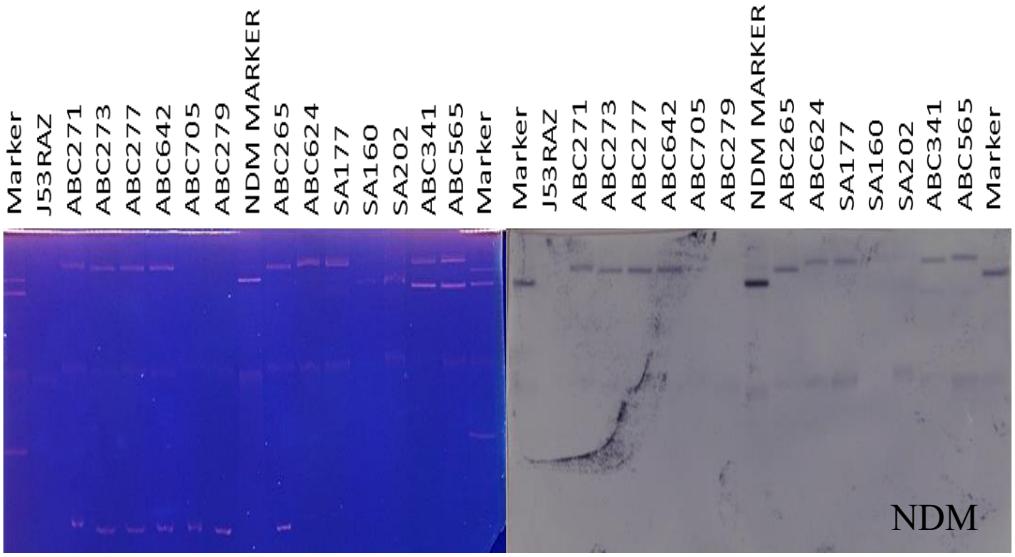
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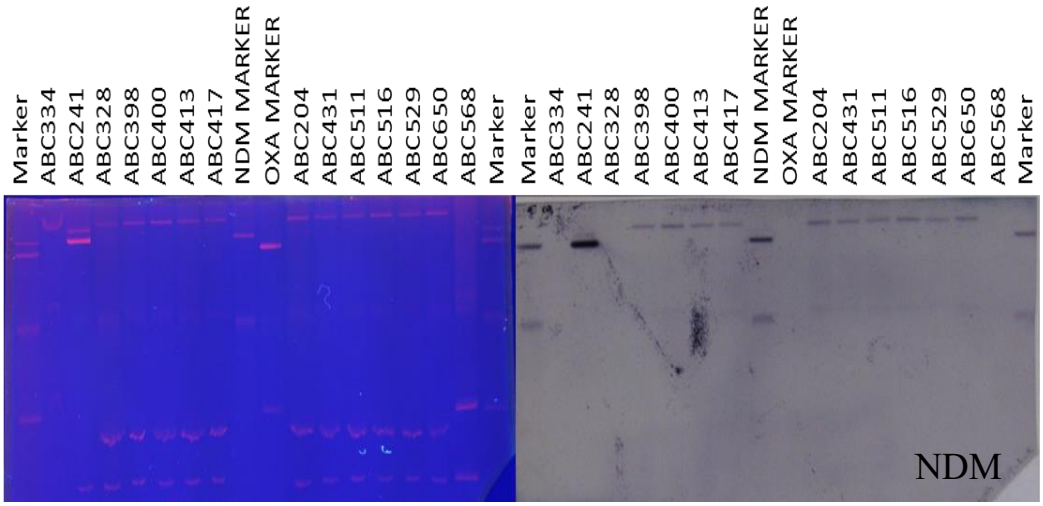
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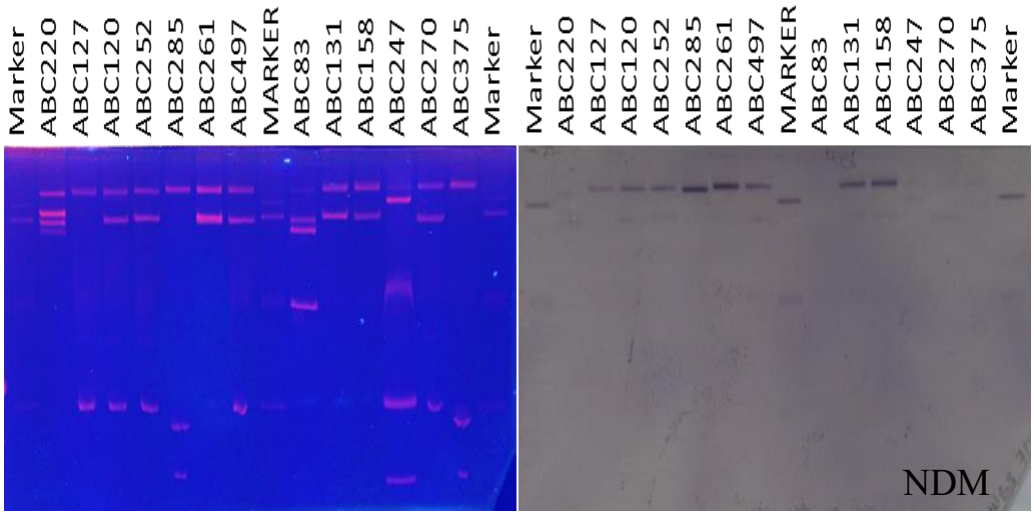
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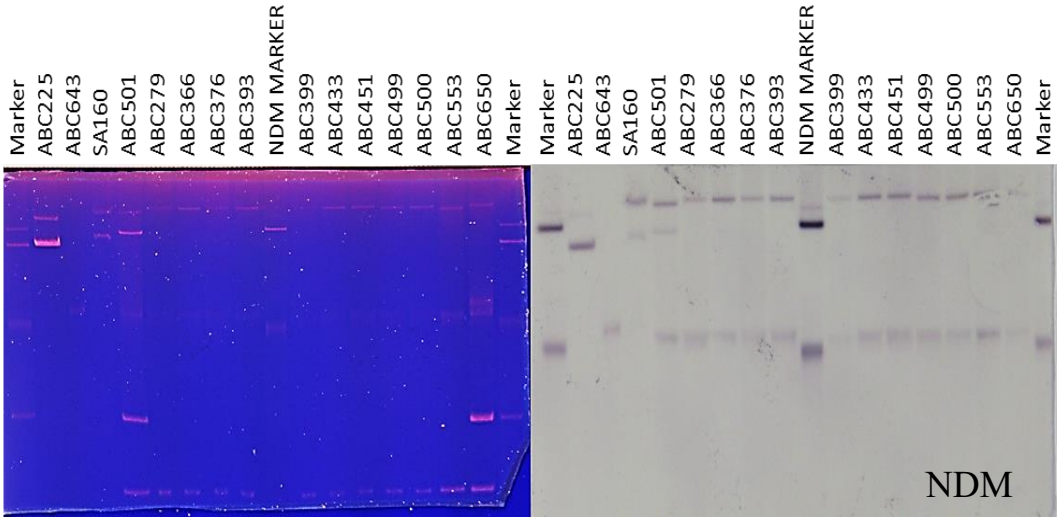
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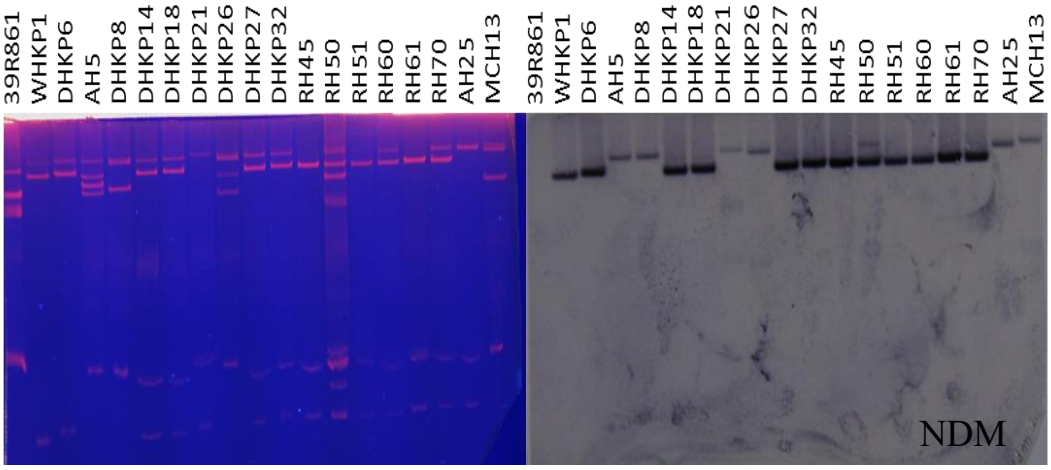
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RH60
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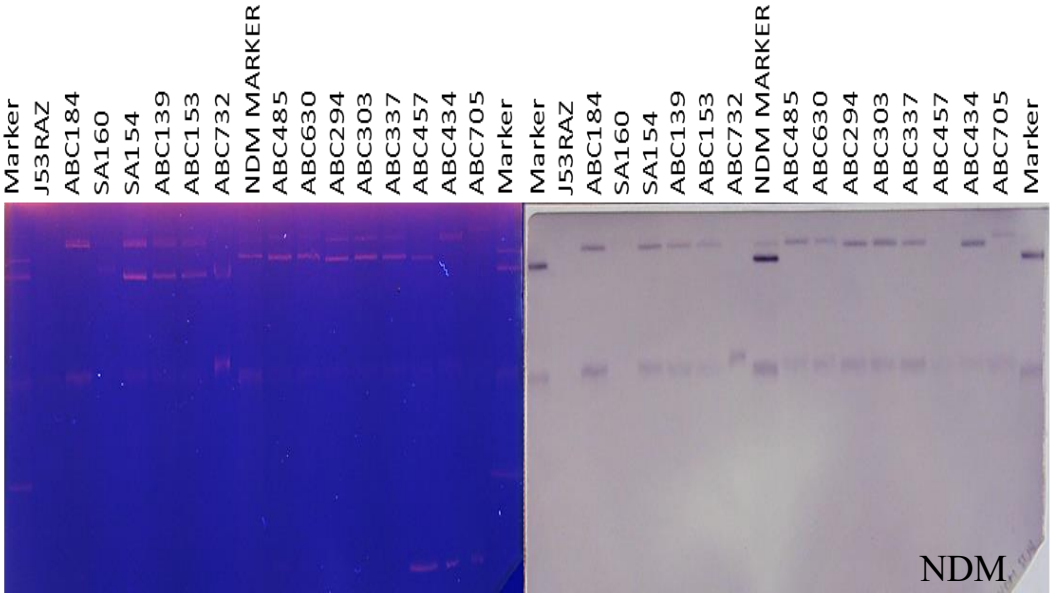
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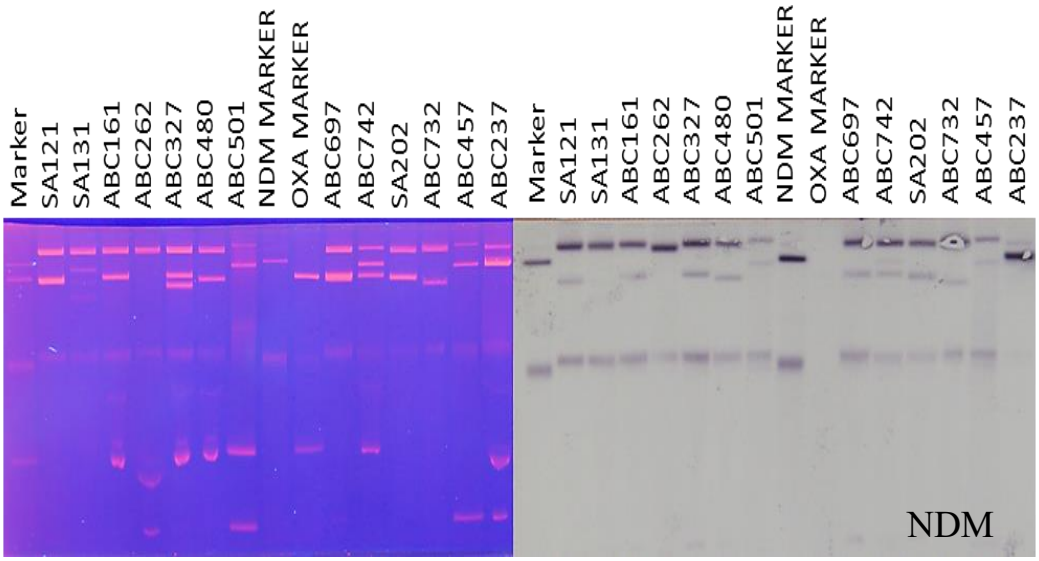
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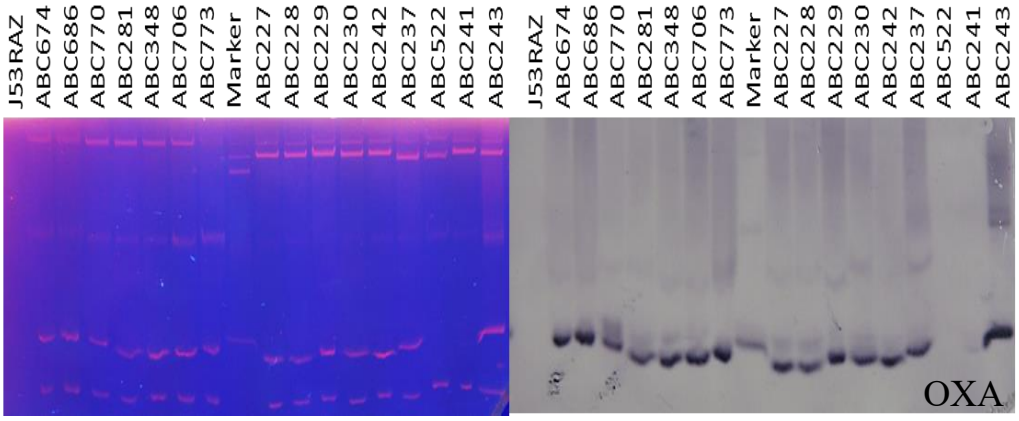
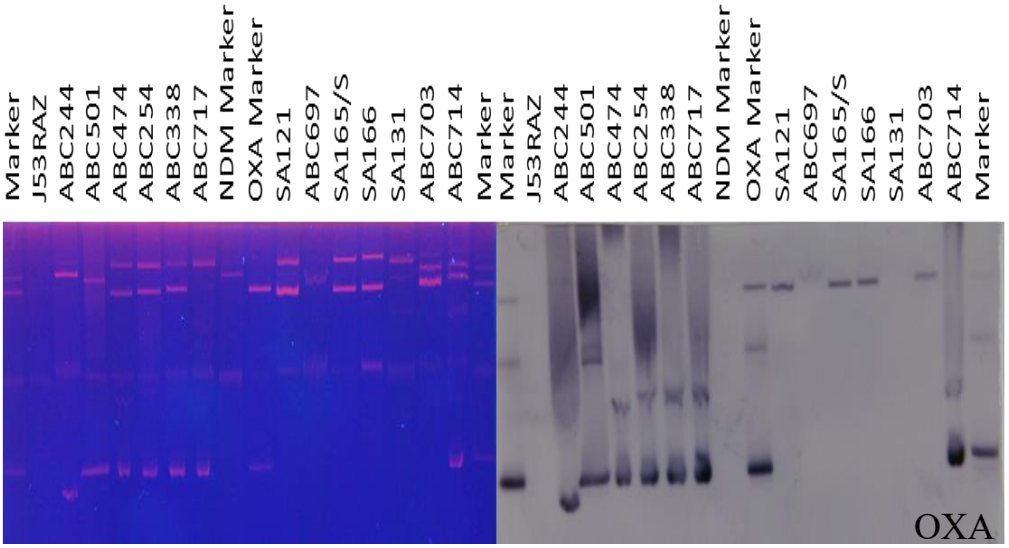
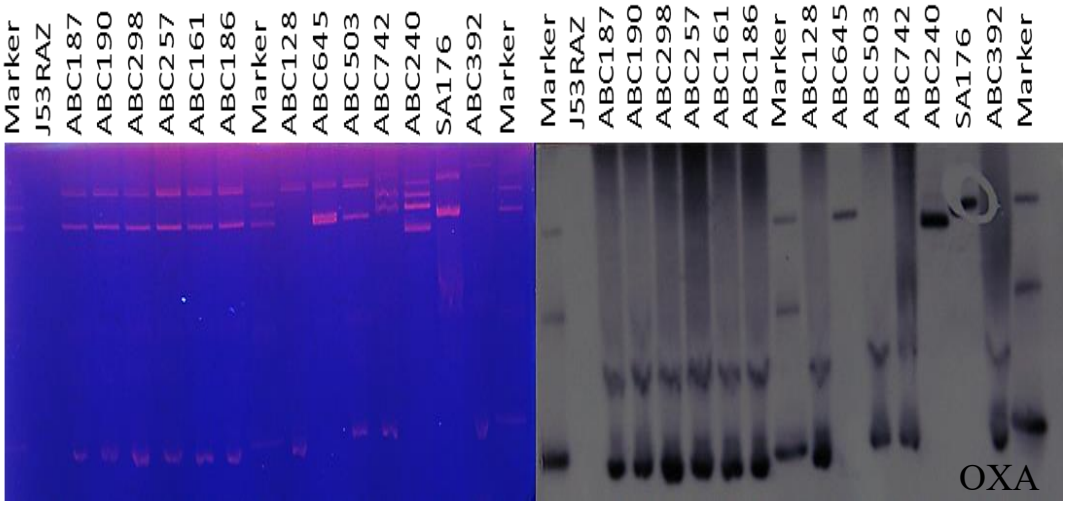
II. OXA-48-like hybridization



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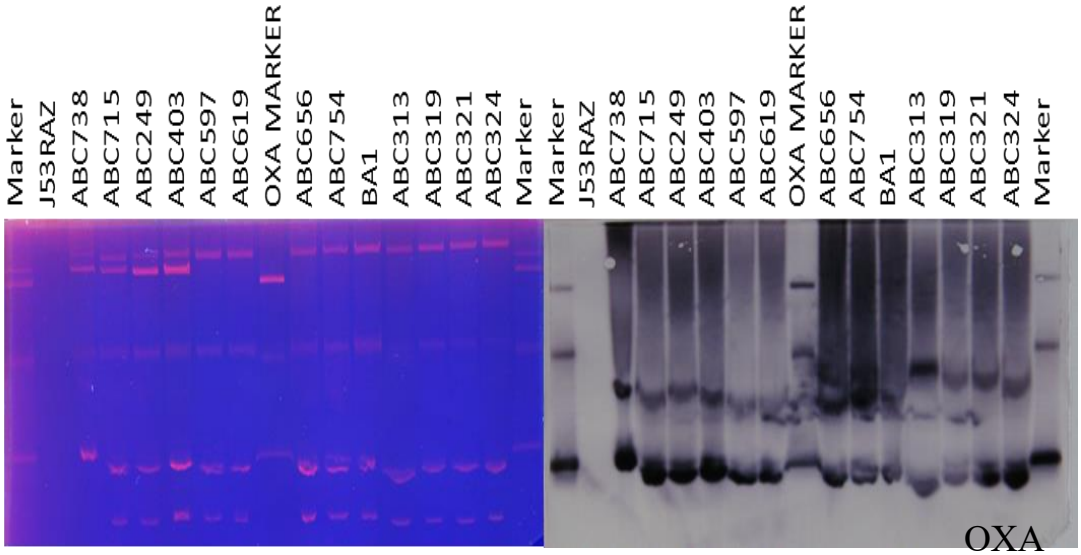
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ABC324
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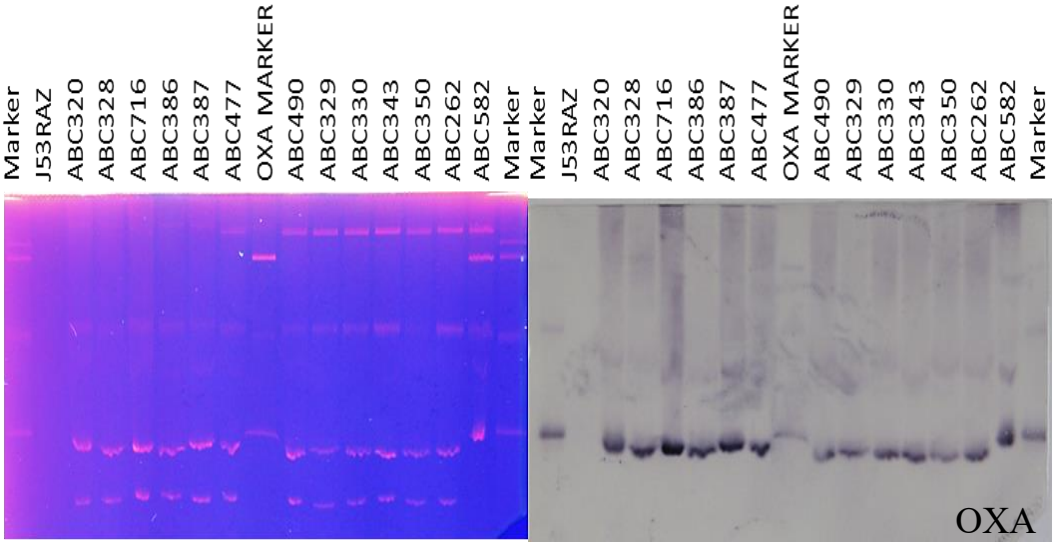
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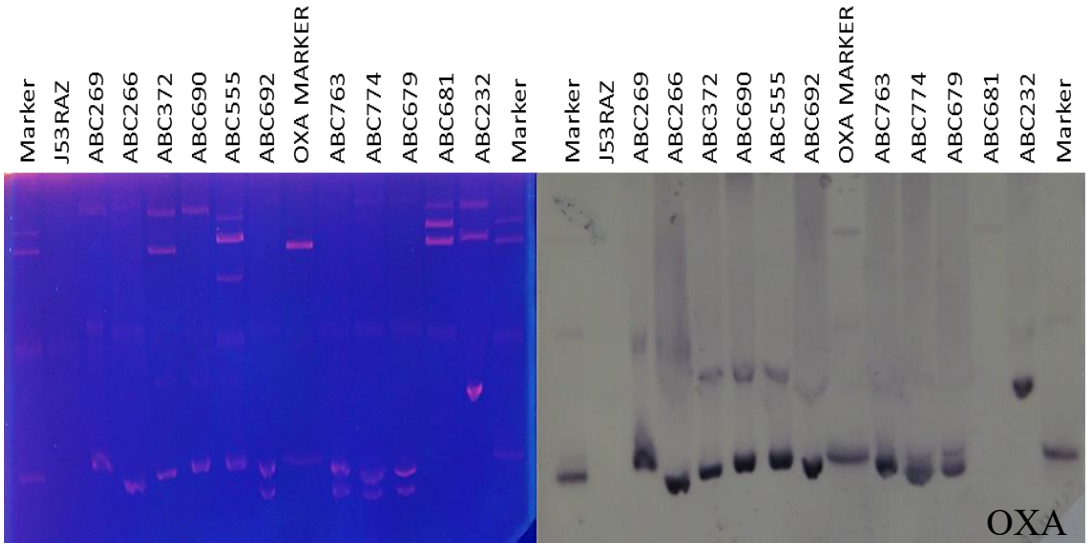
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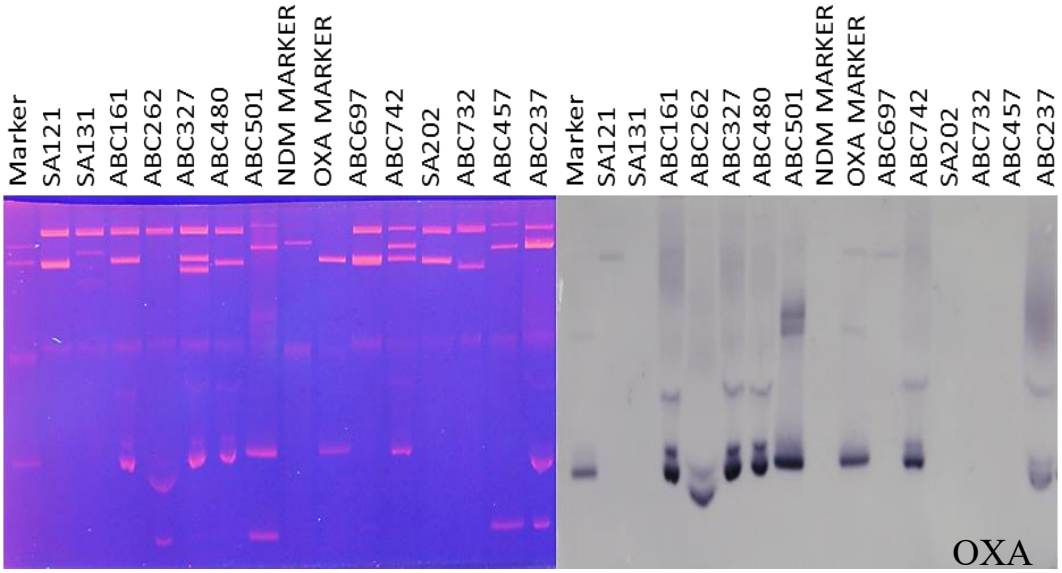
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ABC237
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SA121
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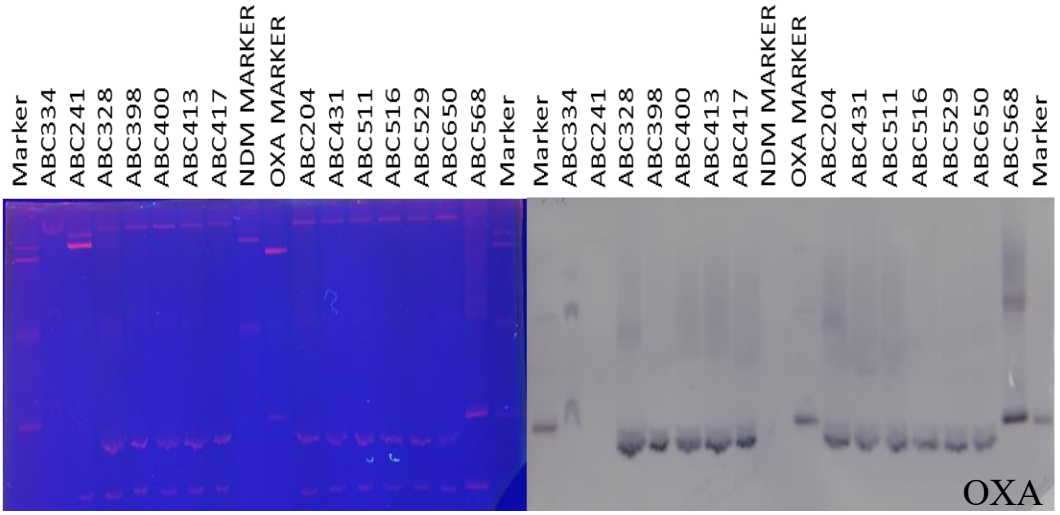
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ABC650
ABC568
Marker
Marker
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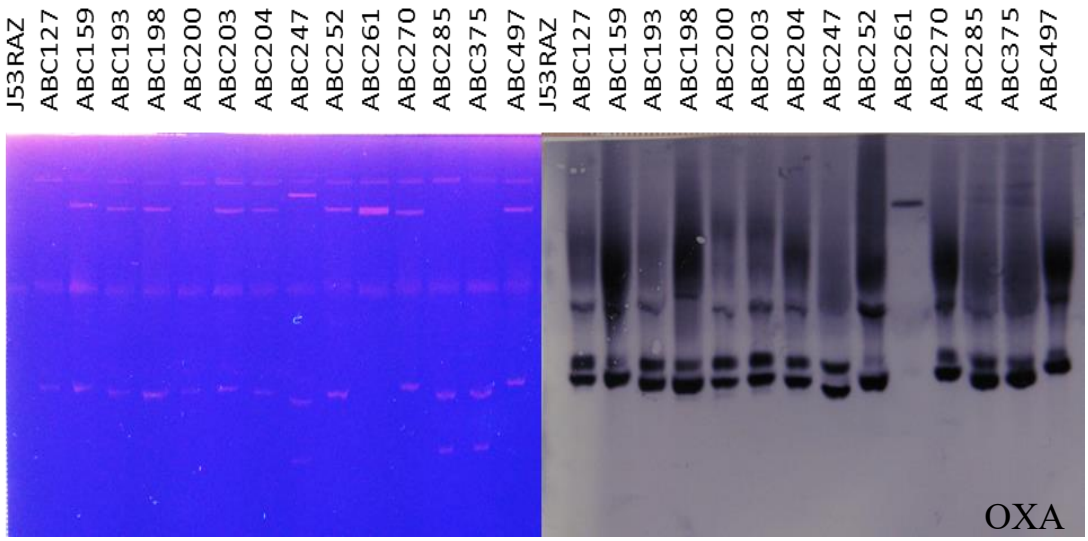
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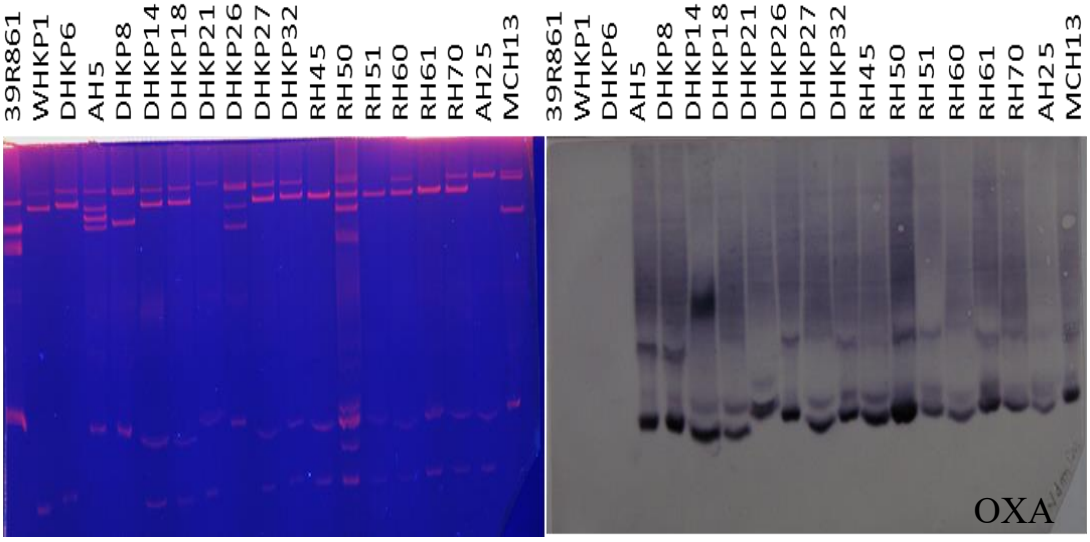
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RH51
RH60
RH61
RH70
AH25
MCH13
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WHKP1
DHKP6
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DHKP14
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RH45
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RH51
RH60
RH61
RH70
AH25
MCH13
OXA