CARBAPENEMASE PRODUCING *Enterobacteriaceae:* MOLECULAR EPIDEMIOLOGY AND ASSESSMENT OF ALTERNATIVE THERAPEUTIC OPTIONS

By

LAURA JIMENA ROJAS COY

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Department of Microbiology and Molecular Biology

CASE WESTERN RESERVE UNIVERSITY

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CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the dissertation of
Laura Jimena Rojas Coy

candidate for the degree of **Doctor of Philosophy*.**

Committee Chair
Dr. Arne Riestch

Committee Member
Dr. Liem Nguyen

Committee Member
Dr. Anthony Wynshaw-Boris

Committee Member
Dr. Robert A. Bonomo

Date of Defense
March 22, 2018

*We also certify that written approval has been obtained
for any proprietary material contained therein.

DEDICATION

To my mother Maria Grelly and my father Daniel for raising me believing that with hard work I could achieve anything I set my mind to. To my loving brother Cami for constantly looking up to me, inspiring me to always do my best; and finally to my adorable little sister Angie for her infinite an unconditional love. I love you all so much.

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PREFACE

The goal of this work is to understand the dissemination of antimicrobial resistance determinants in clinically important Gram negative bacteria (GNB). To that end we applied genomics to determine local and global epidemiology of important carbapenem resistance determinants, and applied this knowledge to design novel therapies. This dissertation will explore antimicrobial resistance of clinically important resistant Gram negative bacteria by two main approaches

a) Molecular Epidemiology

b) Biochemical and therapeutic approaches for alternative treatment options.

My research will involve a multi-disciplinary program which involves:

1) Exploring structure-activity based design and antibacterial activity of novel inhibitory compounds that mimic β-lactams and inactivate class A β-lactamases, and the efficacy of novel combination therapies using clinically available antibiotics (Chapters Two and Three);

2) Utilizing genomic tools to understand the molecular basis of the dissemination of the three most important β-lactam resistance determinants amongst GNB: bla_{KPC}, bla_{NDM-1} and bla_{OXA-48} (Chapters Three and Four);

3) Investigating the prevalence, impact on mortality and resistance mechanisms of bactericidal cationic peptides Polymyxin/Colistin (Chapter five).

Synthesizing the data obtained from these projects will advance our knowledge about dissemination of antimicrobial resistance and development of novel therapies.

Carbapenemase Producing *Enterobacteriaceae:* Molecular Epidemiology and Assessment of Alternative Therapeutic Options

Abstract

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The global spread of GNB resistant to nearly all available antibiotics is currently one the most pressing issues in bacterial resistance. The speed of dissemination and the lack of development of new antimicrobial drugs are rapidly outpacing available treatment options. This complex problem requires innovative solutions combining knowledge about molecular epidemiology to determine the applicability of existing and potential new antimicrobial therapies; as well as about the mechanisms of resistance to develop novel compounds or antibiotic combinations. β-lactams are the most important class of antibiotics used to treat infections caused by both Gram-negative and Gram-positive bacteria. Carbapenems, often considered as the "last resort" antibiotics, are used to treat the most resistant Gram-negative pathogens. Unfortunately, bacteria have developed resistance mechanisms including production of β-lactamases, enzymes that selectively hydrolyze the β-lactam ring, rendering the antibiotics ineffective against their natural targets.

Herein, we study the molecular basis of the dissemination of the three most important carbapenem resistance determinants amongst GNB:

bla$_{KPC}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$. Our underlying hypothesis is that carbapenemase gene dissemination is driven by a combination of horizontal transmission via mobile genetic elements and clonal spread. By conducting an extensive genomic analysis of the *K. pneumoniae* population, we show that convergence of horizontal dissemination and clonal dissemination is key for the endemicity of *bla*$_{KPC}$ in Colombia. On the contrary, horizontal dissemination is the main driver of the uncontrolled expansion of *bla*$_{NDM-1}$ in Colombia, and *bla*$_{NDM}$ and *bla*$_{OXA-48}$ in the US. In the face of the expansion of this carbapenem producing *Enterobacteriaceae* (CPE), new therapies and drugs are needed. Therefore, a new boronic acid transition state inhibitor (BATSI) was designed and tested in combination with cefepime; the efficacy of the novel ceftazidime-avibactam and aztreonam was tested for the treatment of metallo-β-lactamase producers, and insights into the clinical impact of the accuracy of the methods to determine colistin resistance are given. Lastly, we hypothesized that alterations in bacterial LPS composition that are linked to polymyxins resistance impacts survival.

Results compiled in this dissertation provide valuable lessons on the epidemiology of CPE, towards the development of novel drugs and testing of novel combination therapies. Moreover, we uncovered a significant link between resistance mediated by cell wall modifications and survival.

CHAPTER 1 - INTRODUCTION

β-lactams and β-lactam resistance mechanisms

Antimicrobial resistance (AMR) is a complex global public health challenge. In spite of being a natural phenomenon in microorganisms, AR is increasingly accelerated by the selective pressure exerted by use and misuse of antimicrobial agents in humans and animals. The current lack of new antimicrobials on the horizon to replace those that become ineffective brings added urgency to the need to protect the efficacy of existing drugs. The problem is so serious that even the achievements of modern medicine may be at risk. Without effective antimicrobial agents against common community-acquired infections, postoperative surgical site infections, or infections in patients particularly vulnerable such as cancer or organ transplanted, the infection is likely to become life-threatening and may be fatal (1).

β-lactam antibiotics target bacterial penicillin binding proteins (PBPs) exerting a bactericidal effect by interfering with peptidoglycan synthesis (i.e., construction of the cell wall) (2). Over the past 60 years, these antibiotics have been the most widely used, and represent approximately 60% of all of the antibiotics used (by weight) in human and veterinary medicine. Until very recently, the cephalosporins were the antibiotics of choice for treating common GNB caused infections (Figure 1-1). However, soon after their widespread use, pathogens resistant to cephalosporins

(i.e., extended spectrum β-lactamases (ESBLs) and AmpC-producing strains) emerged and unfortunately, many ESBL producing bacteria became "common community-acquired pathogens" (3). Treatment of these community-acquired ESBLs required the use of carbapenems (Figure 1-1) (e.g., imipenem, meropenem, doripenem, and ertapenem), which are dipolar compounds (zwitterions) that rapidly enter GNB through outer membrane proteins (OMPS or porins). In many instances, carbapenems were regarded as the most potent β-lactam antibiotics and “life-saving drugs”.

For a brief time, clinicians were comforted by the knowledge that β-lactamases hydrolyzing carbapenems were infrequent. However, the clinical landscape rapidly changed. Now, we are in the midst of a global crisis of carbapenem-resistant GNB where bacterial β-lactamases emerged as the primary mechanism responsible for this phenotype (4). The global dissemination of carbapenem-resistant GNB is a significant source of morbidity and mortality and carbapenemases are severely limiting the treatment of infections caused by bacteria possessing these enzymes (5).

In GNB, β-lactamases can be classified into two groups according to the nucleophile present within the active site allowing them to hydrolyze the β-lactam ring: (i) class A, class C and class D β-lactamases contain a serine in the active site (class A enzymes can be inactivated by β-lactamase inhibitors, including clavulanic acid, sulbactam and tazobactam); and (ii) class B MBL, which contain one or more zinc atoms in the active site and

are inhibited by chelators (e.g., EDTA). Importantly clinical compounds are currently not available to inhibit MBLs (Figure 1-2) (28).

Dissemination of Antimicrobial Resistance

The dissemination of AMR in GNB is largely attributed to inter- and intra-specific DNA exchange. The horizontal transfer of plasmid-located resistance genes is considered the prevalent mechanism at the origin of acquisition of resistance in bacterial pathogens causing community- or hospital-acquired infections (6). Plasmids are extra-chromosomal circular fragments of DNA that replicate autonomously in a host cell and require independent mechanisms for maintenance and coinheritance into daughter cells (7). Plasmids have co-evolved with bacteria, providing intact functional genes that assist in adapting to harsh conditions and unsuitable environments, as they often contain various combinations of virulence, fitness, and AR genes. Once a resistance plasmid is transferred to and replicates in a new bacterial host, the presence of antimicrobial agents will create selective pressure that will discriminate for a bacterial population with such resistance plasmids. However, in the absence of antimicrobial agents, the resistance plasmid may be retained only transiently by the bacterium if it is unstable within the host. The long-term stability and persistence of resistance plasmids in various bacterial populations during the absence of antibiotic selection pressures is receiving surprisingly little attention. Very little is known about the impact of plasmid stability on the long-term survival and host range of resistance

plasmids, especially in the absence of selection pressure created by antimicrobial agents (8).

Plasmids are classified based on incompatibility (Inc) since the 1970s, which is defined as the inability of plasmids sharing similar replication and partition systems to be propagated stably in the same host cell line. Classification into an Inc group is based on the amino acid sequence of the replication initiation (Rep) protein, however whether the plasmid shows incompatibility with the same Inc group plasmid within the same host cell line is not necessarily confirmed by conventional methods (7). Newer plasmid classification methods are based on the molecular characteristics of the replicons instead (replicon typing), which consist in analyzing the nucleotide sequences of not only the origin of replication (ori) but also those that encode specific replication initiator proteins (Rep) that bind the ori and their regulating factors (9). Currently, 27 Inc groups are recognized in *Enterobacteriaceae* by the Plasmid Section of the National Collection of Type Culture (Colindale London, UK). Since 2005, a PCR-Based Replicon Typing (PBRT) scheme has been available, targeting the representative replicons of major plasmid incompatibility groups and replicase genes identified on resistance plasmids circulating among *Enterobacteriaceae* (9, 10) (Table 1-1). More recently, a PBRT has been described for plasmids identified in *Acinetobacter* spp., where twenty-seven replicase genes, completely different from those circulating in

Enterobacteriaceae are detected in a convenient way by defining 19 distinct groups in 6 PCR multiplexes (11).

This typing method is quite successful, however difficulties include: (i) plasmids frequently carry multiple replicons, and it is therefore difficult to classify the plasmid into single replicon group; (ii) detailed information about Inc groups or Rep types is limited among several microbial taxonomies especially *Enterobacteriaceae*, and iii) it is difficult to identify replication regions for the other types of plasmids (7). Therefore, not all plasmids can be typed with this technique; those plasmids that do not show results with incompatibility typing are often referred to as non-typeable. The most accurate method to characterize a plasmid is based on the determination of the full-length DNA sequence and to date, more than 11500 *Enterobacteriaceae* plasmids have been fully sequenced (http://www.ncbi.nlm.nih.gov; search parameters: enterobacteriaceae[Organism] AND plasmid[filter] NOT shotgun).

Very often multiple physically linked genetic determinants, conferring resistance to different classes of antibiotics can be identified on the same plasmid, conferring selective advantage to the bacterial recipient when several antimicrobials are simultaneously administered. Plasmids conferring multi-drug resistance are usually large (>50 kb), self-conjugative and encode sophisticated mechanisms controlling their copy number, by regulating the rate of replication (6). Plasmids that belong to different incompatibility groups exhibit variable behavior characteristics

(e.g., narrow-bacterial-host-range plasmids versus broad-host-range plasmids and high-copy-number plasmids versus low-copy-number plasmids). The classification of broad-host-range versus narrow-host-range is based on the diversity of the bacterial hosts into which plasmids can be transferred and then successfully maintained in a sustainable manner. The narrow-host-range group of plasmids most often belongs to incompatibility group F (IncF), which contains different replicons (e.g., FIA, FIB, and FII), while the broad-host-range group includes IncA/C, IncL/M and IncN (among others). Broad-host-range plasmids can easily be transferred between different species, while narrow-host-range plasmids tend to be restricted to certain species or even clones within species. This is of special relevance in high-risk clones, as there is a predominance of narrow-range plasmids (specifically IncF with certain β-lactamases [e.g., *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{KPC-2/3}}$]) in these clones. IncF plasmids are diverse, complex, are present in low copy numbers and use post-segregational killing and addiction systems to ensure their propagation among high-risk clones. These plasmids were recently termed "epidemic resistance plasmids" due to their propensity to acquire resistance genes and rapid dissemination among the *Enterobacteriaceae*. AMR determinants on epidemic plasmids provide a selective advantage to high-risk clones and are likely to play a central role in their success (8).

Plasmids also contribute to bacterial genetic diversity by acquiring and losing genes through several types of mobile elements, such as insertion

elements and transposons, and horizontal exchange among bacterial populations by conjugation or mobilization. Transposition is the mobilization of a DNA sequence from one DNA site to another. This mechanism is facilitated by transposons (Tn), insertion sequence (IS) elements and the most recent, IS common region (ISCR) elements (Figure 1-3). IS elements are genes encoding a transposase enzymes capable of copying the DNA sequence of the IS element into new DNA locations. Transposons can be classified into two subgroups i) composite, consisting of two IS elements flanking a sequence and the two IS element sequences that work in tandem to mobilize the DNA sequence between the two flanking IS elements; ii) unit transposons, that work in a similar mechanism, but utilize other enzymes in addition to the transposase such as recombinases, to mobilize the sequence within the unit transposon. ISCR are transposases highly associated with Class 1 integron capture systems and utilize a rolling circle mechanism to mobilize DNA (12).

Impact of carbapenem resistant *Enterobacteriaceae*, the importance of carbapenemases and the threat to β-lactam therapy in the World

For the past three decades, imipenem and the other carbapenems (meropenem, ertapenem and doripenem) were trusted by physicians to effectively treat severe infections caused by suspected drug-resistant bacteria in very ill patients. Regrettably, the utility of this class of antibiotics is now severely compromised by the emergence of resistance

(13). In GNB, one of the most important concerns is the increasing resistance to β-lactams, especially carbapenems, the drugs of "last resort" to treat severe community and hospital acquired infections. The most common mechanism of resistance to such agents is the production of carbapenemases, β-lactamases that hydrolyze carbapenems. Carbapenemases have emerged as a critical clinical problem in the past fifteen years. In the 1980s and 1990s, only a few β-lactamases could inactivate carbapenems, and these were limited to rare strains of *E. cloacae* (e.g., NMC-A and IMI-1), *Serratia marcescens* (e.g., Sme-1), *Bacillus cereus* (e.g., MBLs I and II), *Stenotrophomonas maltophilia* (e.g., MBL L1), and *Bacteroides fragilis* (e.g., MBL CcrA) (20). However, in a very short time, serine carbapenemases (especially KPC) and MBLs (such as NDM) emerged and became more frequent (23).

Enterobacteriaceae are a family of more than 70 bacteria including *Klebsiella pneumoniae* and *E. coli* that normally live in the digestive tract. In the US, *Enterobacteriaceae* account for 27% of health care associated infections (14). During the past decade, this family of bacteria has become increasingly resistant to carbapenems, and more hospitalized patients are getting lethal infections; for example, carbapenem resistant *Enterobacteriaceae* (CRE) kill up to half of the patients who get bloodstream infections from *K. pneumoniae* (15). According to the CDC, in the past decade the percentage of CRE in the US increased by fourfold, with carbapenem resistant *Klebsiella pneumoniae* showing a sevenfold

increase (16). The number of US facilities reporting CRE, is rising steadily and includes 4% of acute hospitals and 18% of long term acute care facilities (16). In addition to spreading among patients, often on the hands of health care personnel, CRE bacteria can transfer their resistance to other bacteria within their family. This type of spread can create additional life-threatening infections for patients in hospitals and potentially for otherwise healthy people. Currently, almost all CRE infections occur in people receiving significant medical care in hospitals, long-term acute care facilities, or nursing homes. During just the first half of 2012, almost 200 hospitals and long-term acute care facilities treated at least one patient infected with these bacteria (17).

Although the term "CRE" is useful as descriptor of our current epidemic, the considerable diversity of bacteria expressing that phenotype and the complex mechanisms that determine carbapenem resistance are notable. Nonetheless, the chief determinant of carbapemem-resistance in *Enterobacteriaceae* are carbapenemases (13). Three groups of carbapenemases—KPC, NDM, and OXA-48—are currently considered to be the ones of major epidemiologic and clinical significance among this family.

Klebsiella pneumoniae carbapenemase (KPC): a major threat worldwide

By far, the most frequently identified class A carbapenemases are the KPC enzymes (18). KPC was first described in a Kpn isolate identified in North Carolina (US) in 1996 as part of a surveillance program (19). KPC β-lactamases can hydrolyze all β-lactams, including carbapenems, cephalosporins, cephamycins, monobactams, and clavulanic acid (18). To date, 24 different variants are identified (Table 1-2) (http://www.lahey.org/Studies/), however KPC-2 and KPC-3 are the most prevalent. Susceptibility testing shows that most of the times strains possessing bla_{KPC} are multidrug-resistant (MDR). In addition to the carbapenemase, these strains are also resistant to aminoglycosides due to the presence of genes encoding aminoglycoside modifying enzymes (AMEs, e.g., *aac(3)-Ia*), or ribosomal methyl transferases (including *armA,* and *rmtA*) and quinolones (i.e., mutations in *gyrA* and *parC* or the presence of *aac6'-Ib-cr,* an AME that modifies quinolones) (20).

Although Kpn is still the most common carrier of KPC, there is an increasing number of other KPC-producing GNB (Table 1-2) (29), which raises significant concern as poor outcomes and high mortality rates (47-66%) in patients having infections by these bacteria, are reported (11). To complicate things further, silent dissemination of KPCs is described, highlighting the necessity for surveillance efforts (53).

Since their discovery 18 years ago, KPC carrying GNB have spread worldwide (Figure 1-4); however, their local epidemiology and clinical characteristics vary. Some countries became endemic (e.g., Israel, Greece, Brazil and Colombia) whereas others largely continue to possess only imported cases (e.g., Australia, New Zealand, and Canada). However, exceptions, such as the UK, experienced a plasmid expansion instead of clonal expansion (11).

The molecular epidemiology of KPC-producing strains indicates that the most heavily affected countries describe clonal expansion of KPC-producing Kpn, the predominant species, suggesting a unique fitness and selective advantage beyond resistance (21). *bla*$_{KPC}$-Kpn dissemination is primarily associated with a single multilocus sequence type (ST), ST258, and its related variants, which is the predominant clone worldwide. To date, *bla*$_{KPC}$ is found in more than 115 different STs (close to 7.5% of all Kpn STs reported), showing a broad heterogeneous distribution (Figure 1-5). Nevertheless, the vast majority of KPC-Kpn isolates worldwide belong to what is now known as clonal group (CG) 258 a large group containing 43 different STs, with ST258 and ST512 being the two predominant sequence types. CG258 is reported in more than 25 countries from four continents, including the majority of the KPC epidemic countries mentioned previously. The widespread presence of isolates belonging to this clonal group suggests a clonal dissemination in most locations, highlighting the importance of early detection combined with infection

control measures as a key strategy to control infections caused by KPC-harboring organisms. As an example, KPC-Kpn ST258 causes 90% of all infections in Israel and is responsible for >77% of outbreaks in the USA (18, 21-24). By contrast, the spread of other non-CG258 KPC-Kpn STs are largely limited to certain geographic regions, for example, the recently emerged MDR ST442 in Southern Brazil (22).

The spread and success of KPC-producing GNB is multifactorial as dissemination of the *bla*$_{KPC}$ can be mediated by different molecular mechanisms, from the mobility of small genetic elements, namely the promiscuous Tn*4401* transposon that "jumped" to numerous plasmids (usually conjugative), to the horizontal transfer of plasmids, and via clonal spread (21). The most common *bla*$_{KPC}$-containing mobile element is Tn*4401*, a Tn3-based transposon, 10 kb in length, delimited by two 39-bp imperfect inverted repeat (IR) sequences, that harbors *bla*$_{KPC}$, a Tn3 transposase gene (*tnpA*), a Tn3 resolvase gene (*tnpR*), and two insertion sequences, ISKpn6 and ISKpn7 (Figure 1-6 **a**). Five Tn*4401* isoforms (a–e) were identified, differing by 68–255 bp deletions up-stream of *bla*$_{KPC}$ as shown in Figure 1-6 **a** (25). *bla*$_{KPC}$ is also found in other non-Tn*4401* mobile elements (NTEs) that contain genetic remnants of Tn*4401* from Kpn isolates in certain regions such as China and Argentina, as well as in other non-Kpn species, summarized in Figure 1-6 (21). *bla*$_{KPC}$ is typically plasmid-borne, and is carried on variety plasmids of different incompatibility (Inc) groups, including IncFII, FIA, I2, A/C, N, X, R, P, U,

W, L/M and ColE1 (Table 1-3). The epidemiology associated with *bla*$_{KPC}$ plasmids indicates that certain incompatibility groups harboring Tn*4401* are more predominant, for example IncFII plasmids, because they are commonly low in copy number, harbor multiple replicons, and are widely distributed in different species of *Enterobacteriaceae* (21).

New Delhi Metallo-ß-lactamase, an emerging MBL

In parallel to the crisis with KPC, NDM MBLs are also rapidly rising in prevalence. Although retrospective analyses of stored cultures identified the gene encoding this enzyme (*bla*$_{NDM}$) in Indian *Enterobacteriaceae* isolates from 2006 (26), the first documented case of infection caused by an NDM carrier occurred in Sweden in 2008, by a Kpn isolate in a patient that was previously admitted into an Indian hospital (27). Since its first description, NDM carbapenemases were reported in over 40 countries worldwide, encompassing all continents except Antarctica (Figure 1-7) (28, 29).

The dissemination of *bla*$_{NDM}$ demonstrates a complex epidemiology involving the spread of a variety of species of *bla*$_{NDM}$-carrying bacteria and the inter-strain, inter-species and inter-genus transmission due to a genetic environment that is readily transferable, (to date found on the chromosome, plasmids, and in integrons) proving to be more complex and apparently, more unpredictable than KPC (29). Thought to have a chimeric origin likely generated in *A. baumannii*, (30) NDM, with 16 variants to date (http://www.lahey.org/Studies/), confers resistance to all β-lactams

(penicillins, cephalosporins, carbapenems, and the clinically available β-lactamase inhibitors). Interestingly, the hydrolytic profile of MBLs does not typically include monobactams (*e.g.* aztreonam). Nevertheless, as is the case for KPC-harboring isolates, NDM-bearing strains often exhibit a MDR profile (28). An association with other AR determinants is also observed in almost all NDM producers. Those associated resistance determinants include AmpC cephalosporinases, ESBLs, other types of carbapenemases (OXA-48-, VIM-, and KPC-types), and resistance to aminoglycosides (16S RNA methylases), to quinolones (Qnr), to macrolides (esterases), to rifampicin (rifampicin-modifying enzymes), to chloramphenicol, to bleomycin, and to sulfamethoxazole, leaving only colistin, tigecycline, and fosfomycin as therapeutic options (28). As a result, infections caused by bacteria possessing NDM are associated with significant in-hospital morbidity (longer mean hospital stays and ICU stays) and mortality (31). To complicate things further, isolates containing bla_{NDM} can be carried without the presence of disease (colonizers) making it difficult to devise effective screening programs or track dissemination (32).

Although most of NDM-producing bacteria are *Enterobacteriaceae*, this MBL has also been reported in *Acinetobacter* spp. and in rare cases *P. aeruginosa.* Among the *Enterobacteriaceae,* NDM production is most often reported in Kpn and *E. coli*, involved in both hospital- and community-acquired infections including urinary tract infections, peritonitis, septicemia, pulmonary infections, soft tissue infections, and device-

associated infections. However, this MBL is also frequently described in other enterobacterial species including *K. oxytoca*, *E. cloacae*, *C. freundii*, *P. mirabilis*, and *Providencia* spp; and rarely on isolates of S*almonella* spp. and *Vibrio cholera (28).*

As opposed to what is observed for *bla*$_{KPC}$, *bla*$_{NDM}$ is not directly associated with certain clones, plasmids, or transposons. As an example, *bla*$_{NDM}$ is reported in at least 11 different STs of both *E. coli* and Kpn to date, indicating a high level of inter-lineage and inter-species gene transfer. Despite reports of chromosomal location in certain isolates, *bla*$_{NDM}$ is primarily associated with multiple separate acquisition events mediated by highly heterogeneous plasmids of different sizes and from incompatibility groups with both broad and narrow host ranges, such as IncF, IncA/C, IncL/M, IncH, IncN and IncX3 (Table 1-4) (28, 29).

The diversity of genetic features associated with the *bla*$_{NDM}$ gene may explain its current high rate of spread worldwide. With the exception of *A. baumannii*, where Tn*125* appears to be the main vehicle for dissemination of *bla*$_{NDM}$ (33), in the rest of species the only common features in the genetic environment are IS*Aba125* element and a bleomycin resistance gene (*ble*$_{MBL}$) identified upstream and downstream, respectively, of the *bla*$_{NDM-1}$ gene (Figure 1-8) (29, 34, 35). The recently reported association of *bla*$_{NDM-1}$ with a class 1 integron ISCR1, one of the most commonly used mechanisms for the spread of antibiotic resistance across species, may also explain the rapid expansion of this gene (36).

Tn*125* is the platform on which *bla*$_{NDM}$ is theorized to be originally mobilized from *Acinetobacter* spp into plasmids and then facilitating mobilization into other species such as those found in the *Enterobacteriaceae* family. However, a range of truncated Tn*125* structures of different lengths are reported (Figure 1-8); in spite of their varying lengths certain Tn*125* structures are still repeatedly observed in different plasmid backbones. Interestingly, various IS elements were identified directly upstream of *bla*$_{NDM-1}$ when the 5' -end IS*Aba125* is truncated. These IS elements may have inserted close to *bla*$_{NDM}$ to provide a one-ended transposition mechanism to mobilize *bla*$_{NDM}$ into new plasmid backbones. That close proximity of *bla*$_{NDM}$ to a transposition mechanism may explain why *bla*$_{NDM}$ is reported on various different plasmid backbones, which in turn, also allowed the spread to various bacterial hosts (37).

OXA-48: "The phantom menace"

OXA-48 belongs to the class D β-lactamases following the Ambler classification. According to the NCBI's Bacterial Antimicrobial Resistance Reference Gene Database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047), this class currently groups 568 different variants, originally named oxacillinases due to their preferred penicillin substrate (they commonly hydrolyze the isoxazolylpenicillin oxacillin much faster than classical penicillins, i.e. benzylpenicillin)(38). Among class D β-lactamases, only 3 subgroups have

the capability of hydrolyzing carbapenems (39). The first two subgroups share only a 60% identity and possess a weak activity against imipenem; the first group includes OXA-24, OXA-25, OXA-26, and OXA-40, and the second group consists of OXA-23 and OXA-27, that share 99% amino acid identity (40-44). The third group includes OXA-48 and its variants, which have a stronger activity against imipenem and share less than 46% amino acid identity with any other oxacillinases (45). These class D carbapenemases are of increasingly high clinical relevance, as they contribute to the evolution towards extensively or pan-drug resistant phenotypes of already difficult-to-treat pathogens such as *Acinetobacter baumannii* and *Klebsiella pneumoniae* (46).

The OXA-48 carbapenemase was first reported in a *Klebsiella pneumoniae* isolate from a 54-year-old man with a urinary tract infection and skin burns from Istanbul (Turkey) in 2001 (45). Since then, several variants (differing from OXA-48 by one to five amino acid substitutions and/or by a four-amino acid deletion) have emerged and disseminated and is currently considered the third globally distributed group of carbapenemases (47, 48) (Table 1-5). Outbreak reports of OXA-48-*like*–producing *Enterobacteriaceae* are concentrated in European and Mediterranean countries including North Africa, whereas sporadic cases have been identified in many other countries worldwide, commonly related to travel to the Mediterranean or Southeast Asia (Figure 1-9) (47).

It has been proposed that OXA-48 originated in *Shewanella* spp., a waterborne environmental bacterium. A chromosome-located β-lactamase gene from a *Shewanella oneidensis* encoding a carbapenem-hydrolyzing Ambler class D β-lactamase, OXA-54, was found to share 92% amino acid identity with the plasmid-encoded OXA-48 originally found in *Klebsiella pneumoniae* (49). Likewise, several other variants of *bla*$_{\text{OXA-48}}$ genes have been identified in *Shewanella xiamenensis* strains including *bla*$_{\text{OXA-181}}$ (50), *bla*$_{\text{OXA-199}}$ (51), and *bla*$_{\text{OXA-204}}$ (52). Comparison of the surrounding sequences of *bla*$_{\text{OXA-48}}$ to those of *bla*$_{\text{OXA-54}}$ strongly suggest that mobile genetic elements might have been involved in the mobilization of *Shewanella* spp. chromosomal carbapenemase to plasmids, which have then spread to other bacterial species. Specifically, although the insertion sequence IS1999 found upstream of the plasmid-located *bla*$_{\text{OXA-48}}$ gene in *K. pneumoniae* was not found in *S. oneidensis*, 21 out of 26 bp that separated IS1999 from the start codon of *bla*$_{\text{OXA-48}}$ were identical to the DNA sequence located upstream of the *bla*$_{\text{OXA-54}}$ gene. Noncoding sequences of 236 and 303 bp found immediately downstream of the *bla*$_{\text{OXA-54}}$ and *bla*$_{\text{OXA-48}}$ genes, respectively, did not share nucleotide identity. However further downstream, the 252 bp of the 3′ end of a gene encoding a putative LysR-type transcriptional regulator was found in *S. oneidensis*. This 252-bp sequence shared 88% nucleotide identity with the sequence located further downstream of *bla*$_{\text{OXA-48}}$ (49).

The widespread dissemination of *bla*$_{\text{OXA-48-}like}$ is mediated by the rapid spread of broad host-range conjugative plasmids (Table 1-6), usually harboring these carbapenemase genes within a composite transposon, namely Tn1999 (53). Tn1999, is a composite transposon with two copies of IS1999 flanking the carbapenemase gene (45). IS1999 encodes a putative transposase of 402 amino acids (17) and belongs to the IS4 family, like other members of this family it is delimited by short imperfect inverted repeat sequences (IR). IS1999 is 1,328 bp long, has 21-bp imperfect terminal repeats, and generates a 9-bp target site duplication after transposition (54). Several transposon variants have been described to date (Tn1999.2-Tn1999.5; Figure 1-10), and usually involve rearrangements or truncation by other transposons, indicating the plasticity of these mobile genetic elements, and explaining why in some cases *bla*$_{\text{OXA-48-}like}$ genes have also been found in the chromosome of several *Enterobacteriaceae* (55, 56). Tn1999.2 was identified in clinical *K. pneumoniae* isolates from Turkey and differs to Tn1999 by the insertion of IS1R within the IS1999 located upstream of *bla*$_{\text{OXA-48}}$ (57). The Tn1999.3 was identified in an *E. coli* isolate from Italy, with a second copy of IS1R located downstream of *bla*$_{\text{OXA-48}}$ (58). Tn1999.4 was found in *E. coli* and *E. cloacae*, and is composed of Tn1999.2 truncated by Tn2015 transposon; this latter is comprised of ISEcp1, *bla*$_{\text{CTX-M-15}}$, and a truncated Tn2-type transposase gene (59). Finally, Tn1999.5, is a novel variant of the Tn1999.2 transposon in which the lysR gene encoding a transcriptional

regulator was truncated by the ISKpn19 element (60). Due to its high transfer efficiency to any enterobacterial species (61), the backbone most commonly associated with the spread of OXA-48–is an IncL/M-type plasmid with integration of the *bla*$_{\text{OXA-48}}$ gene through the acquisition of a Tn1999 composite transposon (6). Along with IncF, IncA/C, IncI, IncHI2, and IncX3 and ColE-like replicons, the IncL/M plasmids are currently one of the six major resistance plasmid families identified in clinically relevant *Enterobacteriaceae*, and are now commonly identified among both environmental and clinical isolates (6).

Among *K. pneumoniae*, *bla*$_{\text{OXA-48-}\textit{like}}$ genes are found in multiple STs; however, some dominant clones, including ST101, ST395, ST405, ST11, ST14, and ST15, are the most commonly associated with outbreaks in endemic regions. ST101 was the most commonly observed ST in the Mediterranean area (62, 63). Within the *E.coli* population the dissemination of OXA-48 is mostly polyclonal, however the acquisition by already successful clones has been already observed e.g. ST 131 in Spain or the emerging global epidemic clone, ST38 that dominates in North Lebanon, in the UK, in Finland and in France (55, 64-66)[41, 193, 194], [56].

β-lactam Inhibitors

β-Lactamases catalyze the hydrolysis of the β-lactam amide bond to generate ring-opened products that are no longer capable of inhibiting the transpeptidase activity of PBPs. Based on amino acid sequence identity,

these enzymes are classified into four classes A, B, C and D (67). Classes A, C and D group enzymes that use an active-site serine nucleophile and a transient covalent reaction intermediate to catalyze the hydrolysis of β-lactams. These so-called "serine β-lactamases" acylate β-lactam antibiotics and use strategically positioned water molecules to catalyze the hydrolysis of the acylated β-lactam. Finally, deacylation occurs to form the cleaved inactive β-lactam product and to regenerate the active enzyme (Figure 1-11) (68). In contrast, class B groups the Metallo-Beta-Lactamases (MβLs), which catalyze hydrolysis of β-lactams through a non-covalent mechanism in which one or two equivalents of bound zinc ions promote formation of a nucleophilic hydroxide that substitutes for the Ser residue used by class A, C, and D β-lactamases (69).

Two strategies for combating β-lactamase-mediated resistance have been designed. The first contemplates the design of substrates that reversibly and/or irreversibly bind the enzyme with high affinity, but form catalytically incompetent conformations that are poorly hydrolyzed (e.g. extended-spectrum cephalosporins, monobactams, and carbapenems). The second strategy is the development of mechanism-based or irreversible "suicide inhibitors" (70). Example of these, are commercially available serine β-lactamase-inhibitors, such as sulbactam, tazobactam, and clavulanic acid, that can permanently inactivate the β-lactamase by trapping a covalent adduct with the active-site Ser residue of these enzymes (68). Noteworthy, these inhibitors only work against serine β-

lactamases since the absence of a covalent intermediate during the catalytic mechanism of MβLs renders them ineffective against these enzymes.

Several years after the introduction of β-lactamase-inhibitors, strains of *E. coli* and *K. pneumoniae* resistant to the clinically used combinations of β-lactam/ β-lactam inhibitors (BLIs) e.g. amoxicillin-clavulanate, ticarcillin-clavulanate, were identified (71-74). Later studies revealed that the inhibitor resistant phenotype may be the result of several mechanisms; i) production of β-lactamases not susceptible to the inhibitors, such as AmpCs, KPC or MβLs, ii) enzyme hyper production, or ii) point mutations on the *bla* genes that result in the production of Inhibitor Resistant (IR) enzymes. For instance, the most common substituted residues in IR TEM (IRT) and IR SHV are Arg244, Asn276, Arg275, Met69, and Ser130 (75-78). To overcome the ever-more common presence of IR β-lactamases and to preserve the existing β-lactams, novel β-lactamase inhibitors have been developed. Some of these novel compounds are β-lactams derivatives such as monobactams, methylidene penems, oxapenems, tricyclic carbapenems, and penicillin and cephalosporin sulfones. However, non-β-lactams derivatives are the most promising novel inhibitors. Among them, two groups seem the most promising, reaching clinical development: diazabicyclooctanone (DBOs) and Boronic acid transition state inhibitors (BATSIs).

Diazabicyclooctanes (DBOs) were first investigated as β-lactam mimics in the mid-1990s proving to be a rich source of β-lactamase inhibitors (BLI). However, DBO chemistry has proved to be difficult, with many analogues requiring 12 or more synthetic steps. This has made the progress fairly slow, but the series has proved a rich source of BLIs, with two compounds entering the clinic: Avibactam and Relebactam. The five-membered ring of a DBO contains an amide group that targets the active-site serine of the β-lactamase via a carbamylation reaction, therefore DBOs are very potent inhibitors of class A and class C β-lactamases (including KPCs and ESBLs not inhibited by marketed BLIs) and have variable activity against class D β-lactamases (79). Avibactam (Figure 1-12) is the first member of the DBOs which in combination with the cephalosporin ceftazidime, was recently approved for complicated intraabdominal and urinary infection in adults (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm435629.htm. Remarkable properties of avibactam include the prolonged deacylation rate (days), which suggests the presence of a very stable and long-lived intermediate species (80); as well as the extremely efficient inhibition, as just 1–5 molecules of avibactam are required to inhibit one molecule of β-lactamase compared to 55–214 for tazobactam and clavulanic acid (80, 81). Ceftazidime/Avibactam (CZA) demonstrates *in vitro* activity towards bacteria possessing class A and C β-lactamases, such as extended-spectrum β-lactamases (ESBLs), *Klebsiella pneumoniae*

carbapenemase (KPC), and AmpC cephalosporinases, as well as activity against some class D β-lactamases (82). Specifically, it slowly inhibits OXA-48, since access of the inhibitor to the serine residue in the active site is constrained and regeneration of DBO is extremely slow, resulting in inhibition of the enzyme for the effective life of the bacterial cell (83). Noteworthy, CZA does not demonstrate *in vitro* activity against isolates containing MβLs, however combination with aztreonam has proven effective in some cases (84, 85). Relebactam (MK-7655), paired 4:1 with imipenem/cilastatin, has also proven to have microbiological activity (86). Out of 394 *Enterobacteriaceae* isolates non-susceptible to imipenem (collected from 45 countries, as part of the SMART study), 251 (64%) were rendered susceptible by the addition of relebactam (87). Isolates known to produce KPC carbapenemases, class A ESBLs or class C cephalosporinases in conjunction with porin changes were inhibited at 0.12–1 μg/ml of imipenem with the addition of 4 μg/ml of relebactam. Interestingly, growth inhibition of CRE strains producing OXA-48 was not consistent, even with high concentrations of relebactam. Finally, as with other DBOs, combination with relebactam did not potentiate the activity of imipenem against CRE producing VIM, IMP and NDM MBLs (88).

In addition to the DBOs, another scaffold has been explored for the inhibition of β-lactamases: Boronic Acid Transition State Inhibitors. BATSIs form reversible, dative covalent bonds with serine proteases and inhibit these enzymes by forming adducts that resemble the geometry of

the tetrahedral transition state of the β-lactamase hydrolytic reaction (89-91). Vaborbactam (Figure 1-12) the first of these inhibitors to progress to late stage clinical development, binds covalently to the active site serine in many serine β-lactamases, most notably the class A ESBLs and KPC enzymes and the class C cephalosporinases. In August 2017, the first boronic acid inhibitor combination, vaborbactam combined with the carbapenem meropenem, was approved for clinical use by the FDA (92).

A recent report describes a set of cyclic boronates that act as dual action inhibitors of serine β-lactamases and MBLs by mimicking a common high-energy tetrahedral intermediate. In addition, the study reveals that this cyclic boronates can potently inhibit some PBPs via the same mechanism. These compounds thus represent a promising line of investigation not only for the protection of –lactam antibiotics from both MBLs and/or SBLs, but also for direct inhibition of PBPs (93).

These two classes of compounds add up to the structural classes of BLIs currently available beyond clavulanic acid and the penicillanic acid sulfones. This molecular diversity is encouraging, as it is less likely for inhibitors in the different classes to be cross-resistant due to mutations in the target enzymes (92).

Colistin as a last resort antibiotic for Carbapenem Resistant *Enterobacteriaceae*

The global spread of Gram-negative bacteria (GNB) resistant to nearly all available antibiotics is currently the most pressing issue in bacterial resistance (94). In the last decade, the crisis in drug resistance has spread worldwide. The speed of dissemination and the lack of development of new antimicrobial drugs are rapidly outpacing available treatment options (1, 24). CRE are usually resistant to all commercially available β-lactams, including carbapenems, penicillins, cephalosporins and combinations with β-lactamase inhibitors. Even though aztreonam cannot be hydrolyzed by MBLs, most of the times MBL-producing *Enterobacteriaceae*, also carry ESBLs or other cephalosporinases that can hydrolyze aztreonam (95). Susceptibility to fluoroquinolones is rare among while susceptibility to sulfas and aminoglycosides may be unpredictable and can vary according to geographic location and strain type (96). Additionally, there is increasing resistance to all aminoglycosides due to the prevalence of 16S rRNA methylases, especially associated with NDM-producing CRE (97). Antibiotics with reliable activity against CRE (>85% susceptibility) typically include tigecycline, fosfomycin and polymyxins B and E (or colistin). Nonetheless, there are important concerns regarding the limited efficacy of these options because of their pharmacologic characteristics, and reports of increasing resistance, toxicity and adverse events (13). In the last few years, the use of combination therapy has been adopted in

light of the decreasing activity of currently available options (i.e., colistin, tigecycline and fosfomycin) and their apparent selection of resistance when used as monotherapy against CRE, as well as empirical evidence suggesting that it might offer a survival benefit when compared to monotherapy (98, 99).

Colistin mode of action and resistance in carbapenem resistant K. pneumoniae

Carbapenem resistant *Enterobacteriaceae* (CRE) pose an urgent risk to global human health. CRE that are non-susceptible to all commercially available antibiotics threaten to return us to the pre-antibiotic era. Carbapenem-resistant Kpn, especially isolates that produce KPC and belong to clonal complex 258 (CC258), are challenging pathogens due to the limited treatment options, high mortality rates, and potential for rapid dissemination in health care settings. Colistin (polymyxin E) and polymyxin B (Figure 1-13) are bactericidal cationic polypeptides synthetized by a Gram-positive species (namely *Paenibacillus polymyxa*), which act by disrupting the negatively charged outer membrane of GNB (100). Polymyxins are positively charged and thus readily bind to the outer membrane displacing divalent cations such as Ca^{2+} and Mg^{2+} from the phosphate groups of lipid A. As a result, they change the permeability of the bacterial cell membrane, thereby causing leakage of cell contents and finally cell death (101). Colistin sulfate and colistimethate sodium (a prodrug that is hydrolyzed to colistin sulfate) are the commercially

available forms of this agent, long kept as reserve because of serious nephrotoxicity and neurotoxicity issues and the availability of less toxic antibiotics. However, polymyxins are among the few agents that retain activity against CRE, therefore are now often considered as the last option to treat these and other MDR GNB. As a likely consequence of increased use, infections caused by polymyxin-resistant (polymyxin B- or colistin-resistant) strains are increasingly reported.

Chromosomally mediated colistin/polymyxin resistance

The primary cause of colistin resistance is change in the overall negative charge of the outer membrane, which is weakened by changing the phosphate groups of lipid A to 4-amino-4-deoxy-l-arabinose and/or phosphoethanolamine. Acquired colistin resistance usually results from modification of the lipid A, following mutational upregulation of the endogenous lipid A modification systems. *Proteus mirabilis* and *Serratia marcescens* are both intrinsically resistant to polymyxins due to the constitutive expression of the arnBCADTEF operon and/or the eptB gene, which causes addition of phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) cationic groups to the LPS (102-104). In *K. penumoniae*, inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the signaling system PhoQ-PhoP, which in turn activates the PmrAB system responsible for modification of the lipopolysaccharide polymyxin target, is found to be one of the most common mutational mechanisms responsible for polymyxin resistance among clinical isolates

of carbapenem resistant Kpn (Figure 1-14). However individual mutations in the genes encoding components of these two-component regulatory systems may result in resistance as well. A number of different genetic alterations were observed in the *mgrB* locus, including insertional inactivation by various insertion sequences, point mutations, and small or even large deletions (105). However, several studies demonstrated that inactivation of *mgrB* by insertion sequences appears to be the most common mechanism of *mgrB* alteration in KPC-Kpn (106-109). Among insertions by IS elements, the IS5 family is the most frequent element found to inactivate *mgrB*, followed by the IS1 family. Insertions can occur in either the promoter or the coding region of *mgrB*, with most insertions occurring between nucleotides 74 and 75 (109). This observation reinforces the hypothesis regarding the existence of a specific hot spot for IS-like elements insertion in the *mgrB* gene making interesting to assess whether this mechanism of *mgrB* inactivation is facilitated by the presence of ISs on KPC-encoding plasmids. Another two-component regulatory system linked to colistin resistance in *K.pneumoniae* is encoded by the *crrAB* (colistin resistance regulation) operon. This operon codes for two proteins: CrrA, the regulatory protein and CrrB, the sensor protein kinase. Inactivation of the *crrB* gene leads to overexpression of the *pmr*AB operon, thus causing activation of the *pmr*HFIJKLM operon leading to the production of L-Ara4N and consequently colistin resistance (110). Mutations that derive in CrrB inactivation activate PmrAB via the newly

named crrC (originally reported as ORF H239_3062). The now phosphorylated PmrAB then upregulates the transcription of arnBCADTEF and *pmr*C (111). This illustrated the existing cross-communication between two-component regulatory systems, in this case crrC connects crrAB and *pmr*AB together (109).

Colistin resistance in Kpn has been reported from numerous regions, including Europe, North America, South America, Asia and South Africa. Noteworthy, the highest overall colistin resistance rate (10.5–20%) in clinically isolated Kpn strains was reported in Greece, a well-known KPC-endemic country. In Italy, where KPC-producing Kpn is also endemic, a remarkable dissemination of colistin resistant KPC-Kpn was recently reported at a countrywide level. More recently, clonal outbreaks of colistin-resistant, KPC-producing Kpn were reported from the United States and Hungary both attributed to the most ST258 clone, the predominant KPC-associated clone worldwide (112, 113).

Plasmid-Mediated Resistance to Polymyxins

Recently, the emergence of transmissible, plasmid-mediated colistin resistance mechanism was reported in China in a porcine *E.coli* isolate collected in 2011. This resistance is mediated by the Mobilizable Colistin Resistance gene (*mcr*), encoding a phosphoethanolamine transferase (that adds a phosphoethanolamine moiety to lipid A) found on a 64015 bp IncI2 plasmid (114). Following these initial findings, the *mcr-1* gene has been reported worldwide extending to all continents (Figure 1-15) in various

genera of *Enterobacteriaceae* (*Escherichia, Klebsiella, Enterobacter, Cronobacter, Salmonella, Shigella*, and *Kluyvera*) isolated from the environment, vegetable and meat foods, animals, and patients (115). To date, several variants have been reported in different bacterial species, predominantly in *E.coli* and *Salmonella* spp (Table 1-7). The genetic vehicles involved in the dissemination of the *mcr-1* gene has been investigated extensively. This gene has been found in plasmids of various sizes (58 to 251 kb) and 13 different backbones including IncHI2, IncP, IncY, IncFI, IncFIB, IncFIBK, IncFIIB, IncHI2A, IncF, IncI2 and IncX4, these last two being the dominant plasmid types, accounting for 47% and 36% of the isolates, respectively (115, 116). Additionally, it has been shown that *mcr-1* is located within a 2,600-bp genetic structure, defined as the "*mcr-1* cassette," that might have been mobilized by transposition, and which carries its own promoter sequences driving the expression of mcr-1(117). It has been proposed that the dissemination of *mcr-1* started with a single mobilization event of mcr-1 by a composite transposon containing the abovementioned "*mcr-1* cassette" composed by *mcr-1* (1,626bp) and a putative open reading frame encoding a PAP2 superfamily protein (765bp), flanked by two IS*Apl1* insertion sequences (118). IS*Apl1*, originally described identified in the pig pathogen *Actinobacillus pleuropneumoniae,* is a member of the IS30 family of insertion sequences, which uses a "copy –paste" mechanism with a targeted transposition pathway requiring the formation of a synaptic complex between an inverted repeat (IR) in the

transposon circle and an IR-like sequence in the target. After the initial formation of this composite transposon, these flanking IS*Apl1* elements were lost over time, leading to the stabilization of *mcr-1* in a diverse range of plasmid backgrounds and subsequent spread through plasmid transfer (116, 118) (Figure 1-16). The earliest *mcr-1*-harboring strain was collected from chickens in China 30 years ago when colistin first started to be used in food-producing animals (119). However, *mcr-1* then did not reappear until 2004, which suggests that in spite of an early emergence, the gene stayed in a long dormancy period until the formation of the composite transposon, subsequent mobilization, and global spread. Phylogenetic reconstructions suggest that such global spread has been achieved through multiple translocations, and is mainly driven by trade of meat, and to a lesser extent by a global movement by colonized or infected humans(116).

Table 1-1. Major plasmid incompatibility groups and replicase genes (replicons) identified on resistance plasmids circulating among *Enterobacteriaceae*

Replicon	Inc Group	Reference plasmid	Species	GenBank Accession
HI1	IncHI1	R27	*Salmonella typhi*	AF250878
HI2	IncHI2	R478	*Serratia marcescens*	BX664015
HIB-M	N/A	pNDM-MAR	*K. pneumoniae*	JN420336
FIB-M	N/A	pNDM-MAR	*K. pneumoniae*	JN420336
I1γ	IncI1γ	R64	*S. enterica Typhimurium*	AP005147
Iα	IncIα	R621a	*S. enterica Typhimurium*	NC_015965
I2	IncI2	R721	*Escherichia coli*	AP002527
M	IncM	pCTX-M3	*Citrobacter freundii*	AF550415
L	IncL	pOXA-48	*Klebsiella pneumoniae*	KM406491
K	IncK	R387	*Escherichia coli*	M93063
B/O	IncB/O	p3521	*Escherichia coli*	GU256641
A/C	IncA/C	pRA1	*Aeromonas hydrophila*	FJ705807
N	IncN	R46	*Escherichia coli*	NC_003292
N2	IncN2	P271A	*Escherichia coli*	JF785549
W	IncW	R721	*Escherichia coli*	AP002527
P1	IncP1-alpha	pBS228	*N/S*	BN000925
T	IncT	Rts1	*Proteus vulgaris*	AP004237
U	IncU	pRA3	*Aeromonas hydrophila*	DQ401103
R	N/A	pK245	*Klebsiella pneumoniae*	DQ449578
X1	IncX1	pOLA52	*Escherichia coli*	EU370913
X2	IncX2	R6K	*Escherichia coli*	M65025
X3	IncX3	pIncX-SHV	*K. pneumoniae*	JN247852
X4	IncX4	UMNF18_32	*Escherichia coli p*	CP002895
FIA	IncF	Plasmid F	*Escherichia coli*	AP001918
FIB	IncF	Plasmid F	*Escherichia coli*	AP001918
FII	IncFII	NR1	*Escherichia coli*	DQ364638
FIIS	IncFIIS	pSLT	*S. enterica Typhimurium*	AE006471
FIIk	IncFIIK	pKPN3	*Klebsiella pneumoniae*	CP000648
FIB KN	IncFIIK	pKPN-IT	*Klebsiella pneumoniae*	JN233704
FIB KQ	IncFIIK	pKpQIL-IT	*Klebsiella pneumoniae*	JN233705

Table 1-1. (continued). Replicons and major plasmid Inc families circulating among *Enterobacteriaceae* identifiable by PCR-Based Replicon Typing (PBRT). Of note, small plasmids (2-15 kb) that do not control copy number and replication, do not contain replicons and thus are not detected by PBRT. Bacteria may contain more than one plasmid species within the same cell and plasmids may contain more than one replicon (multireplicon plasmids). Replicons that are not yet assigned to any known Inc group are reported as "N/A: not assigned". N/S: not specified. Adapted from (10, 120)

Table 1-2. KPC variants described to date with corresponding species and geographic location where each one was first reported

Variant	Country	Species	Ref
KPC-2	USA	*Klebsiella pneumoniae*	(19)
	USA	*Klebsiella oxytoca*	(121)
	Colombia	*Pseudomonas aeruginosa*	(122)
	USA	*Pseudomonas putida*	(123)
	USA	*Salmonella enterica*	(124)
	USA	*Enterobacter spp.*	(125)
	China	*Serratia marcesens*	(126)
	USA	*Raoultella planticola*	(127)
	Portugal	*Kluyvera georgiana*	(128)
	USA	*Proteus mirabilis*	(129)
	Greece	*Citrobacter koseri*	(130)
KPC-3	USA	*Klebsiella pneumoniae*	(131)
	Puerto Rico	*Acinetobacter baumannii*	(132)
	USA	*Raoultella ornithinolytica*	(127)
KPC-4	Scotland	*Enterobacter cancerogenus*	(133)
KPC-5	Puerto Rico	*P. aeruginosa*	(134)
KPC-6	Puerto Rico	*Klebsiella pneumoniae*	(135)
KPC-7	USA	*Klebsiella pneumoniae*	(136)
KPC-8	Puerto Rico	*Klebsiella pneumoniae*	(137)
KPC-9	Israel	*Klebsiella pneumoniae, E.coli*	(138)
KPC-10	Puerto Rico	*A. baumannii*	(132)
KPC-11	USA	*Klebsiella pneumoniae*	(139)
KPC-12	China	*Klebsiella pneumoniae*	[a]
KPC-13	Thailand	*E. cloacae, E. coli, Citrobacter freundii*	(140)
KPC-14		*Klebsiella pneumoniae*	[a]
KPC-15	China	*Klebsiella pneumoniae*	(141)
KPC-16	Taiwan	*Klebsiella pneumoniae*	(142)
KPC-17		*Klebsiella pneumoniae*	
KPC-19	Italy	*Klebsiella pneumoniae*	[a]
KPC-21	Portugal	*E. coli*	[a]
KPC-22	Taiwan	*Klebsiella pneumoniae*	[a]
KPC-24	Chile	*Klebsiella pneumoniae*	[a]
KPC-25	USA	*Klebsiella pneumoniae*	[a]

[a]No associated publication; variant assigned according to Genbank accession number

Table 1-3. Completely sequenced *bla*$_{KPC}$-bearing plasmids in *K. pneumoniae*

Inc type	Size (bp)	Location	Year[d]	KPC	ST	Genetic element [e]
N[a]	55,417	Greece	2009–2010	KPC-2	340	Tn*4401*b
FIIK [b]	78,227	Italy	2011	KPC-3	258	Tn*4401*a
ColE1	23,753	New York City	2005	KPC-2	–	Tn*4401*a
ColE1	25,284	New Jersey	2010	KPC-3	258	Tn*4401*b
FIA	73,635	New Jersey	2010	KPC-3	258	Tn*4401*d
FIA	73,636	New Jersey	2010	KPC-3	258	Tn*4401*d
FIA	139,941	New Jersey	2010	KPC-3	963	Tn*4401*d
FIIK	86,518	Taiwan	2012	KPC-2	11	NTE_{KPC-Ib}
FIIK1	165,295	New York City	2010	KPC-2	258	Tn*4401*a
FIIK1	317,154	China	2010	KPC-2	11	NTE_{KPC-Ia}
FIIK1, R	107,748	Italy	2011	KPC-2	101	Tn*4401*a
FIIK2	21,138	–	–	KPC-2	–	Tn*4401*a
FIIK2	113,622	Greece	–	KPC-2	–	Tn*4401*a
FIIK2	113,637	Israel	2006	KPC-3	258	Tn*4401*a
FIIK2	113,640	Greece	–	KPC-2	–	Tn*4401*a
FIIK2	113,640	Greece	–	KPC-2	–	Tn*4401*a
FIIK2	115,300	Italy	2010	KPC-3	258	Tn*4401*a
FIIK2	116,047	Greece	–	KPC-2	–	Tn*4401*a
FIIK2, R	111,195	China	–	KPC-2	–	NTE_{KPC-Ib}
FIIK5, R	151,188	China	2006–2007	KPC-2	–	NTE_{KPC-Ia}
I2	77,801	New Jersey	2005	KPC-3	258	Tn*4401*b
N	53,081	Brazil	2005	KPC-2	–	Tn*4401*b[c]
N	54,605	Brazil	2009	KPC-2	–	Tn*4401*b
N	65,549	China	–	KPC-2	–	NTE_{KPC-Ic}
N	70,655	New York City	2005	KPC-2	–	Tn*4401*b
N	75,617	New York City	2005	KPC-3	–	Tn*4401*b
N	83,712	New Jersey	2005	KPC-4	834	Tn*4401*b
P	42,848	China	2006	KPC-2	–	NTE_{KPC-Ia}
X3	42,447	New York City	2011	KPC-2	258	Tn*4401*a
X3	45,574	Southern Brazil	2009	KPC-2	442	NTE_{KPC-Ic}
X3	53,286	France	2009	KPC-2	258	Tn*4401*a
X5	47,387	New Jersey	2006	KPC-5	429	Tn*4401*b

Adapted from (21). [a]IncN plasmid backbone, but lack the IncN *repA* replicon gene. [b]IncFIIK2 but lack the IncFII *repA* gene. [c]256 bp insertion in *tnpA* in Tn*4401*. [d]Year of isolation. [e]Genetic elements harboring *bla*$_{KPC}$ in Figure 1-6.
Abbreviation: –, data not available

Table 1-4. Reported plasmid backbones and species carrying *bla*$_{NDM}$

Plasmid backbone (Inc type)	Co-harboring resistance genes			Isolate species (ST type)	Country of isolation
	β-lactamase (*bla*)	Quinolone resistance	Aminoglycoside resistance		
A/C	-	-	-	*Providencia stuartii*	Afghanistan
	-	-	-	Kpn (ST14)	Kenya
	-	-	-	*E. coli*	Canada
	-	-	-	*E. coli* (ST38)	Japan
	bla$_{CMY-16}$ *bla*$_{OXA-10}$	-	-	*E. coli* (ST10)	France
	-	-	*rmtA*	Kpn (ST147)	Switzerland
	bla$_{OXA-10}$ *bla*$_{CMY-16}$	*qnrA6*	-	Kpn (ST25)	Switzerland
	bla$_{CMY-6}$	-	*rmtC*	Kpn (ST14)	Kenya
	bla$_{OXA-10}$ *bla*$_{CMY-16}$	-	*armA*	*P. mirabilis*	Switzerland
	-	-	-	*E. coli*	India
	-	-	-	*V. cholerae*	India
	-	-	-	*C. freundii*	India
	-	-	-	*A. baumannii*	Switzerland
	bla$_{SHV-12}$ *bla*$_{CTX-M-15}$	*aac(6')-lb-cr, qnrS*	-	Kpn (ST147)	Canada
N	Unknown	Unknown	Unknown	Kpn	UK
N2	-	-	-	*E. coli*	Australia
	-	-	-	Kpn	Singapore
HI1	-	-	-	*C. freundii*	India
	-	-	-	*E. coli* (ST156)	Spain
Hl1B	*bla*$_{TEM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{SHV-12}$ *bla*$_{OXA-1}$	-	*armA*	Kpn	Oman
	bla$_{SHV-12}$	-	*armA*	Kpn	Oman
	bla$_{OXA-1}$ *bla*$_{CTX-M-15}$	-	*armA*	Kpn	Oman
L/M	-	-	-	*E. coli*	China
	bla$_{TEM-1}$	-	-	Kpn (ST14)	Oman
	bla$_{TEM-1}$	-		Kpn	Oman
F	*bla*$_{TEM-1}$		*armA*	*E. coli*	Switzerland
	-		*armA*	*E. coli* (ST648)	India
	bla$_{TEM-1}$ *bla*$_{CTX-M-14}$	-	-	*E. coli*	Oman
FII	*bla*$_{OXA-1}$	-	*aacA4 aadA2 aac2*	*E. coli* (ST131)	India
FIIA	*bla*$_{SHV-12}$ *bla*$_{CTX-M-15}$	*aac(6')-lb-cr*	-	*Kpn (ST340)*	Canada

FIIs	*bla*$_{TEM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-9}$	-	-	Kpn	Oman
H	-	-	-	*Kpn (ST15)*	Morocco
X3	-	-	-	Kpn	China
P	-	-	-	*E. cloacae*	India

Modified from (37). Kpn: *K.pneumoniae*; ST: Sequence type; Inc type: Plasmid backbone/incompatibility type (Inc) via Carattoli replicon typing; ND: Not defined; –: Not present

Table 1-5. Representative OXA-48 variants described to date with corresponding species and geographic location where each one was first reported

Variant	Substitutions	Country	Species	Ref
OXA-162	Thr213Ala	Turkey	*Klebsiella pneumoniae*	(143)
OXA-163	Ser212Asp ΔArg214, ΔIle215, ΔGlu216, ΔPro217	Argentina	*Klebsiella pneumoniae* *Enterobacter cloacae*	(144)
OXA-181	Thr104Ala, Asn110Asp, Glu168Gln, Ser171Ala	India	*Klebsiella pneumoniae*	(145)
OXA-204	Gln98His, Thr99Arg	Link to North Africa	*Klebsiella pneumoniae*	(146)
OXA-232	Arg214Ser	France (patient transferred from India to Mauritius)	*Klebsiella pneumoniae*	(147)
OXA-244	Arg214Gly	Spain	*Klebsiella pneumoniae*	(148)
OXA-245	Glu125Tyr		*Klebsiella pneumoniae*	
OXA-247	Tyr211Ser Asp212Asn	Argentina	*Klebsiella pneumoniae*	(149)
OXA-370	Gly220Glu	Brazil	*Enterobacter hormachei*	(150)
OXA-405	ΔThr213 - Glu216	France	*Serratia marcescens*	(151)
OXA-436	Val3Ala, Phe10Leu, Leu11Met, Ala13Thr, Ser14Thr, Ile15Met, Thr36Ser, Ser40Thr, Lys51Thr, Asn58Asp, Thr104Ala, Asn110Asp, Val154Leu, Glu168Gln, Ser171Ala, Gly201Ala, Thr213Val, Val226Ile, Met237Thr, Ser244Ala, Asp245Glu, Ala252Thr, Glu256Ala	Denmark	*Enterobacter asburiae*	(152)

OXA-535	Val3Ala, Phe10Leu, Leu11Met, Ala13Thr, Ser14Thr, Ile15Met, Thr36Ser, Ser40Thr, Lys51Thr, Asn58Asp, Thr104Ala, Asn110Asp, Glu125Gly, Val138Leu, Glu153Gln, Ser171Ala, Gly201Ala, Thr213Val, Val226Ile, Met237Thr, Ser244Ala, Asp245Glu, Ala252Ser, Glu256Ala	France (patient transferred from Africa)	*Shewanella* sp.	(153)

Table 1-6. Reported plasmid backbones and species carrying *bla*$_{\text{OXA-48-}like}$

Inc type	Size (bp)	Geographic origin	Year of isolation	Species isolated from	Genetic context of *bla*$_{\text{OXA-48 like}}$	GenBank accession No.
L/M	61,881	Istanbul, Turkey	2001	*Klebsiella pneumoniae*	Tn*1999.1*	JN626286
L/M	63,434	Tripoli, Lebanon	2011	*Raoultella planticola*	Inverted Tn*1999.2*	LN864821
L/M	84,252			*Klebsiella pneumoniae*	Inverted Tn*1999.2*	LN864819
L/M	62,014			*Klebsiella pneumoniae*	Inverted Tn*1999.2*	KP659188
L/M	49,151			*Citrobacter freundii*	Inverted Tn*1999.2*	LN864820
L/M	63,578	Ireland	NS	*Klebsiella pneumoniae*	Tn*1999.2*	KC335143
L/M	63,581	Bern, Switzerland (transfer from Belgrade, Serbia)	2013	*Klebsiella pneumoniae*	Tn*1999.2*	KM406491
L/M	62,592	Nancy, France	2010	*Klebsiella pneumoniae*	Tn*1999.2*	KC757416
L/M	167,203	Nancy, France	2010	*Klebsiella pneumoniae*	Tn*1999.2*	KC757417
L/M	71,446	Sydney, Australia (patient recently returned from Egypt	2010	*Klebsiella pneumoniae*	Tn*1999.1*	KC354801
L/M	72,127	Gaza, Palestine	2012	*Proteus mirabilis*	Inverted Tn*1999.2*	KP025948
L/M	69,471	Dubai, United Arab Emirates	2012	*Escherichia coli*	Tn*1999.2*	CP015071.1
L/M	63,543	Rabat, Morocco	2012	*Escherichia coli*	Tn*1999.2*	CP015075.1
N2	71,171	Buenos Aires, Argentina	2011	*Escherichia coli*	ΔIS*1326*-IS*Shfr9*-Tn*3*-IS*4321*-*bla*$_{\text{OXA-163}}$-IS*6100*	CP015078.1
IncHI2	314137	Denmark	2013	*Enterobacter asburiae*	IS91-flanked 7.3Kb *Shewanella spp.* region	KY863418

Modified from (154)

Table 1-7. MCR variants described to date with corresponding species and geographic location where each one was first reported

Variant	AA identity MCR-1	Country	Species	Source	Plasmid		Ref#
					Inc type	Size	
MCR-1.2	SNV (Q3L)	Italy	*Klebsiella pneumoniae*	Rectal swab	IncX4	33Kb	(155)
MCR-1.3	SNV (I138V)	China	*Escherichia coli*	Poultry	IncI2	60Kb	(156)
MCR-1.4	SNV (D440N)	China	*Escherichia coli*	Sewage	--	--	KY041856.1
MCR-1.5	SNV (H452Y)	Argentina	*Escherichia coli*	Urinary tract	IncI2	60Kb	(157)
MCR-1.6	SNV (R536H)	China	*Salmonella enterica serovar Typhimurium*	Healthy human	IncP	47Kb	(158)
MCR-1.7	SNV (A215T)	China	*Escherichia coli*	Sewage	--	--	NG_054678
MCR-1.8	SNV (Q3R)	Brunei	*Escherichia coli*	Poultry	--	--	NG_054697
MCR-2	81%	Belgium	*Escherichia coli*	Porcine/ bovine	IncX4	35Kb	(159)
MCR-3	35%	China	*Escherichia coli*	Porcine	IncHI2	261Kb	(160)
MCR-4	35%	Italy	*Salmonella enterica serovar Typhimurium*	Porcine	ColE10	8.7Kb	(161)
MCR-5	36.11%	Germany	*Salmonella enterica subsp. enterica serovar Paratyphi B*	Chicken meat	ColE	12Kb	(162)

SNV, single nucleotide variant; # if publication is not available, Genbank accession number is provided

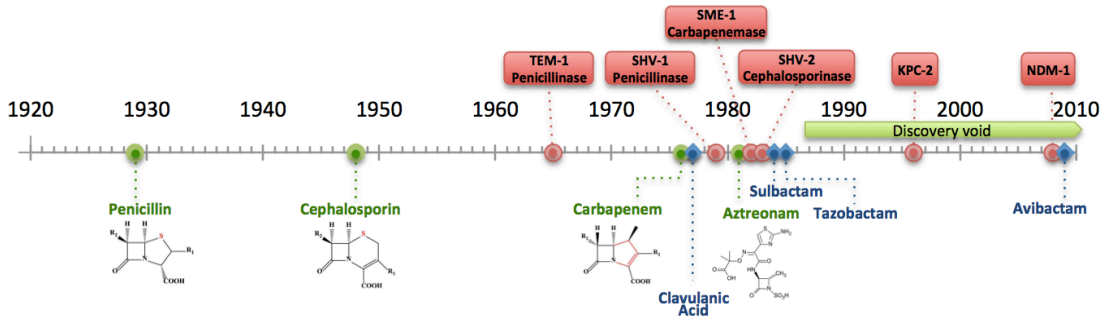


Figure 1-1. Timeline of antibiotic discovery and emergence of resistance due to β-lactamases

In green, major groups of β-lactam antibiotics and their structure; in blue, commercially available β-lactamase inhibitors; in red, major classes of β-lactamases.

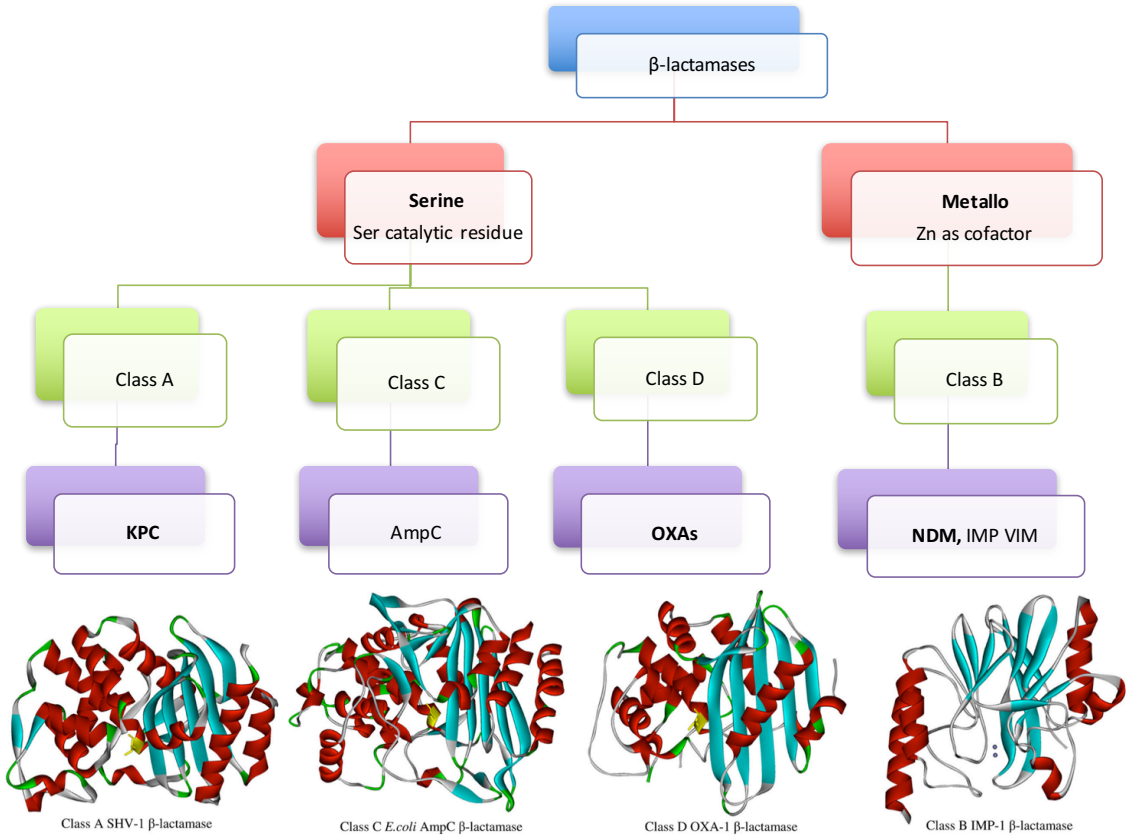


Figure 1-2. Ambler classification of B-lactamases.

This molecular classification is based on the amino acid sequence and divides β-lactamases into class A, B, C, and D. Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis. Figure adapted from (68)

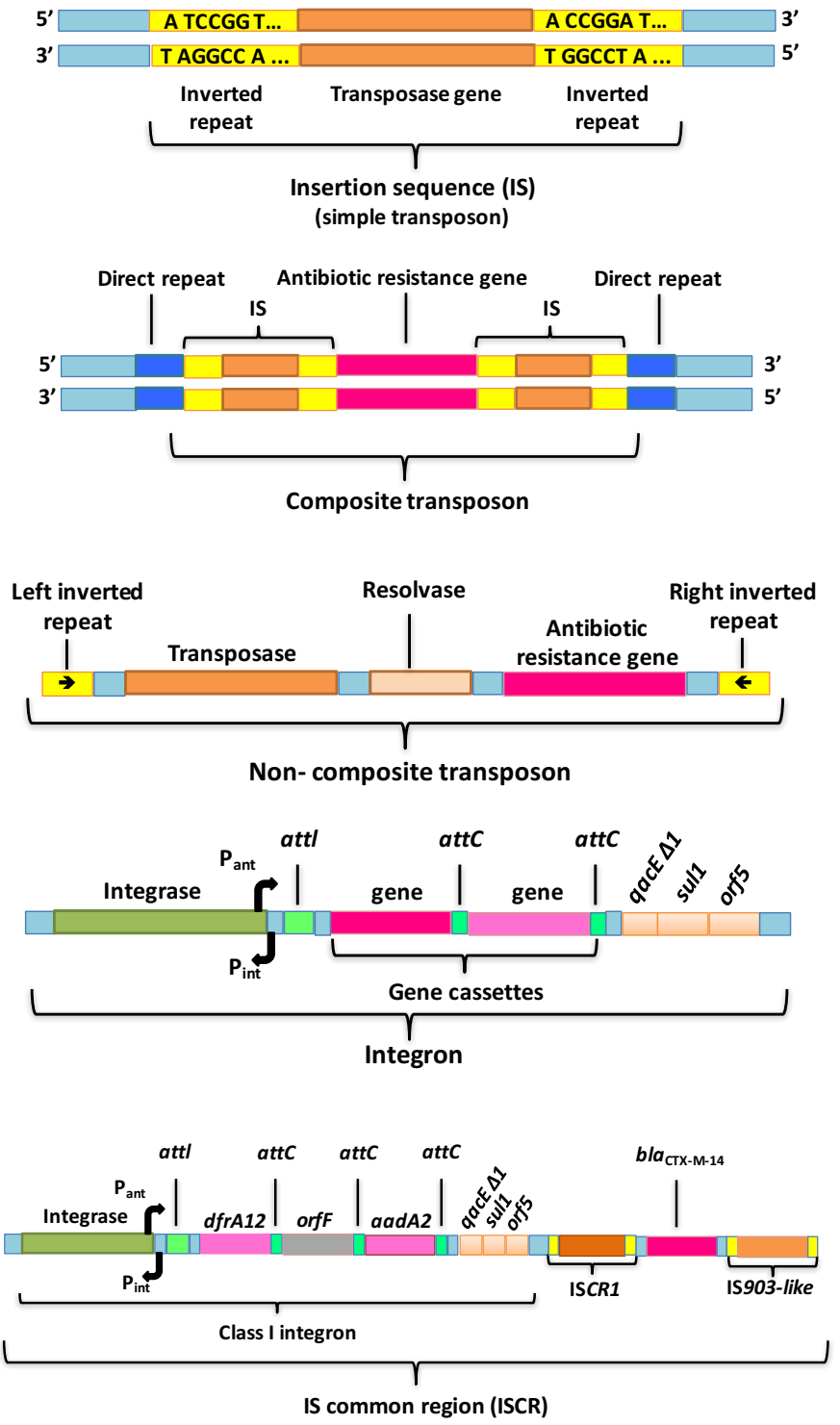


Figure 1-3. General structure of mobile genetic elements commonly involved in transposition of antimicrobial resistance genes among Gram negatives.

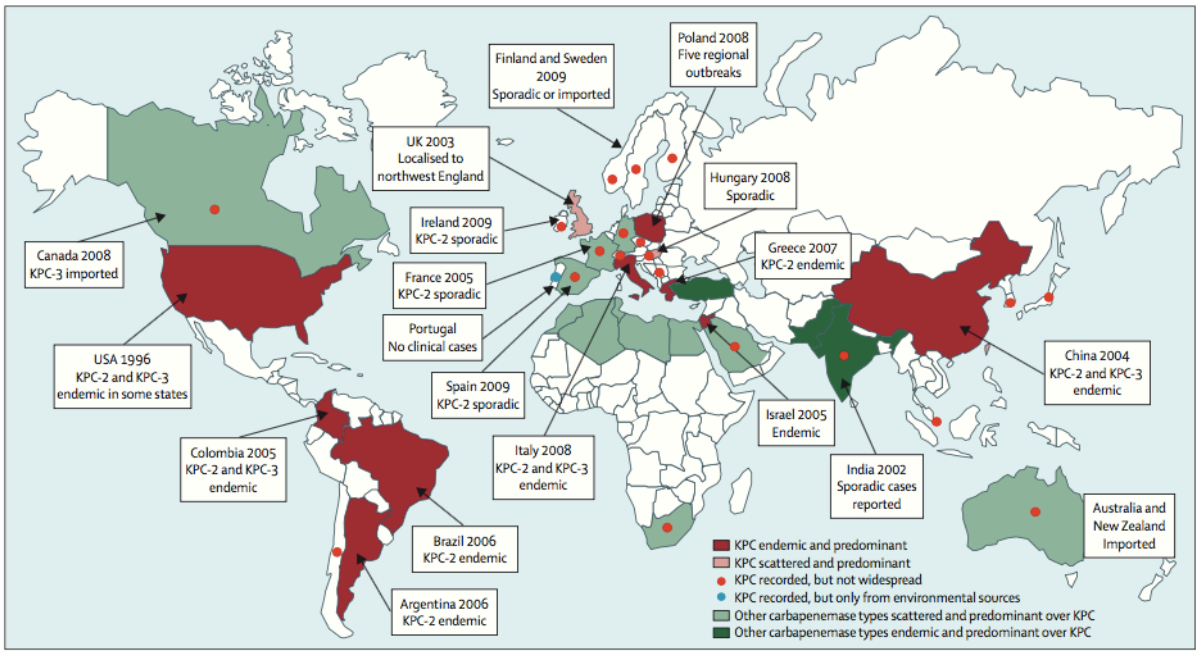


Figure 1-4. Epidemiological features of KPC producers by country of origin.

Figure from (163)

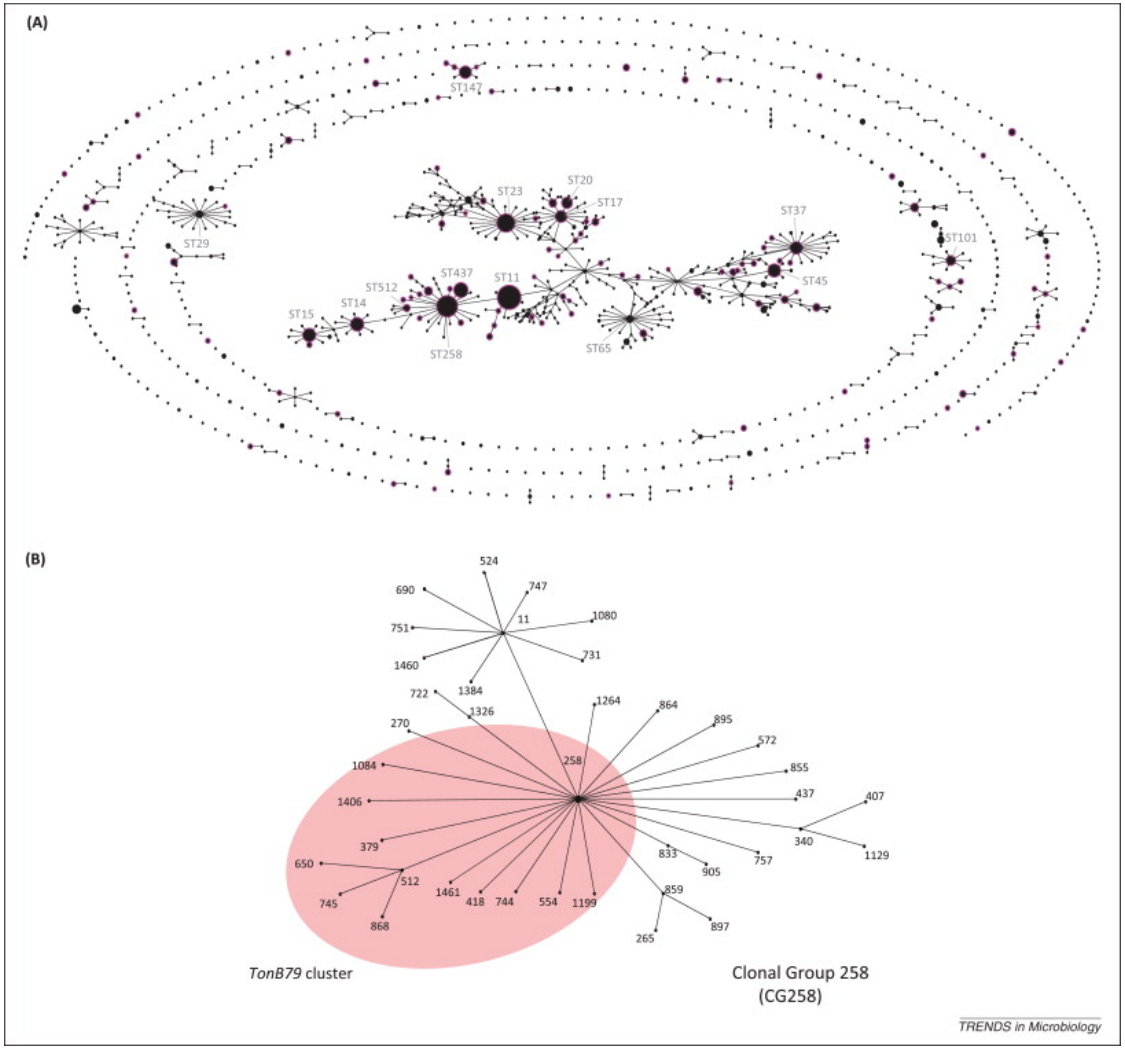


Figure 1-5. Population structure of KPC-Kpn.

Figure 1-3. (cont) (A) The figure represents the population structure of the *K. pneumoniae* MLST database (http://www.pasteur.fr/mlst) as of April 1, 2014, depicted graphically by eBURST v.3 (http://eburst.mlst.net), and shown in the context of all of the 1,536 STs from 1,924 isolates. KPC-Kp STs are highlighted by a pink halo. (B). Population structure of CG258, the most epidemiologically relevant clonal group. The pink shading highlights the STs of CG258-tonB79 cluster. Figure from (21).

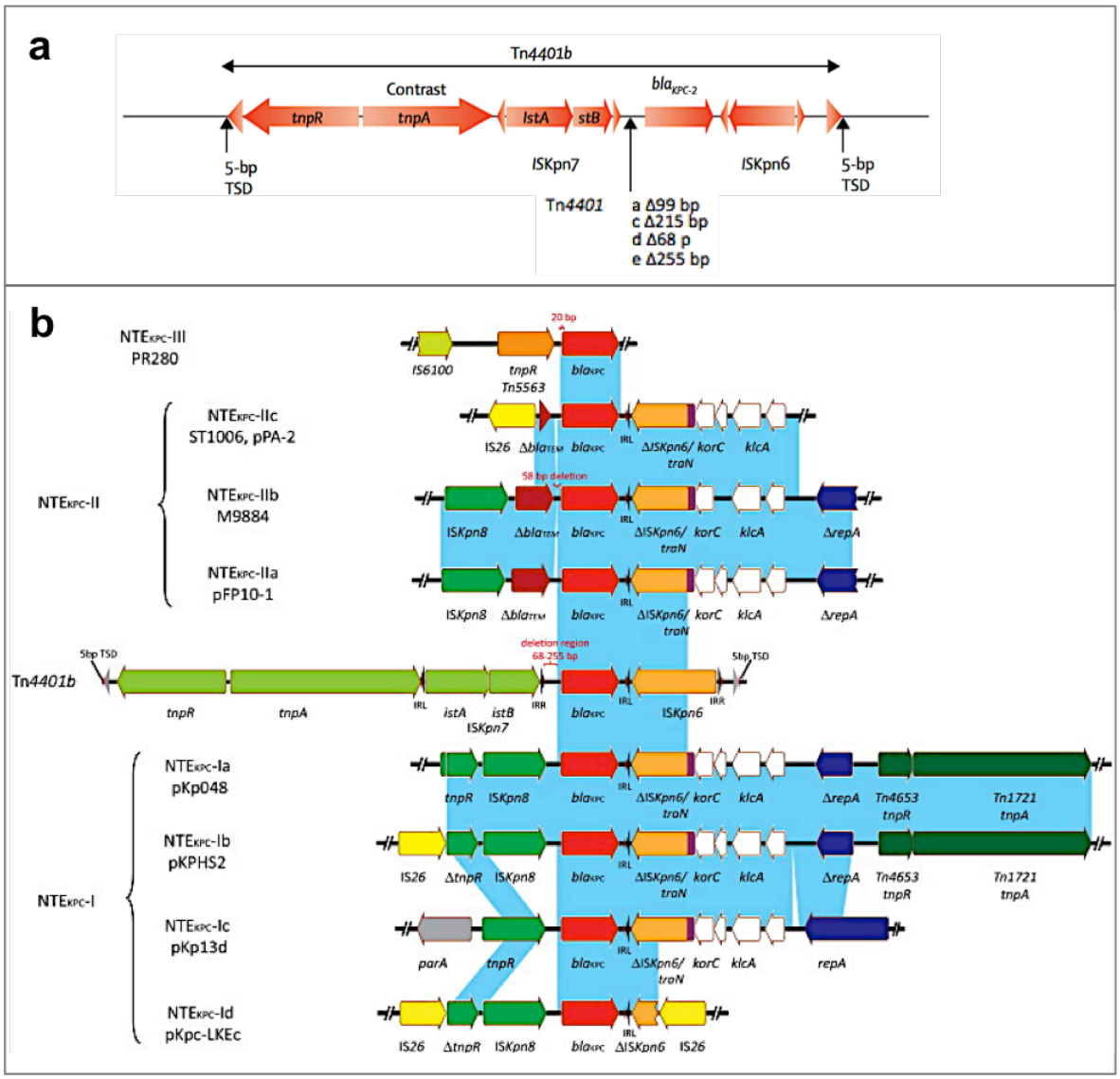


Figure 1-6. Genetic elements harboring *bla*$_{KPC}$ (a.Tn*4401* and b. NTEKPC).

Based on the insertion sequence upstream of *bla*$_{KPC}$, NTE$_{KPC}$ can be divided into three groups: NTE$_{KPC}$-I, no insertion; NTE$_{KPC}$-II, insertion of Δ*bla*$_{TEM}$; and NTE$_{KPC}$-III, insertion of Tn5563/IS6100. NTE$_{KPC}$–I can be further classified as -Ia (prototype, pKp048), -Ib (pKPHS2), -Ic (pKp13d) and -Id (pKPC-LKEc) based on the insertion sites of upstream and/or downstream of IS26 and the presence of ISKpn8. NTE$_{KPC-II}$ can be subgrouped as -IIa (pFP10-1, and *bla*$_{KPC}$-harboring plasmids from strain M9196 and M11180), -IIb (from strain M9884 and M9988), and -IIc (pPA-2), based on the differences of the length of Δ*bla*$_{TEM}$ and the deletions. Light-blue shading denotes shared regions of homology.
Figure reproduced from (21).

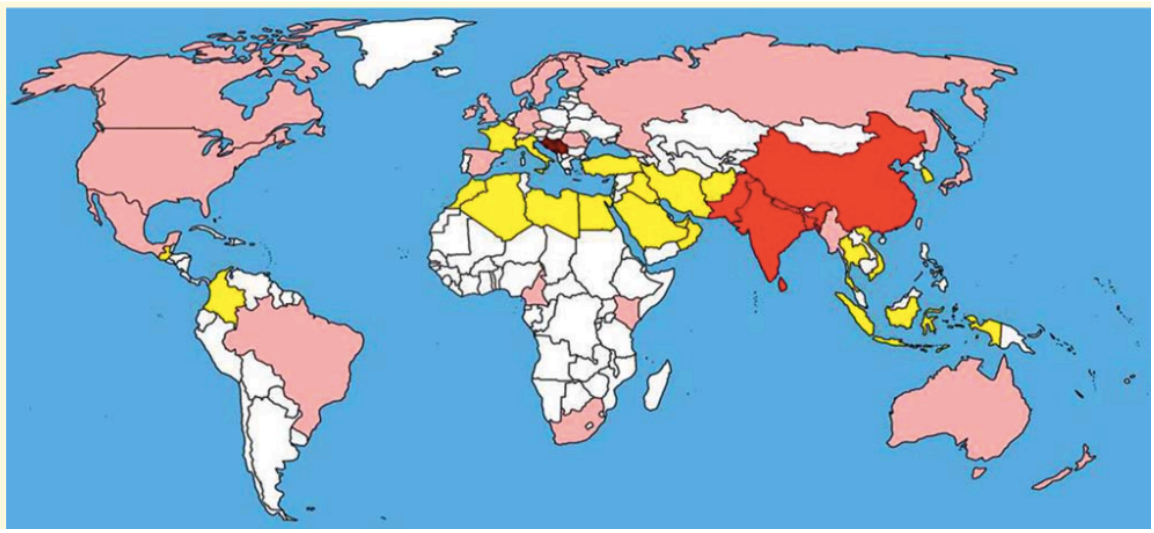


Figure 1-7. International dissemination of *bla*$_{NDM}$

Red: Initial reservoirs (Indian sub- continent and China); Dark red: Secondary reservoirs (Balkan states); Yellow: 'Expanded' reservoirs; Light red: Countries reported cases linked with international travel Figure reproduced from (37).

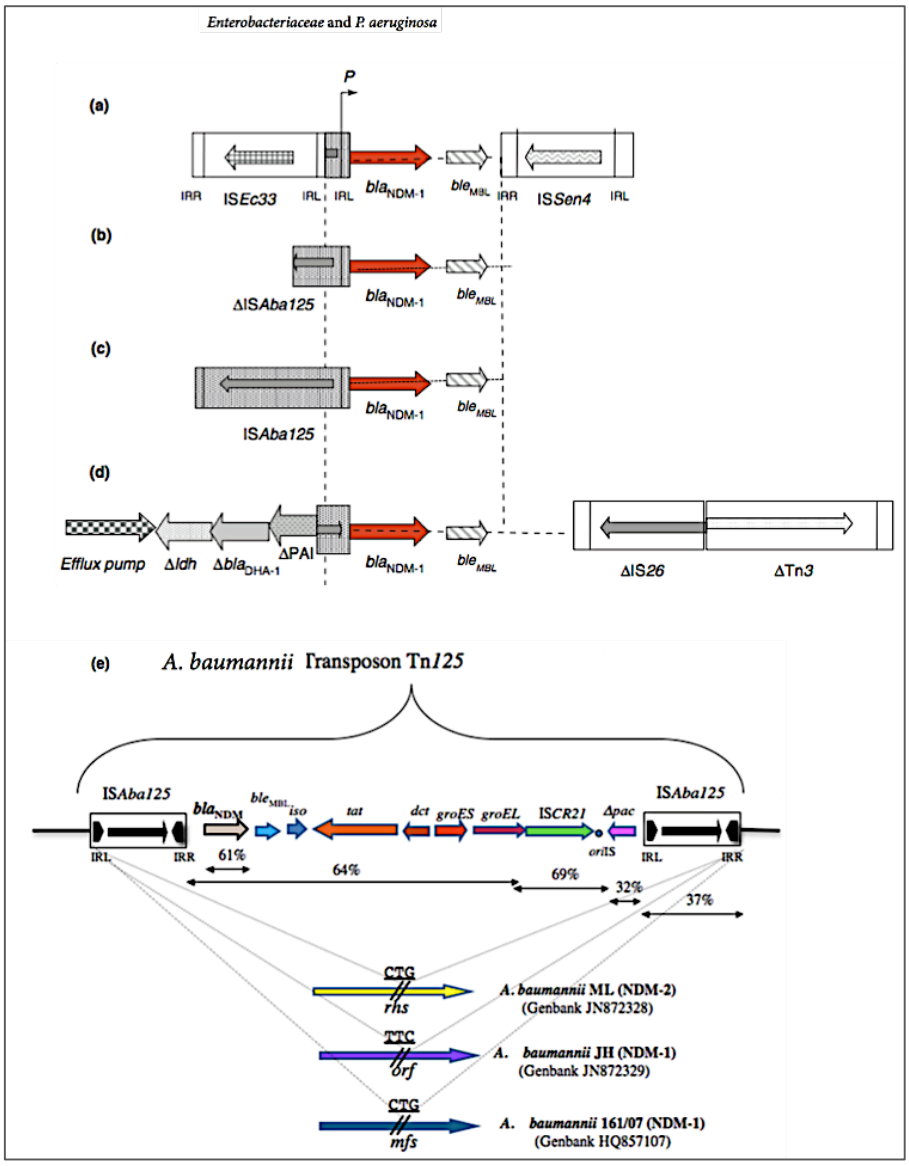


Figure 1-8. Schematic map of *bla*$_{NDM-1}$-associated genetic structures identified among Gram-negative clinical isolates

Legend in next page

Figure 1-6. (cont) (a) Structure identified from *E. coli* 271 in which the ISEc33 and ISSen4 elements were identified on both extremities of the bla_{NDM-1} gene. (b) Structure in which ISAba125 is present as a truncated element, but without ISEc33 (present in Kpn Kp7 and 601). (c) Structure in which ISAba125 is present as a full element with ble_{MBL} (bleomycin resistance) also being present. (d) Structure initially identified in Kpn 05-506. (e) Structure of Tn125 found in *A. baumannii*. The 3-bp Tn125 target sites identified in each isolate are underlined and uppercase. GC content is indicated in percentages. Genes and their corresponding transcription orientations are indicated by horizontal arrows. The bla_{NDM-1} promoter is indicated (P). Abbreviations: bla_{DHA-1}, plasmid-mediated cephalosporinase gene; ble_{MBL}, bleomycin resistance gene; IRL, inverted repeat left; IRR, inverted repeat right; IS, insertion sequence; *ldh*, lactate dehydrogenase gene; *pai*, phosphoribosyl anthralinate isomerase gene; Tn3, transposon Tn3; *iso,* phosphoribosylanthranilate isomerase; *tat,* twin-arginine translocation pathway signal sequence protein; *dvt,* divalent cation tolerance protein; *Δpac,* truncated phospholipid acetyltransferase; *rhs,* Rhs protein; *orf,* unknown open reading frame; and *mfs,* major facilitator superfamily metabolite/H^+ symporter. Figure adapted from [48].

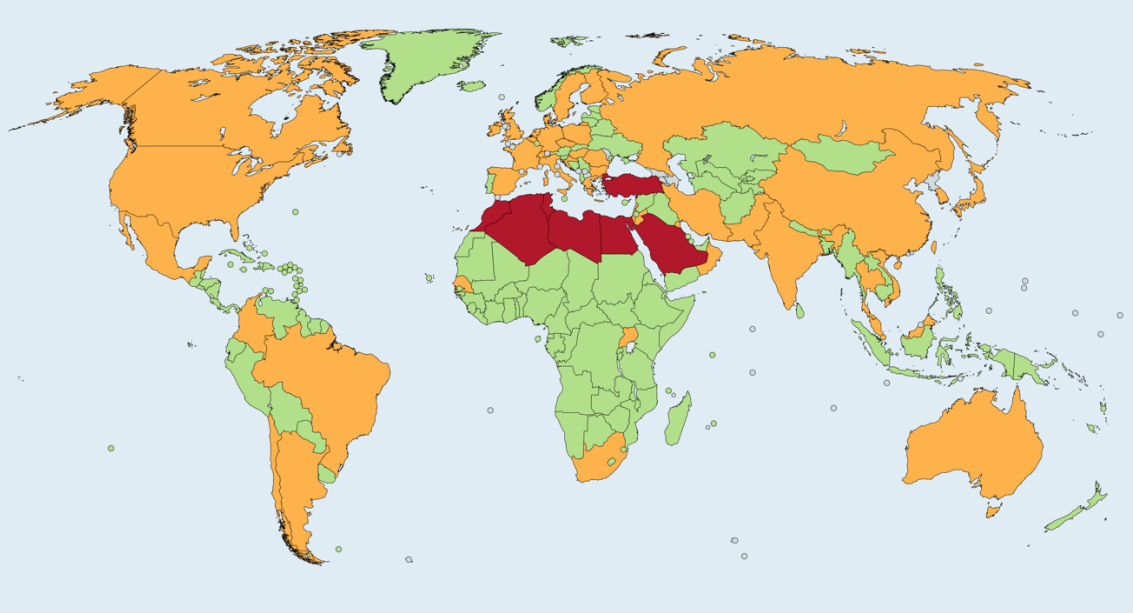


Figure 1-9. International dissemination of *bla*$_{\text{OXA-48-}like}$ as reported in the literature until January 2018.

Orange indicates countries where isolates harboring *bla*$_{\text{OXA-48-}like}$ have been reported; red indicates initial reservoirs and current endemic regions

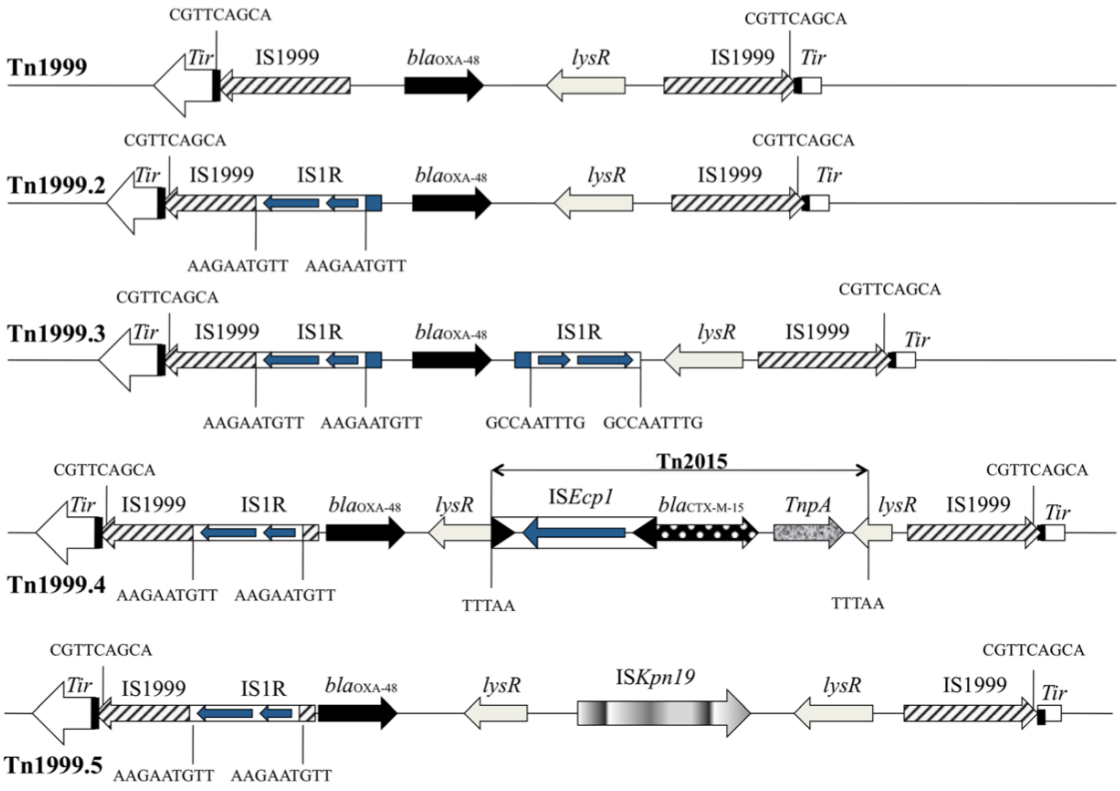


Figure 1-10. Genetic environments of the *bla*$_{\text{OXA-48}}$-harboring Tn1999-like transposon structures in *Enterobacteriaceae* isolates

Reproduced from (48).

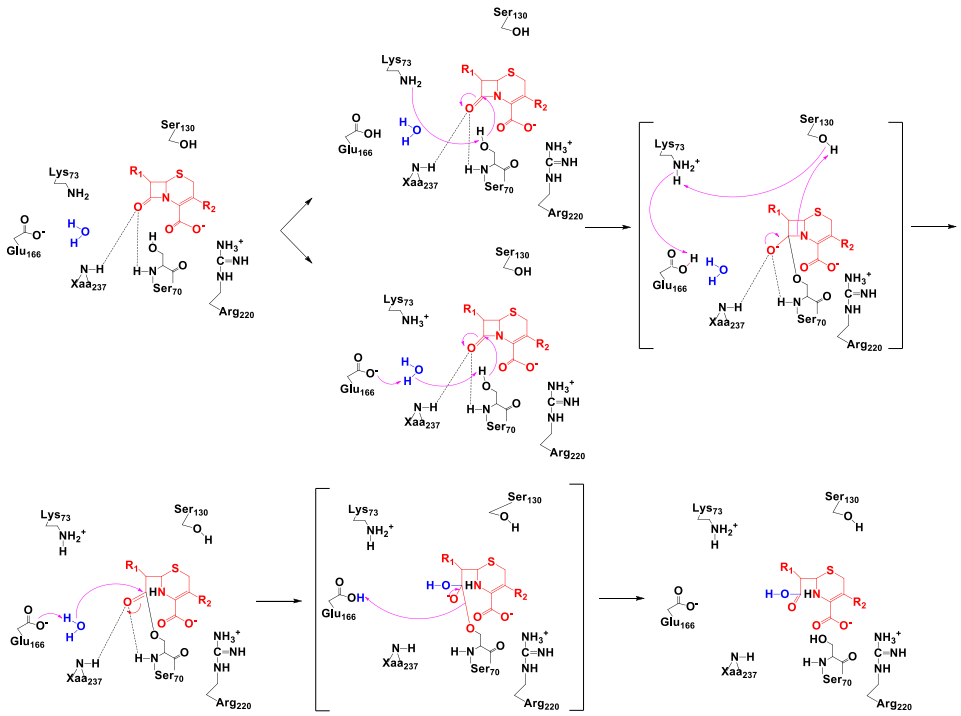
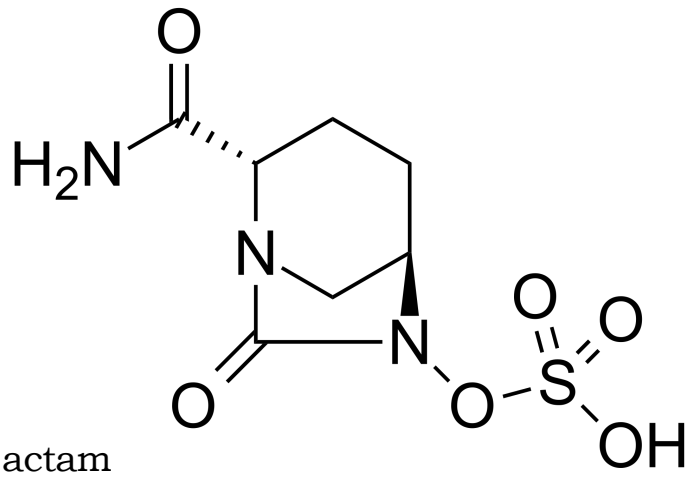
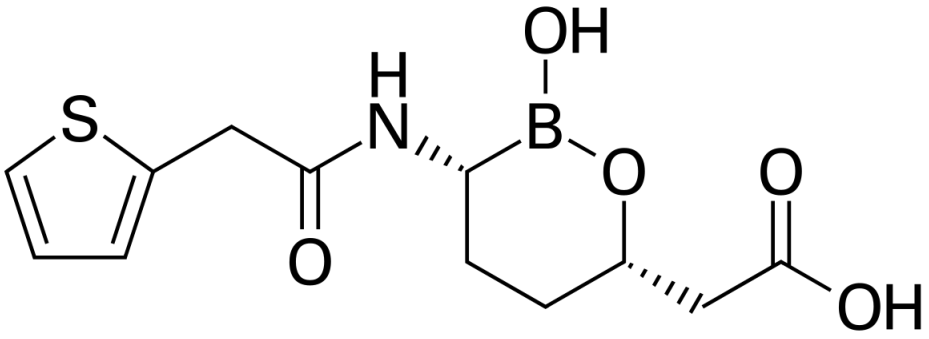


Figure 1-11. Proposed reaction mechanism for a cephalosporin with a class A serine β-lactamase.

The carbonyl of β-lactam sits in the oxyanion hole (electrophilic center) formed by the backbone nitrogens of Ser-70 and Xaa-237. The carboxylate forms a salt bridge to a positively charged amino acid, e.g. Arg-220. Either Lys-73 directly removes a proton from Ser-70 or Glu-166 removes a proton from a water molecule (*blue*), which activates the nucleophilic Ser-70 to initiate acylation. Unprotonated Ser-70 attacks the β-lactam bond forming an acyl-enzyme. A proton shuttle starting with the β-lactam and Ser-130 to Lys-73 followed by Glu-166 results in an unprotonated Glu-166. Unprotonated Glu-166 removes a proton from a water molecule (deacylation water), which attacks the acyl-enzyme complex resulting in water being added across the bond and deacylation of the inactivated β-lactam. Reproduced from (164).



Avibactam



Vaborbactam

Figure 1-12. Chemical structure of avibactam and vaborbactam, the two most recently approved β-lactam inhibitors.

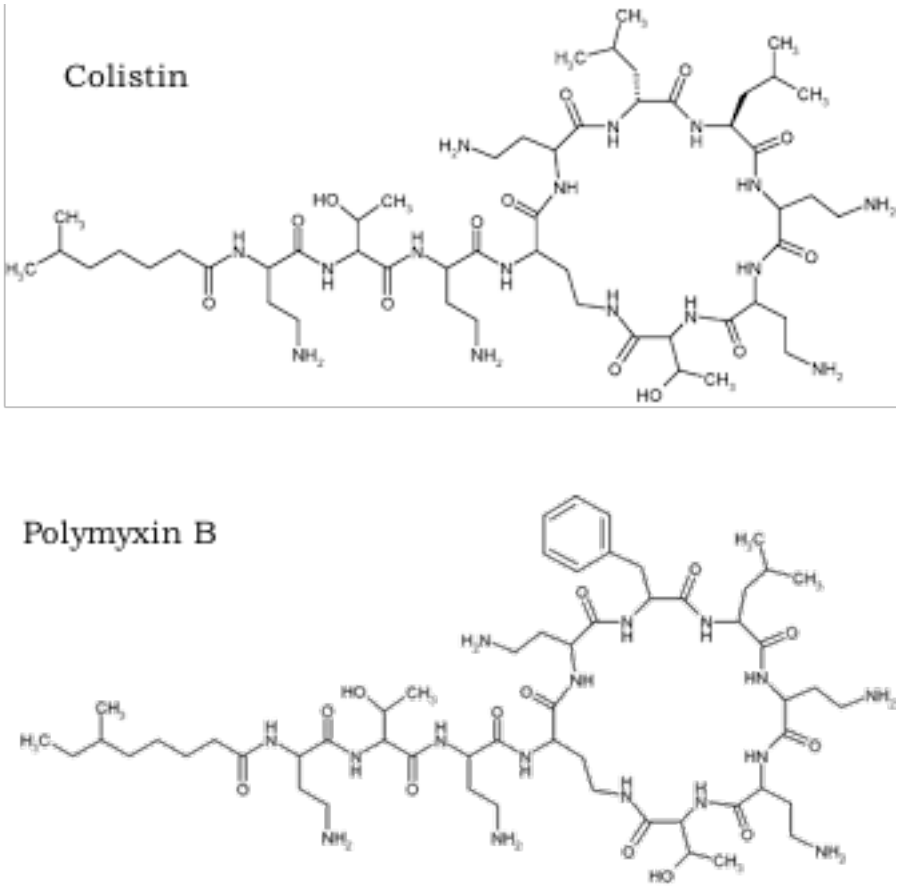


Figure 1-13. Chemical structure of Colistin (Polymyxin E) and Polymyxin B.

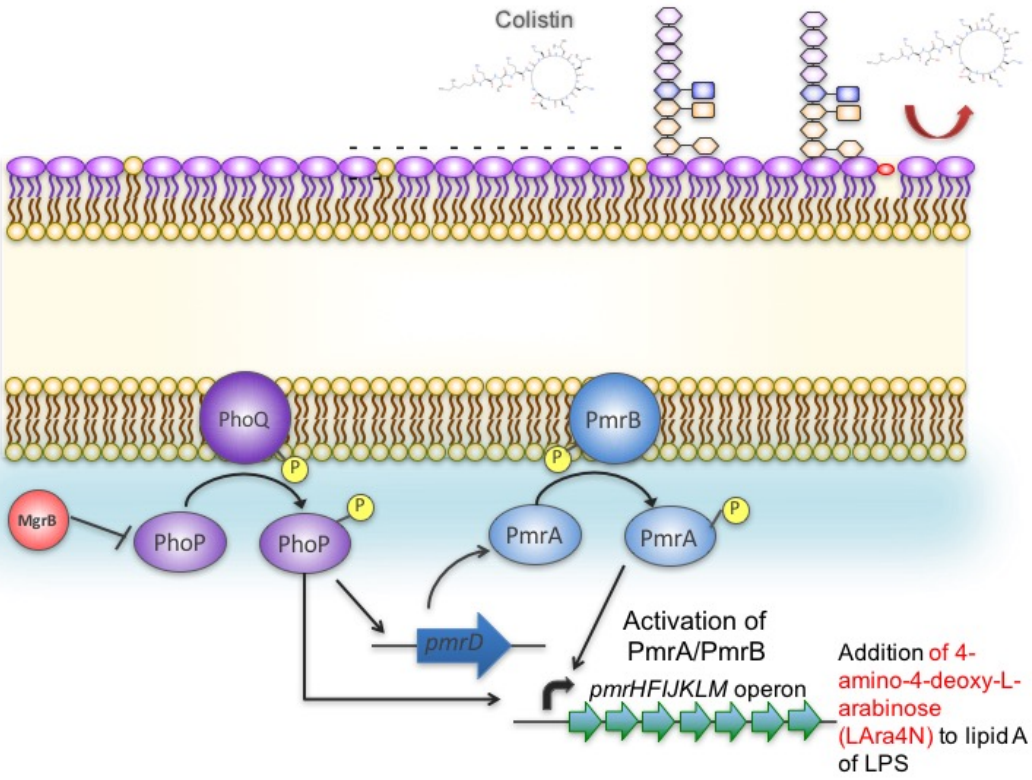


Figure 1-14. Activation of lipopolysaccharide-modifying genes involved in polymyxin resistance in *K. pneumoniae*.

Figure adapted from (165)

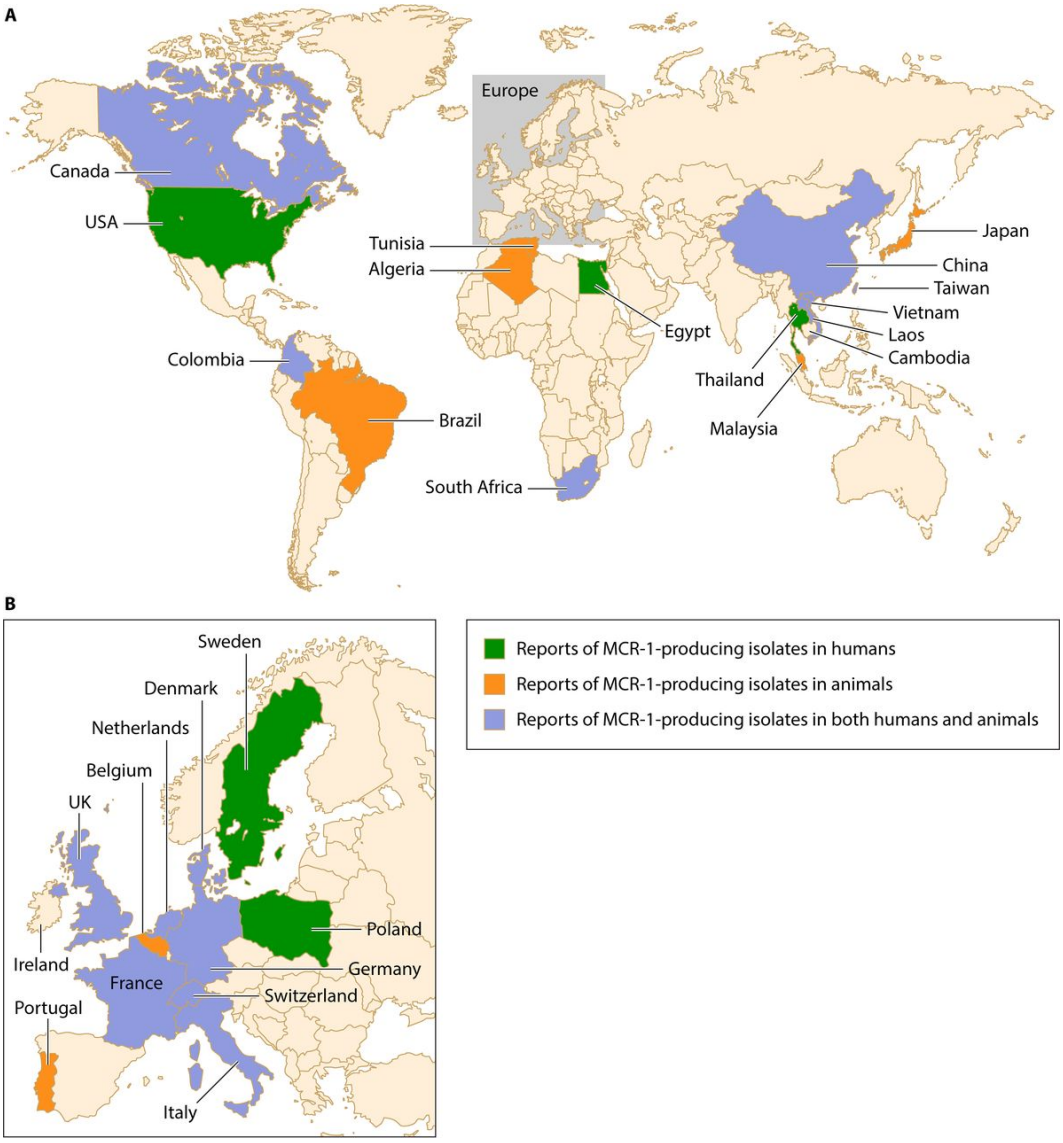


Figure 1-15. Worldwide reports of MCR-1-producing isolates in humans, animals or both

Figure reproduced from (115)

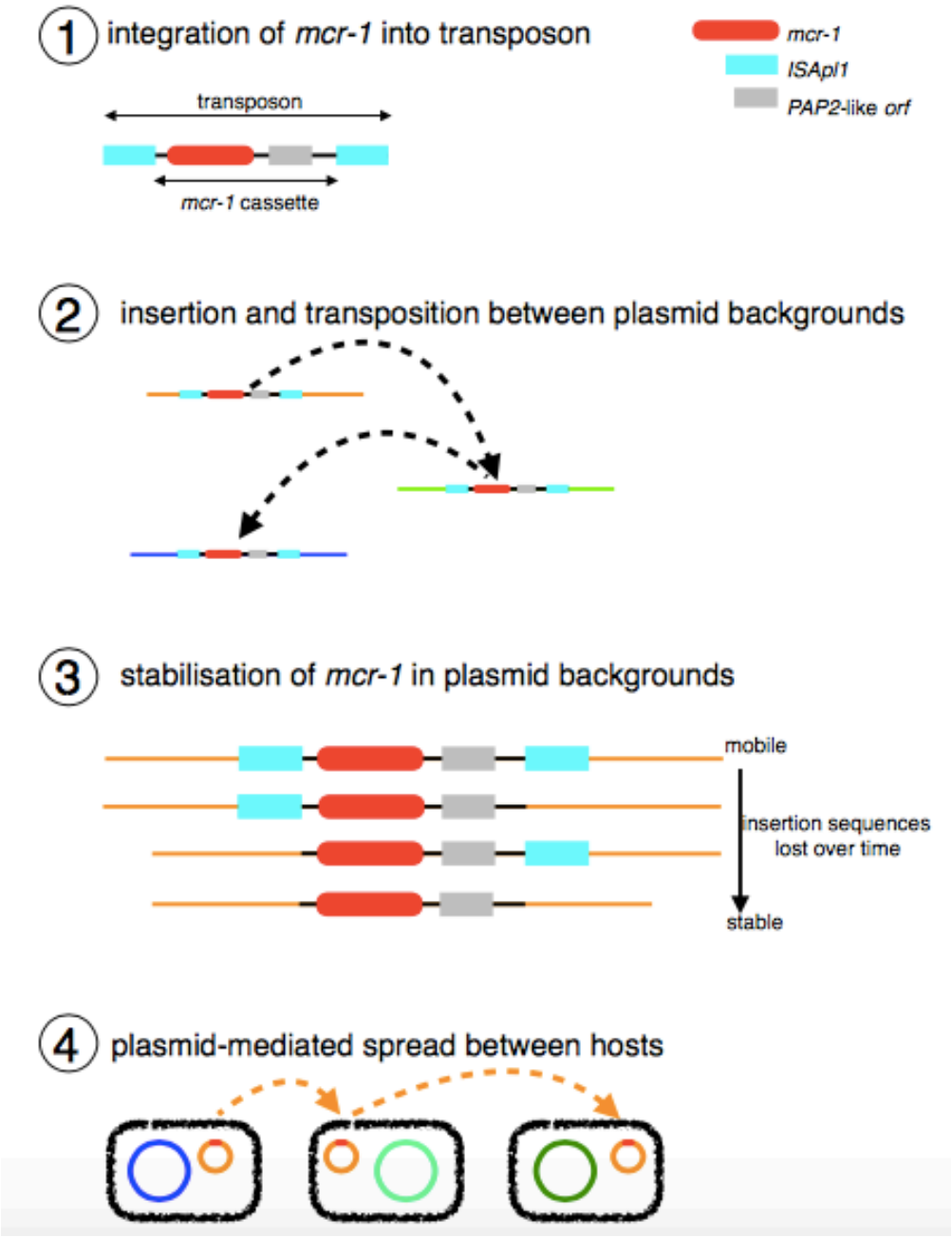


Figure 1-16. Schematic representation of the evolutionary model for the steps in the spread of the *mcr-1* gene

(1) The formation of the original composite transposon, followed by (2) transposition between plasmid backgrounds and (3) stabilization via loss of ISApl1 elements before (4) plasmid- mediated spread.
Figure reproduced from (116)

CHAPTER 2 - BORONIC ACID TRANSITION STATE INHIBITORS AS AN ALTERNATIVE FOR CLASS A BETA-LACTAMASES

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Introduction

The continuous and accelerated emergence of bacterial strains resistant to virtually all antibiotics is one of the most challenging issues in clinical medicine. β lactam antibiotics are ancient, and resistance to them can be found at very low levels and widespread in nature. Unfortunately, the ubiquitous overuse of these agents in the past 80 years, at levels massively in excess of those found in nature, has resulted in the rapid, nearly continuous emergence of resistance mechanisms that are new to clinical medicine. Therefore, novel treatment options for infections produced by multidrug-resistant Gram-negative bacteria are required.

β lactam antibiotics are among the most important antibiotics in all of medicine, and production of β-lactamases is the most common mechanism of resistance to β-lactam agents in clinically important Gram-negative bacteria (38). Based on conserved amino acid motifs, β-lactamases are grouped into four molecular classes. Classes A, C and D β-lactamases hydrolyze the β-lactam ring and form an acyl enzyme through a serine

active site, whereas class B are metallo-enzymes characterized by the presence of one or two Zn^{2+} ion binding sites (38). As of 2015, the number of officially numbered β-lactamases listed on the authoritative website at Lahey Clinic (www.lahey.org/studies) is roughly 1800. The astonishing number of β-lactamases reported might be explained by the increasing selective pressure from the proliferation of β-lactam antibiotics as well as advances in the molecular biology tools to detect them (94).

KPCs are "versatile" class A *β*-lactamases that threaten the use of all current β-lactams as KPCs hydrolyze penicillins, cephalosporins, monobactams, and carbapenems and also are resistant to inhibition by clavulanic acid, sulbactam, and tazobactam (75). These carbapenemases, first reported in *Klebsiella pneumoniae* in 2001 (19), are carried by transposons (Tn*4401* family) and have disseminated to an increasing number of bacterial genera, becoming the major carbapenemase carried by clinically important Gram negatives worldwide (24, 75).

The SHV family of enzymes belongs to the molecular class A of serine β-lactamases and is thought to have emerged in association with the use of ampicillin to treat infections caused by *K. pneumoniae* (94) where they originated as narrow spectrum penicillinases. Although the principal localization of bla_{SHV} in *K. pneumoniae* is chromosomal, bla_{SHV} has been found in many *Enterobacteriaceae* on mobile plasmids (166). Most of the more than 180 plasmid-encoded variants described to date possess extended-spectrum β-lactamase (ESBL) activity, which confers resistance

to expanded-spectrum cephalosporins, such as ceftazidime, as well as monobactams. Some SHV β-lactamases also exhibit resistance to β-lactamase inhibitors (BLIs) such as clavulanate (68) which further reduces the therapeutic options for treatment of infections caused by bacteria harboring these enzymes.

To overcome this growing threat, research is directed towards two strategic targets: (*i*) to obtain β-lactam antibiotics that are intrinsically resistant to β-lactamases; and (*ii*) to identify BLIs that would allow the partner β-lactam to reach the penicillin binding proteins (PBPs), the primary target of β-lactams (167). In this second aim, structure-based design is widely used to discover new BLIs by mimicking interactions observed between the target enzyme and its natural substrates. Of late, great interest was focused on inhibition of common serine β-lactamases (168). Attention to novel BLIs bearing an electrophilic center (phosphonates, aldehydes, trifluoromethylketones, and boronic acids) that can covalently modify the nucleophilic catalytic serine has conceptually advanced our understanding in this field (169).

Among the novel BLIs bearing electrophilic centers, the boronic acid transition state inhibitors (BATSI) merit particular attention as clinically important BLIs. BATSIs possess a boron atom acting as an electrophile that mimics the carbonyl carbon of the β-lactam ring and forms a tetrahedral adduct with the catalytic serine that closely resembles a transition state of the hydrolytic mechanism (168, 170, 171). Presently,

Carbavance® which is combination of a novel boronic acid-based BLI (RPX7009) with meropenem (RPX2014) for intravenous treatment of hospitalized patients with serious infections attests to the interest in this class of compounds (e.g., NCT02168946).

In a parallel quest, BATSIs were studied and optimized for a variety of β-lactamase enzymes (68, 172-177). Mechanistically, the boronic inhibitors act by binding to the active site of the enzyme, where they sterically resemble the quaternary transitional state of the β-lactam hydrolysis reaction and occupy the active site with high affinity, leading to inhibition in a reversible competitive manner (168). Recently, rational inhibitor design efforts were directed towards the inclusion of an R1 side chain on BATSIs that resembles the side groups of known β-lactams (178). This feature is necessary for specific interactions with the β-lactamases. Additionally, BATSIs with a variety of R2 groups were also rationally designed (Scheme 1a and 1b).

Herein, a series of BATSIs targeting KPC-2, SHV-1 and other class A ESBLs, were synthetized and screened. A diverse portfolio was designed to explore optimal SAR. In this group of BATSIs, three regions were selectively modified: The R1 group, the R2 group, and the amide group. Our biochemical analysis and antibiotic susceptibility results provide evidence that a strategy can be developed to create effective inhibitions of SHV and KPC β-lactamases based on SAR. Our goal in this manuscript and other studies planned is to iteratively test novel scaffolds to explore

the importance of different interactions not currently known by the boronic acids in development and to explain their potency by structural studies.

Materials and methods

Synthesis

The BATSIs were synthesized in the desired *R* absolute configuration by stereoselective homologation of (+)-pinandiol boronates. For the triazole-containing BATSIs, a Cu-catalyzed azide-alkyne cycloaddition was performed on the suitable β-azido-boronate (178, 179). Complete description of the organic synthesis of compound *3c* is provided as supplementary data.

Bacterial strains and plasmids

The bla_{KPC-2} gene in the pBR322-*catI* vector (pBR322-*catI*-bla_{KPC-2}) was a kind gift from Dr. Fred Tenover of the Centers for Disease Control and Prevention (Atlanta, GA) (75). The cloning of bla_{SHV-1} into phagemid vector pBC SK(-) was previously described (180). Both plasmids were maintained in *Escherichia coli* DH10B cells.

β-Lactamase purification

The KPC-2 and SHV-1 β-lactamases were expressed and purified as previously described. Briefly, *E. coli* harboring SHV-1 and KPC-2 were grown overnight in lysogeny broth (LB) containing 20 μg/ml chloramphenicol. Cells were pelleted and lysed by stringent periplasmic fractionation. The soluble fractions were run on a preparative isoelectric

focusing platform as previously described (75, 180). Fast protein liquid chromatography (FPLC) on an AKTA purifier was conducted using a gel filtration Superdex 75 column and anion exchange HiTrapQ XL column. Purity of the fractions was assessed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). The protein concentrations were determined by measuring the absorbance at λ_{280} nm and Beer's law using the proteins' extinction coefficient ($\Delta\varepsilon$ = 39,545 $M^{-1}cm^{-1}$ for KPC-2 and $\Delta\varepsilon$ = 31,970 $M^{-1}cm^{-1}$ for SHV-1).

Steady-state kinetics

Steady-state kinetics were performed on an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). Each assay was performed in 10 mM phosphate-buffered saline pH 7.4 (PBS) at room temperature. The inhibition of β-lactamases by BATSIs is hypothesized to follow a slow-reversible process represented according to the following scheme:

$$E + I \leftrightarrow E{:}I \leftrightarrow E\text{-}I^* \leftrightarrow E\text{-}I^{**}$$

Here, E represents the β-lactamase enzyme, I represents the BATSI, E:I is the Michaelis complex, E-I* is the enzyme-inhibitor complex resembling the acylation high-energy intermediate, and E-I** is the de-acylation high-energy intermediate. This model takes into account the crystallographic intermediates captured in two previous studies (173, 175) .

The IC_{50} values were determined after a 5 min pre-incubation of the β-lactamase (7 nM KPC-2 or 2.5 nM SHV-1) and the BATSI used at increasing concentrations as previously described (178). The initial velocities (v_0) of hydrolysis of the indicator substrate, nitrocefin ($\Delta\varepsilon_{482}$ =17,400 M^{-1} cm^{-1}) were measured and the data were fit to Equation 1.

$$v_0 = \frac{V_{\max}[S]}{K_m[1+\frac{I}{IC_{50}}]+[S]} \quad \text{Eq.1}$$

Measurements of apparent association rate constants (k_2/K) for inhibitor binding were carried out in the presence of several concentrations of compound *2b*. Reactions were then initiated by the addition of enzyme (2.5 nM KPC-2 or SHV-1) to a mixture of 50 μM nitrocefin and the BATSI, without preincubation. Inhibitor association rate constants were estimated from linear extrapolation of the observed rate constant for inhibition (k_{obs}), which was obtained by fitting progress curves of nitrocefin hydrolysis to Eq. 2:Eq. 2

$$A = A_0 + v_s t + \frac{(v_0 - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} \quad \text{Eq.2}$$

Here A is absorbance, v_0 is the initial velocity, v_s is the steady-state velocity, and k_{obs} is the apparent rate constant for formation of the enzyme-inhibitor complex. The linear dependence of k_{obs} vs. [I] at limiting inhibitor concentration, approximates second-order rate constant k_2/K . The observed second-order rate constant also includes an adjustment for competition between inhibitor and substrate binding (Eq. 3).

$$k_{obs} = k_{-2} + \frac{k_2}{K_i} \frac{[I]}{(1 + \frac{[S]}{K_m})}$$ Eq. 3

In order to determine the apparent dissociation rate constant (k_{off}), 1 μM SHV-1 or KPC-2 was incubated with 2.5, 5, 10 or 15 μM of compound *2b* for 5 min at 37°C and diluted 4,000-fold in the assay buffer without inhibitor. In the off-rate experiment, v_0 represents fully inhibited enzyme velocity and was estimated measuring a reaction without enzyme. Uninhibited enzyme velocity (v_S) was measured using a reaction with SHV-1 and KPC-2 and no added BATSI. The data were fit to equation 2, where k_{obs} was replaced by k_{off}. The k_{off} value is reported as ±2 SD from three separate determinations.

Antimicrobial susceptibility testing

To assess the permeability and qualitative antimicrobial activity of compound *2b*, the minimum inhibitory concentrations (MICs) for ceftazidime, cefepime, and ertapenem, alone and in combination with *2b*, were determined for *E. coli* DH10B carrying pBR322-*catI*-*bla*$_{KPC-2}$ or pBC SK (-) *bla*$_{SHV-1}$ as well as for clinical isolates containing one or more β-lactamases. The agar dilution method in Mueller-Hinton following Clinical and Laboratory Standards Institute (CLSI) recommendations was used (181).

Molecular Modeling-Docking

The crystal structures of KPC-2 (PDB: 2OV5) and SHV-1 (PDB: 1SHV) were used to generate molecular representations of the interactions of these two β-lactamases with representative BATSIs (*achiral cephalothin*, *1c* and *2b*). Discovery Studio 4.1 (DS 4.1) software (Accelrys, CA) was employed to prepare and optimize the crystallographic structures and to dock the inhibitors into the active site of these enzymes.

Briefly, the minimization was performed in several steps, using the Steepest Descent and Conjugate Gradient algorithms to reach the minimum convergence (0.001 kcal mol^{-1}*Å). The protein was solvated employing a model with periodic boundary conditions. The force-field parameters of CHARMm were used for minimization and the Particle Mesh Ewald method addressed long-range electrostatics. The bonds that involved hydrogen atoms were constrained with the SHAKE algorithm.

The minimized and equilibrated KPC-2 and SHV-1 structures were used for constructing the complexes of the β-lactamases and the BATSI. The ligand structures were built using DS 4.1 Fragment Builder tools. The molecular docking was performed using CDOCKER module of DS 4.1 (182). The protocol uses a CHARMm-based molecular dynamics (MD) scheme to dock ligands into a receptor binding site. Random ligand conformations were generated using high-temperature MD. The conformations were then translated into the binding site. Candidate poses

were then created using random rigid-body rotations followed by simulated annealing. A final minimization is then used to refine the ligand poses. From the multiple possible conformations generated, the candidate ones were manually chosen. The complex between the ligand and the enzymes was created, solvated, and energy minimized. The acyl-enzyme complex was created by making a bond with Ser70, and the assembly was further minimized using conjugate gradient algorithm with periodic boundary conditions to 0.001 minimum derivatives.

Results and Discussion

In this report we examined the SAR of the designed BATSI's against SHV-1 and KPC-2 β-lactamases in biochemical and microbiological assays. The results of kinetics experiments were interpreted using molecular models. After identifying a compound with most desirable properties, we further determined microscopic rate constants and performed antimicrobial susceptibility testing as a proof of concept. We discovered that BATSI containing a thiophene ring as R1 substituent and possessing a triazole with a carboxyl group as a R2 sidechain demonstrated optimal activity against the KPC and SHV β-lactamases of *Klebsiella spp*.

SAR of BATSIs against KPC-2 and SHV-1 β-lactamases and molecular modeling.

After modifying three regions of the BATSI (i.e., the R1 group, the R2 group, and changing the amide group to a sulfonamide, urea or thiourea), we first

explored the structural and affinity contributions of a group of boronic acid inhibitors. Starting with the reference compound, with an amide group and devoid of R1 or R2 side chains, the IC_{50} values were determined to be more than 150 μM for both SHV-1 and KPC-2 β-lactamases (Table 1). The addition of a thiophene ring at R1 position (*achiral cephalothin*) improved the binding to both enzymes, with the greatest increase seen with KPC-2 β-lactamase at ~3 μM vs. 50 μM for SHV-1. The chiral BATSIs that possessed a cephalothin R1 side chain combined with an amide group and various R2 substituents, generally demonstrated greater affinity towards both KPC-2 and SHV-1. Of these thiophene containing BATSIs, seven compounds (*1b*-*2e*) demonstrated IC_{50} values in the nM range (38 - 600 nM). By changing the R1 chain from thiophene to phenyl (compound *1c* vs *3a*), the IC_{50} values were only slightly affected for KPC-2 or SHV-1 (Table 2-1).

To understand how the addition of a thiophene ring lowers the IC_{50} values by 80-fold compared to the reference compound, a molecular representation of KPC-2 with *achiral cephalothin* docked in the active site was generated (Figure 2-2). This model suggests that the lack of interactions with the catalytic part of the enzyme may be compensated by the steric interaction of thiophene ring with W105. We advance that since SHV-1 possesses a Tyr at position 105 instead of a Trp, this may explain why it displayed a higher IC_{50} value for achiral cephalothin compared to KPC-2. In addition, the hydroxyls of the boronate were within hydrogen

bond distance of the oxyanion hole atoms, S70:N and T237:N as well as S130. The addition of a phenyl group (compound *1a*) at the R2 position improved the IC_{50} value for SHV-1 by nearly 10 fold (from 50 to 5.9 μM), suggesting that the addition of a R2 chain was also important for interactions with SHV-1.

Another significant change was observed for the thiophene and amide containing BATSIs with the addition of a carboxyl group to the phenyl side chain at the R2 position (compound *1b*). The IC_{50} values decreased to 0.4-0.6 μM from 2-6 μM with the phenyl group alone (compound *1a*). From our modeling we hypothesize that the carboxyl group is able to make interactions with R220 (KPC-2) and R244 (SHV-1) as observed with the Figure 2-2carboxylate group on β-lactams. By adding an extra carbon (compound *1c*) to compound *1b*, the IC_{50} values were further lowered to 0.1-0.08 μM, most likely due to improved interactions with positions R220 in KPC-2 and R244 in SHV-1 (Figure 2-2).

The triazol-based R2 side chain present on compounds *2a*, *2b*, *2c*, *2d*, and *2e* improved the IC_{50} values up to 75-fold and 380-fold for KPC-2 and SHV-1, respectively compared to *achiral cephalothin*. Regardless of the substituent (carboxyl, phenyl, carboxyphenyl, etc.) attached to the triazole, all of the compounds possessed low IC_{50} values from 0.04-0.43 μM for KPC-2 and 0.13-0.43 μM for SHV-1. Compound *2b* was one of the best inhibitor against KPC-2 and SHV-1 with IC_{50} values of 84 ± 2 nM and 130 ± 2 nM, respectively.

To assess the contribution of the triazol moiety, compound *2b* was docked into the active sites of KPC-2 and SHV-1. The molecular docking generated multiple conformations for *2b* compound (Figure 2-3) suggesting this compound may demonstrate a high conformational flexibility in the active site of KPC-2 and SHV-1. The crystal structure of *2b* (S02030) with the class C β-lactamase, ADC-7 revealed multiple conformations of the compound in the active site as well (183). Based on the possible conformations of *2b* into the active site of ADC-7, a similar binding mode with KPC-2 and SHV-1 enzymes was chosen (Figure 2-3). In this molecular model, the triazol side chain produced a strong dipole moment and behaved as an active linker by forming interactions with position R220 in KPC-2 and R244 in SHV-1 (Figure 2-3).

In the study described in the companion article, the crystal structure of KPC-2 and SHV-1 with 2b (S02030) revealed multiple conformations of 2b in the active site of KPC-2 and SHV-1 (184). The boronic acid preserved the tetrahedral interactions, forming the bond with catalytic serine, positioning one of the boronic acid oxygens in the oxyanion hole (T237:N for KPC-2) and the other toward S130. The amide moiety of 2b interacts with the side chain of N132 as suggested by the molecular model. In the crystal structure, the carboxyl-triazole moiety does not make a salt bridge with either R220 (KPC-2) or R244 (SHV-1) as observed during the MD in the molecular modeling.

When the amide group present in compound *3a* was replaced by a urea, thus switching the benzyl R1 group of *3a* into an aniline, a slight loss of activity was observed (0.23 ± 0.002 μM KPC-2 and 0.9 ± 0.02 μM SHV-1 for *3b* with respect to 0.07 ± 0.01 μM and 0.20 ± 0.005 μM for *3a*, respectively). The loss of activity was much more pronounced replacing the amide with a thiourea (compound *3c*) as displayed by IC_{50} values of 73 ± 4 μM SHV-1 and 32 ± 2 μM KPC-2 (Table 2-1), highlighting the importance of the carbonyl as H-bond acceptor.

Compared to *achiral cephalothin*, the achiral phenyl sulfonamide BATSI (compound *4a*) performed similarly against KPC-2 and SHV-1 (Table 2-1). However, an interesting and unexpected decrease in IC_{50} values was observed by the addition of a carboxyl side chain to the phenyl group (compound *4b*). The presence of a carboxyphenyl group at the R1 side chain was beneficial for achiral BATSIs. Based on the crystal structure of similar compounds with AmpC β-lactamases (PDB: 3O87) (185) we hypothesize that the carboxyphenyl group will flip to interact with position R220 for KPC-2 and residue R244 for SHV-1, and that the sulfonamide group will make interactions with N132 and/or K73 (Figure 2-4).

Susceptibility testing and biochemical analysis of compound 2b.

Among the evaluated BATSIs, compound *2b* possessed one of the most favorable initial biochemical properties against KPC-2 and SHV-1, thus was chosen for antimicrobial susceptibility testing and detailed kinetic analysis. We tested the ability of compound *2b* to restore susceptibility

to cephalothin, cefepime, ceftazidime, and/or ertapenem for *E. coli* DH10B producing KPC-2, SHV variants including inhibitor resistant and ESBLs, as well as clinical isolates with different class A β-lactamases. All strains expressing bla_{KPC}s were resistant to cefepime and ceftazidime, while 7 of 8 were resistant to ertapenem (Table 2-2). When partnered with cefepime or ertapenem, compound *2b* restored the susceptibility to all resistant strains carrying bla_{KPC}s (Table 2-2). The ceftazidime-compound *2b* combination restored susceptibility to all isolates except *K. pneumoniae* VA375, which displayed an intermediate MIC value of 8 mg/L with the combination. Against a panel of 16 strains expressing bla_{SHV}, bla_{TEM}, and/or $bla_{CTX\text{-}M}$ variants, cefepime-compound *2b* restored susceptibility to all isolates tested (Table 2-3). Fourteen of sixteen strains were resistant to cephalothin and when combined with compound *2b* this number decreased to six strains. With ceftazidime, thirteen of sixteen strains demonstrated resistance, but when combined with compound *2b* only five of sixteen remained resistant. The cefepime-compound *2b* combination was the most effective combination tested against all 24 strains expressing various class A β-lactamases.

To obtain a detailed biochemical profile of compound *2b* against KPC-2 and SHV-1, acylation rates (k_2/K) and off rates (k_{off}) were determined. With the KPC-2 β-lactamase, a k_2/K value of $1.2 \pm 0.2 \times 10^4$ $M^{-1}s^{-1}$ was obtained, whereas for SHV-1, $4.7 \pm 0.6 \times 10^3$ $M^{-1}s^{-1}$ was observed (Figure 2-5). These values are comparable with the inactivation efficiency (k_{inact}/K_I) values

reported for a variety of other BLIs against class A β-lactamases (e.g., SHV-1, CTX-M-9, and KPC-2) with magnitudes of 10^4 to 10^6 $M^{-1}s^{-1}$ (75, 186-189). Most importantly compared to the recently Food and Drug Administration approved BLI, avibactam, the k_2/K values for compound *2b* are similar to avibactam with values of 2.2×10^4 $M^{-1}s^{-1}$ for KPC-2 and 6×10^4 $M^{-1}s^{-1}$ for SHV-1 (190, 191).

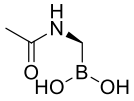
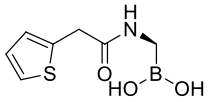
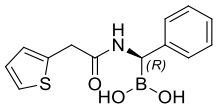
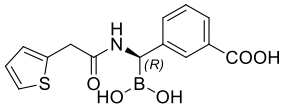
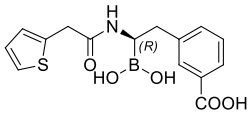
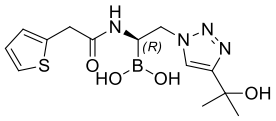
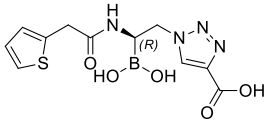
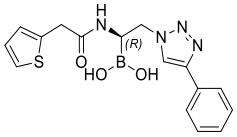
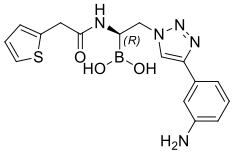
The k_{off} values for compound *2b* were also determined for KPC-2 and SHV-1 at 0.00046 ±0.00002 s^{-1} and 0.0024 ± 0.0001 s^{-1}, respectively (Figure 2-5). The corresponding residence time half-lives ($t_{1/2}=0.693/k_{off}$) were 24.8 ±1 min for KPC-2 and 4.9 ± 0.5 min for SHV-1 respectively. For comparison, the $t_{1/2}$ values for avibactam range from 6-300 min for susceptible β-lactamases (i.e., TEM-1, CTX-M-15, KPC-2, PDC-1, and P99) (191).

Conclusion

Here, novel synthetic strategies investigated three parts of the molecule in the same BATSI. We found that the best R1 side chain was the cephalothin analog. In addition, at position R2 a substituted phenyl or triazole resulted in lower IC_{50} values. Finally, the change of amide into sulfonamide or urea was beneficial (185), while the thiourea was not active. We also discovered that against SHV-1 β-lactamase, and more strikingly against KPC-2, compound *2b* exhibited a time-dependent inactivation, a behavior that was previously observed for other β-lactamases when inactivated with BATSIs (178, 183). By identifying common features of these new BATSIs, novel

SAR studies to target KPC-2 and other clinically important β-lactamases can be performed with the aim to determine key amino acid residues in the catalytic pocket that contribute to molecular recognition. Studies against other important β-lactamases and *in vivo* (animal model) testing are warranted. It has not escaped our attention that benefits and limitations of each new class of BLI must be fully vetted in order to find an optimal inhibitor. Nevertheless, as compounds become more focused and pathogen directed, there is intent to design specific chemotypes to treat particular resistant bacteria (i.e., *K. pneumoniae*). The approach taken here is an important step towards "precision medicine".

Table 2-1. BATSI chemical structures and IC50 data

Compound	Structure R1	R2	IC_{50} (μM) SHV-1	KPC-2
reference			>200	164 ± 5
achiral cephalothin			50 ± 2	2.9 ± 0.1
1a			5.9 ± 0.3	2 ± 0.2
1b			0.40 ± 0.02	0.60 ± 0.05
1c			0.10 ± 0.001	0.08 ± 0.001
2a			0.13 ± 0.002	0.18 ± 0.002
2b			0.13 ± 0.002	0.08 ± 0.002
2c			0.33 ± 0.02	0.06 ± 0.005
2d			0.43 ± 0.07	0.43 ± 0.07

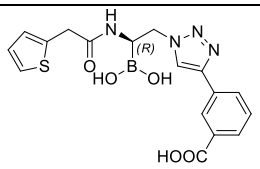
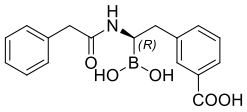
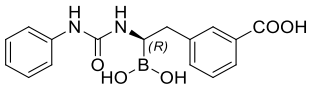
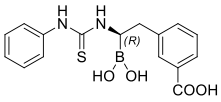
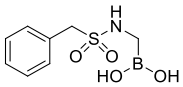
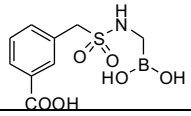
Compound	Structure		IC_{50} (μM)	
	R1	R2	SHV-1	KPC-2
2e			0.14 ± 0.02	0.04 ± 0.01
3a			0.20 ± 0.05	0.07 ± 0.01
3b			0.90 ± 0.02	0.23 ± 0.002
3c			73 ± 4	32 ± 2
4a			118 ± 7	6.2 ± 0.5
4b			0.43 ± 0.07	0.20 ± 0.01

Table 2-2. MICs for *E. coli* DH10B expressing KPC-2 and clinical strains carrying variety of KPCs with cefepime, ceftazidime, and ertapenem alone and in combination with compound 2b at 4 μg/ml

Species	Strain	β-lactamase (s)	MIC[a] μg/ml					
			FEP	FEP -*2b*	CAZ	CAZ -*2b*	ERT	ERT -*2b*
E. coli	DH10B	none	0.06	0.06	0.5	0.5	0.06	0.06
E.coli	DH10B pBR322-*catI*	KPC-2	**16**	0.25	**128**	4	**8**	0.06
K.pneumoniae	Kpn96	KPC-2, TEM-1	**16**	0.12	**64**	2	**16**	0.5
	KonST258	KPC-3, TEM, SHV	**32**	0.5	**64**	2	**4**	0.06
	Kpn ST17	KPC-2, TEM, SHV, CTX-M group 1	**64**	0.12	**32**	2	**8**	0.06
	Kpn VA375	KPC-3, TEM-1, SHV 11/14	**16**	0.5	**64**	**8**	**8**	0.5
	Kpn VA 388	KPC-3, TEM-1, SHV-1	**8**	0.12	**64**	2	**4**	0.06
E.coli	EcoPR261	KPC, CTX-M group 1, SHV	**16**	0.25	**64**	0.2	**16**	0.25
E.coli	Eco pLTCF1	CTX-M group 9	**8**	0.12	**8**	0.5	0.12	0.06

[a] MICs in bold are values that are intermediate or indicate resistance according to the CLSI (181). FEP, cefepime; CAZ, ceftazidime; ERT, ertapenem.

Table 2-3. MIC values in µg/mL for *E. coli* DH10B expressing SHV-1 and clinical strains carrying variety of SHV's and other class A β-lactamases with cephalothin (CEF), cefepime (FEP), and ceftazidime (CAZ) alone and in combination with compound 2b at 4 µg/mL

Species	Strain	β-lactamase(s)	CEF	CEF -*2b*	FEP	FEP -*2b*	CAZ	CAZ -*2b*
E.coli	DH10B	none	**4**	2	0.06	0.06	0.5	0.5
	ATCC BAA-202	SHV-1	**128**	8	**4**	0.25	**32**	8
	ATCC 35218	TEM-1	**8**	2	0.06	0.06	0.25	0.12
	DH10B pBC SK(-)	SHV-1	**128**	4	**2**	0.25	**16**	2
	DH10B pBC SK(-)	SHV-2	**512**	4	**4**	0.06	**64**	4
	DH10B pBC SK(-)	SHV R244S	**4**	2	0.12	0.06	**1**	1
K. pneumoniae	Kpn 266	SHV-5	**512**	2	**4**	≤0.06	**>128**	1
	Kpn 104	TEM-1, SHV-1	**256**	64	**4**	1	**>128**	>8
	Kpn 255	TEM-1, SHV-2	**256**	64	**8**	2	**>128**	>8
	Kpn 158	TEM-1, SHV-5	**256**	32	**8**	0.5	**>128**	>8
	Kpn 9	TEM-1, CTX-M-2	**1024**	64	**16**	0.5	**32**	4
	Kpn 427	TEM-1, SHV-1, CTX-M-3	**>1024**	32	**8**	0.25	**16**	2
	Kpn 160	TEM-1, SHV-2, CTX-M-3	**1024**	16	**8**	0.5	**32**	2
	Kpn 59	TEM-1, SHV-5, CTX-M-2	**256**	4	**4**	0.12	**128**	1
	Kpn 238	TEM-12, SHV-2, CTX-M-2	**512**	32	**16**	0.5	**>128**	>8
	Kpn 34700	CTX-M-15, SHV, TEM	**>1024**	128	**16**	1	**64**	4
E. coli	Eco 29838	CTX-M-14, TEM-1	**1024**	32	**8**	0.25	2	1

MIC values in bold represent values that are intermediate or resistance according to CLSI (192).

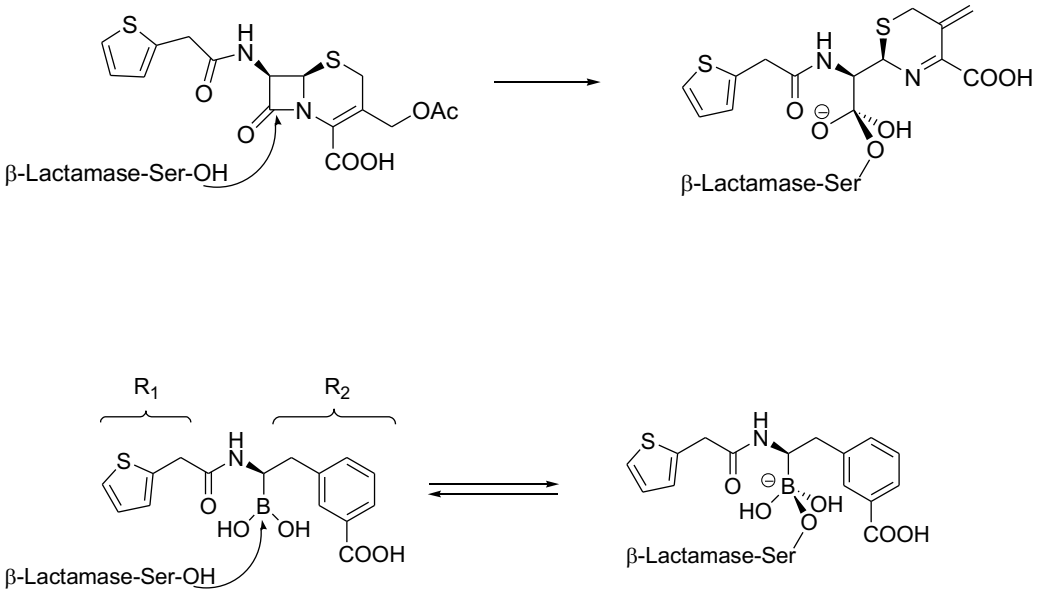


Figure 2-1. Mechanism of action of BATSIs.

BATSIs act by binding to the active site of the enzyme, where they sterically resemble the quaternary transitional state of the ß-lactam hydrolysis reaction and occupy the active site with high affinity, leading to inhibition in a reversible competitive

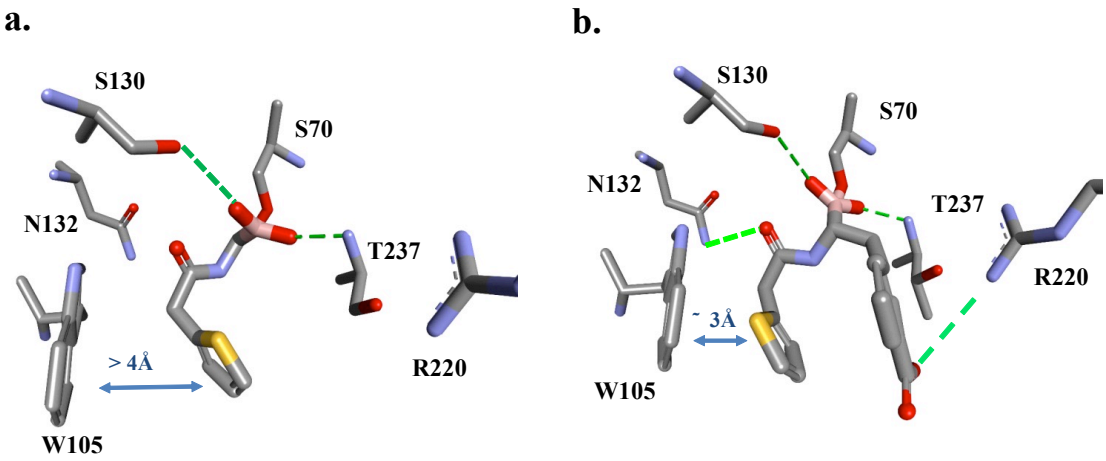


Figure 2-2. Molecular docking of achiral cephalothin (a) and compound 1c (b) in the active site of KPC-2.

The model of achiral cephalothin (missing the R2 side chain) with KPC-2 shows fewer H-bonding interactions (green dashed lines) compared to those of compound 1c. The boron atom preserves the tetrahedral conformation, as observed in the crystal structures (H-bonding distance with S130, S70:N, and T237:N). The addition of *m*-carboxyphenyl group as the R2 side chain in compound 1c creates interactions with R220. The thiophene ring of 1c is 3 Å from Trp 105, making possible steric interactions with phenyl ring, which are more favorable than achiral cephalothin (d > 4 Å). Molecular modeling was performed by Magdalena Taracila at Case Western Reserve University.

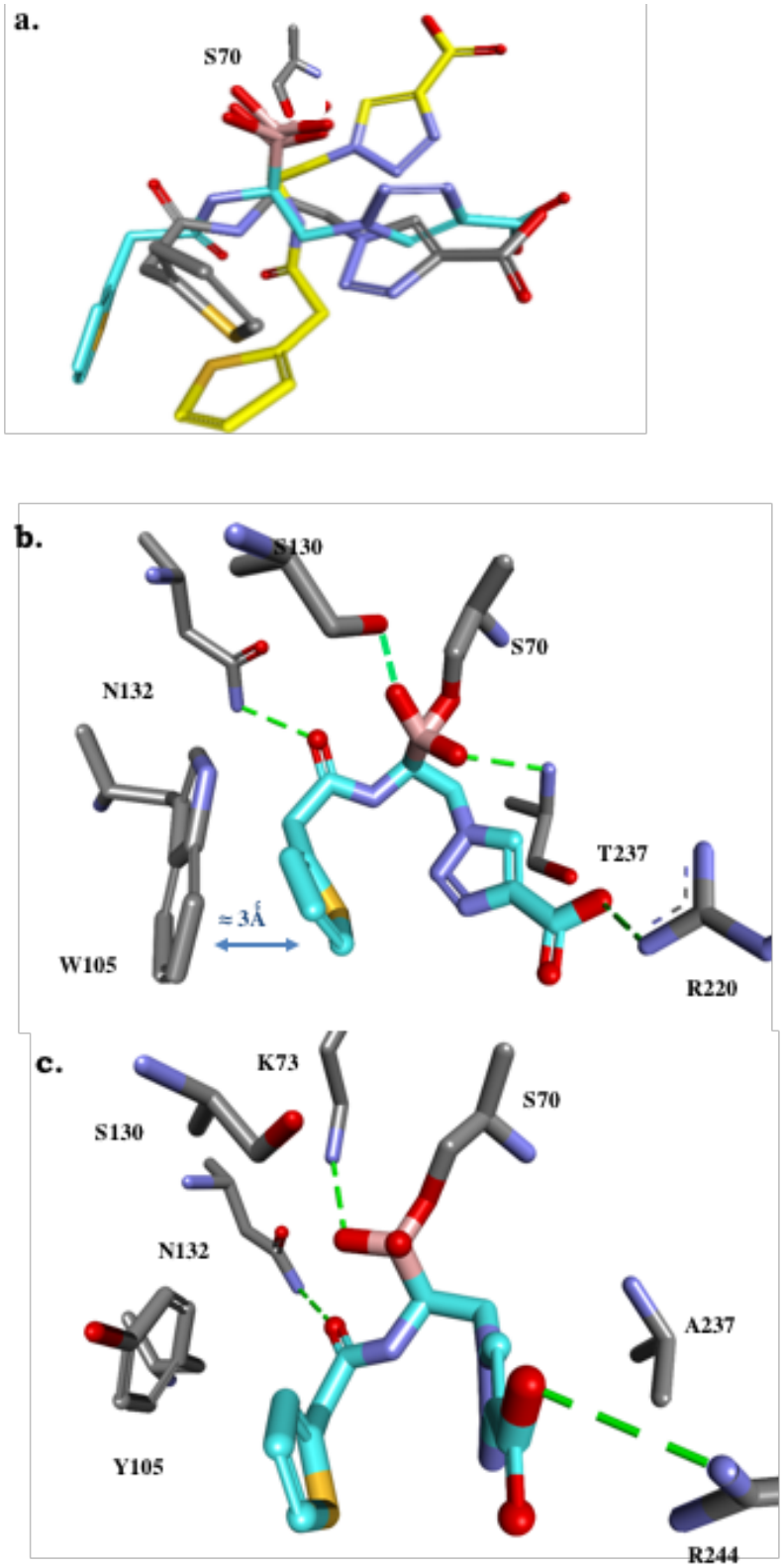


Figure 2-3. Molecular docking of compound 2b into the active sites of KPC-2 (b) and SHV-1 (c).

Figure 2-3 (continued).Using high-temperature MD, molecular docking (CDOCKER) generated multiple boronic acid conformations; shown here are 3 of those (gray, yellow, and cyan) (a). The candidate poses (b and c) were chosen and complex enzyme-compound 2b was created. The *m*-carboxyl group attached to the triazole R2 side chain demonstrated interactions with R220 in KPC-2 and R244 in SHV-1. The triazole moiety enhanced binding due to a strong dipole moment and behaved as an active linker in both KPC and SHV enzymes. The hydroxyl boron atoms preserve the interactions with S130 and T237 for KPC-2 (b), but not for SHV-1 enzyme (c). The MD suggests that hydroxyl borons form H bonds with K73, S130, and A237. Molecular modeling was performed by Magdalena Taracila at Case Western Reserve University.

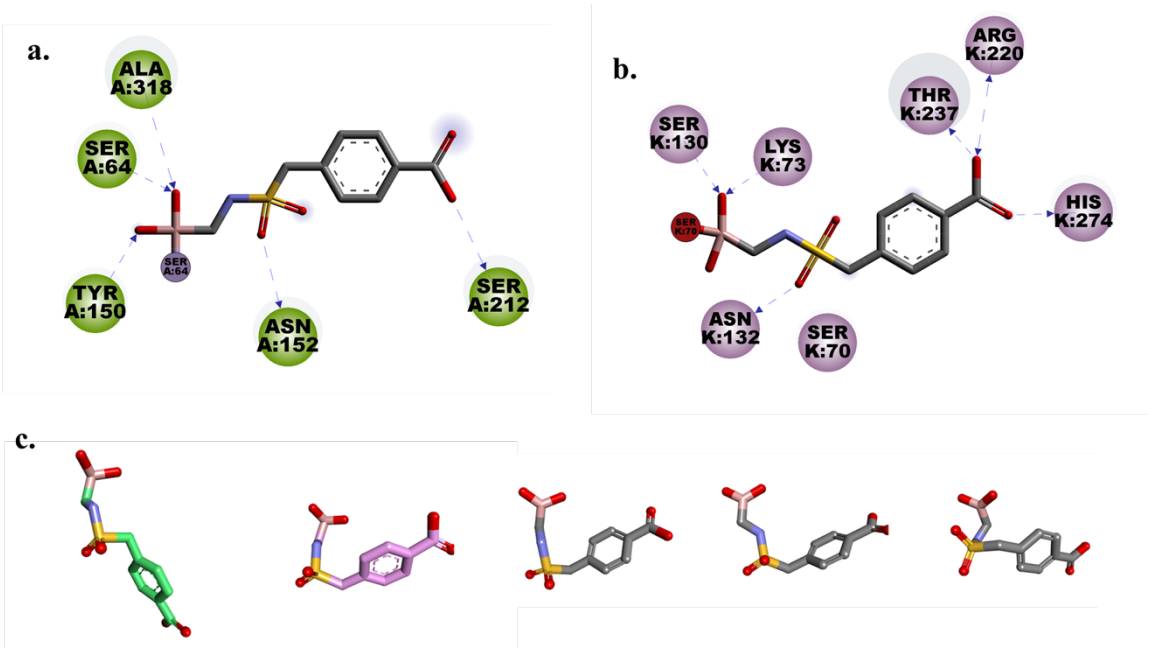


Figure 2-4. Schematic 2D diagram showing possible interactions between BATSI 4d and AmpC -lactamases (PDB code 3O87) (a) and KPC-2 enzyme (b).

Based on the conformational flexibility of compound 4d (c) and the high affinity for KPC -lactamases, the model suggests that the carboxyl group may interact with residue R220. Molecular modeling was performed by Magdalena Taracila at Case Western Reserve University.

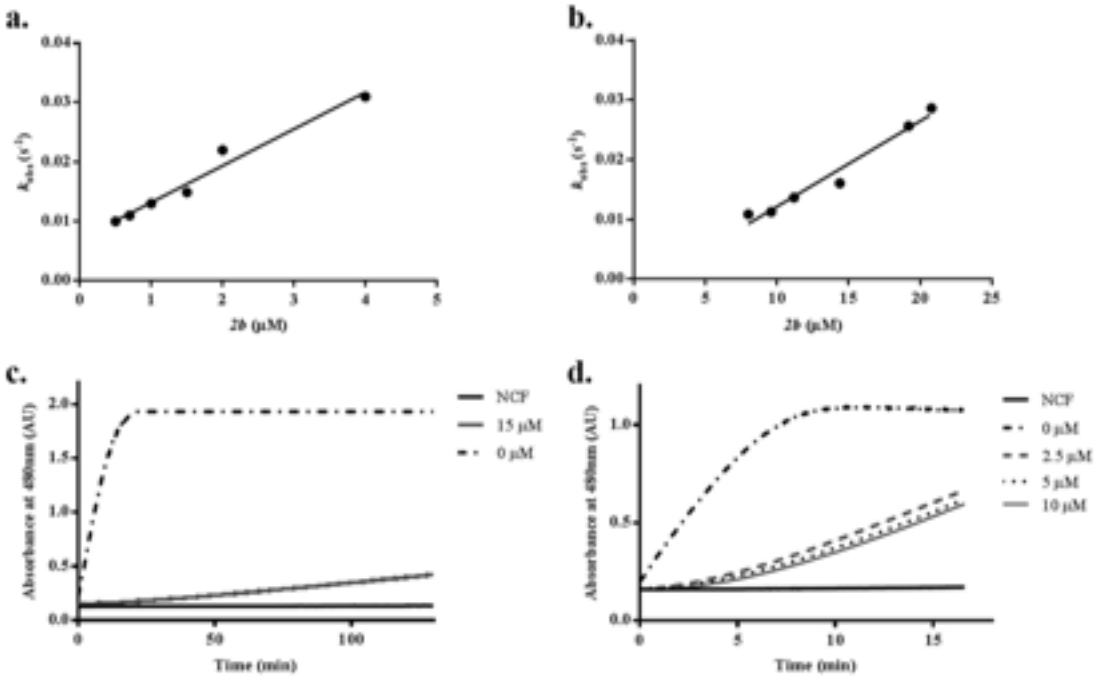


Figure 2-5. Inhibition of nitrocefin hydrolysis by compound 2b of KPC-2 (a) and SHV-1 (b).

Inhibitor association rate constant $k2/K$ was estimated from linear extrapolation of the observed rate constant for inhibition, $k$obs, as function of inhibitor concentration. The dissociation rate, $k$off, for compound 2b for KPC-2 (c) and SHV-1 (d) represents the recovery of activity as a function of time.

CHAPTER 3 - NDM & OXA-48, EMERGING CARBAPENEMASES

The global spread of Gram-negative bacteria (GNB) resistant to nearly all available antibiotics is currently the most pressing issue in bacterial resistance (94). One of the biggest concerns is the increasing resistance to β-lactams, especially carbapenems, the drugs of "last resort" to treat severe community and hospital acquired infections, often caused by *Enterobacteriaceae* (13). Three groups of carbapenemases—KPC, NDM, and OXA-48—are currently considered the most epidemiological and clinical significant among this family. The New Delhi Metallo-beta-lactamase (NDM-1) was first identified in 2008 (27). Since then, bla_{NDM} has emerged as a major global health challenge not only because of its ability to confer resistance to nearly all β-lactam antibiotics, but also due to its rapid spread worldwide. At present, 16 variants are reported in more than 40 countries and it is endemic in several areas (Southeast Asia, the Balkans and the Middle East) (193). On the other hand, the OXA-48 β-lactamase was initially identified in a *K. pneumoniae* isolate from a patient residing in Istanbul, Turkey in 2001 (45). Soon after, this carbapenemase

(the “phantom menace”) rapidly disseminated through Turkish hospitals, with outbreaks in the main cities of the country reaching an endemic level (53, 194). Since then, 11 variants have been designated, and have spread worldwide. In this chapter we present two studies that describe the dynamics of dissemination of *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48-}like}$ in two different geographic regions. These two studies exemplify the complex genetic pathways of resistance dissemination, and underscore the global nature of this growing clinical and public health threat.

Initial assessment of the molecular epidemiology of *bla*$_{\text{NDM-1}}$ in Colombia

At this time, *bla*$_{\text{NDM}}$ is recognized as a major global health threat. Guatemala and Colombia reported the first cases of *bla*$_{\text{NDM-1}}$ harboring isolates in Latin America (195, 196). In both instances, *bla*$_{\text{NDM-1}}$ was discovered in hospital-acquired, clonally-related *K.pneumoniae* (Kpn) isolates that were recovered from patients lacking a travel history. The molecular epidemiology of *bla*$_{\text{NDM}}$ carbapenemases in South America has been investigated only in a limited fashion, and data in Colombia is very scarce. In order to understand the dissemination of *bla*$_{\text{NDM-1}}$ in Colombia, we performed a genomic analysis of four sentinel isolates: *A. baumannii* (*Aba*), *A. nosocomialis* (*Ans*), *E. coli* (*Eco*) *and Kpn* collected in 2012, shortly after the first reported outbreak (196).

Methods

Four isolates were collected between June and October 2012 in hospitals from two different cities, located 300 and 700 km away from Bogota, where the first Colombian *bla*$_{NDM-1}$ was reported. Isolates were sent to the International Center for Medical Research and Training (CIDEIM) as part of a bacterial resistance surveillance program. Species identification was performed by Vitek-2® (bioMérieux, Marcy l'Étoile,France) and confirmed by MALDI-TOF MS on a Microflex LT instrument (Bruker Daltonics GmbH, Leipzig, Germany). Antimicrobial susceptibility testing was conducted using broth microdilution method (Sensititre panels; TREK Diagnostic systems, Westlake, OH, USA) and minimum inhibitory concentrations (MICs) were interpreted according to CLSI guidelines (197), except for tigecycline which was interpreted using the US Federal Drug Administration (FDA) criteria. Modified Hodge test (MHT), 3D bioassay using an imipenem disk, and double-disk synergy test using EDTA (DDST+EDTA), were performed as previously described (197-199).

DNA was isolated with the MasterPure Gram-positive DNA purification kit (Epicenter Biosciences) and subjected to SMRT sequencing on a PacBio RSII. Two SMRT cells of sequence data were collected per genome; sequencing resulted in ~9-10X coverage of error-corrected reads. Assembly was performed by using PacBio's HGAP assembler version 3.1. Plasmid content and *bla*$_{NDM}$ location was corroborated using S1 nuclease / I-CeuI -PFGE and *bla*$_{NDM-1}$ / 16S probe hybridizations (200, 201). Genes were

annotated automatically using NCBI Prokaryotic Genome Annotation Pipeline (202) and manually using BLAST. Resistance genes where identified using ResFinder 2.1 (203), *in silico* MLST and plasmid typing was performed by MLST 1.8 (204) and PlasmidFinder 1.3 (205), respectively

Vitek-2® initially identified isolates as *Alcaligenes faecalis* -6200, Kpn -6234, Eco -6409 and *Acinetobacter baumannii-calcoaceticus complex* -6411. However, MALDI-TOF MS and whole genome BLAST confirmed isolate 6200 as *Acinetobacter baumannii* (Aba) and isolate 6411 as *Acinetobacter nosocomialis (Ans).*

Results and discussion

Phenotypic characterization revealed that two of the isolates (*Eco* and *Ans*) were resistant to all antibiotics tested, including polymyxin B and tigecycline (Table 3-1). On the other hand, the *Aba* isolate was also multidrug resistant but susceptible to polymyxin B, tigecycline and ciprofloxacin. The *Kpn* isolate was susceptible to polymyxin B and exhibited relatively low susceptibility to carbapenems, highlighting once again the difficulty clinical microbiology laboratories have detecting carbapenemase genes that are expressed at low levels (206, 207). None of the isolates tested positive for carbapenemases using the Modified Hodge Test, but tested positive (with the exception of the *Eco* isolate) in the 3D bioassay using an imipenem disk (198). Double-disk synergy testing using EDTA (DDST+EDTA), confirmed the presence of a metallo-β-lactamase for the *Enterobacteriaceae*, but not in the *Acinetobacter* spp isolates.

In order to understand the genetic background of these early *bla*$_{\text{NDM-1}}$ containing strains, the complete chromosome and plasmid sequences were obtained by assembly of Pacific Biosciences SMRT sequence data, with the exception of *Kpn*, where the chromosome was assembled into three ordered contigs (Table 3-2). Genome sequencing results showed that all isolates possessed multiple plasmids (Table 3-2) and revealed that *bla*$_{\text{NDM-1}}$ was localized in one plasmid per strain, as confirmed by S1-PFGE (200). Multilocus Sequence Type (MLST) analysis revealed that *Acinetobacter* spp. isolates belonged to ST322 (*Aba*) and ST464 (*Ans*), none regarded as "high-risk" clones (208). Both *Aba* and *Ans* harbored three plasmids and carried the *bla*$_{\text{NDM-1}}$ on a Tn*125* backbone (Figure 3-1) located on a 47,274 bp plasmid that was 99% similar to plasmid pNDM-BJ01 (JQ001791.1) reported in an *Acinetobacter Iwoffi* isolate from China (33, 209). This plasmid also carried the aminoglycoside phosphotransferase *aph(3')VIIa* gene, and a type IV secretion system (T4SS) gene cluster encoding a P-type T4SS that has been reported to encode a short, rigid pilus characteristic of broad host range conjugative plasmids (210).

We next discovered that a chromosomally encoded class C β-lactamase *bla*$_{\text{ADC-80}}$ was found in both *Acinetobacte*r spp. isolates. This surprising finding is particularly interesting as it highlights the possibility that *bla*$_{\text{ADC}}$ evolved similarly in two different species of *Acinetobacter*. Consistent with the species identification, Aba also harbored the, *bla*$_{\text{OXA-94}}$ (a *bla*$_{\text{OXA-51-like}}$ derivative). None of the other plasmids contained additional resistance

genes, and interestingly resistance islands (AbaRI) were not found in the chromosome or on plasmids harbored by either of the *Acinetobacter* spp. isolates.

In contrast with *Acinetobacter* spp. isolates, both *Enterobacteriaceae* contained multiple resistance genes in two large plasmids (151-198Kb) (Table 3-2 and Figure 3-1), including *bla*$_{\text{CTXM-15}}$, consistent with the previously documented predominance of that ESBL in Colombia (211, 212). The *Kpn* isolate belonged to ST392, previously associated with the dissemination of *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48}}$ and other ESBLs (213, 214). In addition, this isolate also harbored a chromosomal *bla*$_{\text{SHV-11}}$ and *bla*$_{\text{CTX-M-15}}$, the latter located downstream of IS*Ecp1*, as previously reported in other *Enterobacteriaceae* isolates from Spain, Japan, Germany, Netherlands and the United Kingdom (215-218). The largest plasmid of *Kpn* (198Kb) was multireplicon Inc FII/IncFIB type plasmid, and carried antibiotic determinants conferring resistance to aminoglycosides (*aph(3')-Ib, aph(6')-Id, aac(3)-IIa, aac(6')-Ib-cr*), quinolones (*qnrB66*), sulfonamides (*sul2*), tetracycline (*tet(A)*), trimethoprim (*dfrA14*), chloramphenicol (*catB3*) and β-lactams (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{OXA-1}}$). In this case, the second copy of *bla*$_{\text{CTX-M-15}}$ was found to be part of a previously reported structure: a *Tn*3-like transposon also carrying *bla*$_{\text{TEM-1}}$, which has its *tnpA* gene disrupted by IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ (219). In this plasmid, this entire genomic structure is also followed by an IS*26* element, previously shown to have critical role

in the mobilization and reorganization of antibiotic resistance genes in Gram- negative bacteria (220, 221).

The *bla*$_{NDM-1}$-bearing plasmid (178 Kb) contained an IncA/C2 replicon, extensively associated with antibiotic resistance in Gram-negative bacteria (222). The plasmid backbone shares similarity with other plasmids carrying *bla*$_{NDM-1}$ and other β-lactamases in variety of Gram-negative species (Table 3-3). Additionally, this plasmid carried determinants conferring resistance to most antimicrobial classes, including β-lactams (*bla*$_{CARB-2}$), aminoglycosides (*aph(3')-VIa, aacA29, aadA2*), chloramphenicol (*catB3*, *cmlA1*), sulphonamides (*sul2, sul1*), macrolides (*mph(E)*), streptograminB (*strB*), and lincosamide (*msr(E)*).

The *E.coli* isolate belonged to ST10 (phylogroup A), which has been associated with ESBLs and hyper-expressed AmpC enzymes (208). *Eco* harbored most of the resistance determinants in plasmids; only the sulfonamide resistance gene, *sul2*, was present in the chromosome. The IncFIA/IncFIB 151Kb plasmid carried *bla*$_{TEM-1}$, *catA1, sul1, tetB*, and *dfrA7;* while the193Kb IncA/C2 plasmid harbored not only *bla*$_{NDM-1}$, but also *strA, strB, catA1, sul2, sul1, tetB,* and *dfrA1*. Noteworthy, there were 3 tandem repeats of *bla*$_{NDM-1}$ in the 193Kb plasmid, two of them within a Tn*125* structure and the last one lacking the right side copy of IS*Aba125* (Figure 3-2). We interpret this to be the consequence of the insertion of Tn*125* within a Tn*5393*-like structure, as evidenced by the presence of *tnpA* and *tnpR, strA* and *strB*, characteristic of this Tn*3* transposon,

originally described in *Erwinia amilovora* (223), but now found in several Gram-negative species in clinical, ecological and agricultural niches (224-227). This complex array of transposons is followed by aminoglycoside resistance gene (*aadA16)* flanked by transposable genetic elements indicating that this whole region could be serving as a 'hotspot' for the incorporation of genetic determinants either by homologous recombination via IS-elements, site-specific recombination or transposition. A similar 20Kb resistance region is found on environmental plasmid pRSB101 which was originally isolated from bacterial populations residing in the activated sludge compartment of a wastewater treatment plant (228). Furthermore, one of those transposable elements was identified as IS*26*, and found not only flanking the above-mentioned region, but also next to the first copy of $bla_{\text{NDM-1}}$ containing Tn*125*. This would reinforce the hypothesis of a "hotspot region", given the replicative transposition mechanism of IS*26*, and its previously shown critical role in the mobilization and reorganization of antibiotic resistance genes in Gram-negative bacteria (220, 221). Most importantly, although bla_{NDM} in tandem repeats has been observed before, this is to our knowledge the first report of such a structure in *E.coli*. In both previously reported cases it occurred in *Kpn* isolates: the first from a Taiwanese patient with a hospitalization history in New Delhi (250Kb IncFIB/FII plasmid) (229) and the second from an outbreak in a neonatal unit in Nepal (304Kb IncHIB/FIB plasmid) (230).

All strains were nosocomially acquired and isolated from elderly patients with severe systemic infections, three patients, who presented several co-morbidities, died (Table 3-4). Since *i*) evidence of international travel, or travel to Bogota (where the first Colombian *bla*$_{NDM-1}$ was reported) could not be established for any of the patients or their families, and *ii*) given that they originally lived in rural areas or small cities, this emergence in a variety of species in two different geographic locations, is extremely worrisome. Colombia has often been among the first countries in the region to report the circulation of important resistance determinants, including CTX-M-15, KPC and NMC-A, all of which have become widely disseminated, even reaching endemic status, as it the case for KPC (163, 231-233). Even though, information regarding molecular epidemiology of *bla*$_{NDM-1}$ in Colombia is still very limited, the National Institute of Health of Colombia is reporting increased number of patients infected with NDM producing bacteria. Interestingly Kpn and *Providencia rettger*i are the most prevalent *bla*$_{NDM-1}$-expressing Gram-negatives (234, 235). We hypothesize that the rapid spread of this resistance gene (*bla*$_{NDM-1}$) is aided by the circulation of broad-host, transferable plasmids such as IncA/C found in this study.

The widespread dissemination of *bla*$_{NDM}$ in Colombia portends a significant antibiotic resistance problem in Latin America (196). Colombia's situation may be only the "tip of the iceberg", therefore studies assessing the real prevalence of *bla*$_{NDM}$, especially in countries where few reports are

available, are warranted. It is of great importance that the findings of surveillance and genomic studies like the present one, help inform new and more effective infection control and stewardship programs that can be translated into appropriate national policies to prevent an endemic situation.

NDM-5 and OXA-181 β-lactamases: a significant threat continue to spread in the Americas

Carbapenem-resistant *Klebsiella pneumoniae* (CR *Kp*) infections are among the most problematic clinical challenges worldwide, since very few antimicrobials retain activity against them and they are associated with high morbidity and mortality (96, 163). The New Delhi Metallo-β-lactamase (NDM-1) was first identified in 2008 (27). Since then, bla_{NDM} has emerged as a major global health challenge not only because of its ability to confer resistance to nearly all β-lactam antibiotics, but also due to its rapid spread worldwide. At present, 16 variants are reported in more than 40 countries and it is endemic in several areas (Southeast Asia, the Balkans and the Middle East) (193). The NDM-5 variant differs from NDM-1 at amino acid positions Val88Leu and Met154Leu and this metallo-β-lactamase was first identified in a multidrug-resistant *Escherichia coli* ST 648 isolate from a patient in the United Kingdom who had a recent history of hospitalization in India (236). Since then it has been reported in Algeria Spain, Japan, Australia, China and Egypt (237-241).

The OXA-48 β-lactamase was initially identified in a *K. pneumoniae* isolate from a patient residing in Istanbul, Turkey in 2001 (45). Soon after, this carbapenemase (the "phantom menace") rapidly disseminated through Turkish hospitals, with outbreaks in the main cities of the country reaching an endemic level (53, 194). Since then, 11 variants have been designated, and have spread to the Middle East, North Africa and Europe. In addition, several nosocomial outbreaks were reported in Mediterranean countries including France, Spain, Lebanon, Israel, Tunisia, Morocco and Malta, and have also reached an endemic situation (242). OXA-181, a variant of OXA-48 differs by four amino acid substitutions (Thr104Ala, Asn110Asp, Glu175Gln, and Ser179Ala), was initially identified in *Enterobacter cloacae* and *K. pneumoniae* isolates that were recovered in 2007 in India (26). Since then, OXA-181-producing *Enterobacteriaceae* were reported in several other countries on the Indian subcontinent including Bangladesh, Sri Lanka and Nepal (243, 244), and soon after in Canada, France, the Netherlands, New Zealand (in a *K. pneumoniae* from a patient from Nepal), Norway (in a patient from Romania), Oman, Romania, Singapore, South Africa, and the United Kingdom (in a patient from India) (39).

Herein, we report a *K. pneumoniae* isolate harboring $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$ and a paired isolate from the same patient, exhibiting a highly plastic genomic region associated with the IS26 insertion element involved in the loss of $bla_{\text{NDM-5}}$ and several other resistant determinants. This isolates were

detected as part of a surveillance network dedicated to characterizing the *Klebsiella penumoniae* carbapenemase (KPC) epidemic in the US and elsewhere.

A young man underwent sleeve gastrectomy for management of obesity in India in 2014. The surgery was complicated by a post-operative anastomotic leak and the patient was transferred to a US hospital a month after surgery. An abdominal "wash-out" was required, and the patient was placed on bowel rest and started on total parenteral nutrition. However, he developed an enterocutaneous fistula, and was transferred to one of the hospitals participating in CRACKLE (The Consortium on Resistance against Carbapenems in *Klebsiella pneumoniae*) -a prospective, multi-center, observational study in the Great Lakes Region of the US (96) for a Roux-en-Y gastric bypass procedure and resection of the fistula. This surgery was performed 4 months after his initial operation. Cultures sent from abdominal fluid revealed a carbapenem-resistant *K. pneumoniae* (CR *Kp*-1) (Table 3-5). He was treated for a total of 16 days with tigecycline. His symptoms resolved and he was discharged in stable condition off antibiotics, after a 28-day admission.

Check-MDR CT103 XL Assay (Check-Points, Wageningen, Netherlands) was used to detect β-lactamase genes including extended spectrum β-lactamases (*bla*$_{SHV}$ and *bla*$_{TEM}$ ESBLs), plasmid mediated AmpCs, and carbapenemases including *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA-48-like}$, *bla*$_{GES}$, *bla*$_{GIM}$, *bla*$_{SPM}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-24/40-like}$, *bla*$_{OXA-48-like}$. CR *Kp*-1

contained *bla*$_{NDM}$, *bla*$_{OXA-48-like}$, *bla*$_{CTX-M-15-like}$, *bla*$_{SHV-WT}$, and *bla*$_{TEM-WT}$. CR *Kp*2 contained *bla*$_{OXA-48-like}$, *bla*$_{CTX-M-15-like}$ and *bla*$_{SHV-WT}$. PCR amplification and sequencing performed to identify *bla*$_{NDM}$ and *bla*$_{OXA-48-like}$ variants, confirmed that CR*Kp*-1 carried *bla*$_{NDM-5}$ and *bla*$_{OXA-181}$, while CR *Kp*-2 carried only *bla*$_{OXA-181}$.

Conjugation experiments were performed using both *K. pneumoniae* clinical strains as donors and the azide-resistant *Escherichia coli* J53 as a recipient, however transconjugants were not obtained. Therefore, plasmid DNA was extracted (245) and electroporated into *E.coli* DH10B. Transformants were selected on ampicillin containing lysogeny broth (LB) agar and the presence of *bla*$_{NDM-5}$ and *bla*$_{OXA-181}$ was confirmed by PCR amplification. Plasmid typing on CR *Kp*-1 and CR *Kp*-2 isolates, as well as on transformants was performed by PCR-based replicon typing using the PBRT KIT (DIATHEVA, Fano PU, Italy) following the manufacturer's instructions. Only an IncFII amplicon was obtained for both strains and transformants. S1-PFGE followed by southern hybridization with *bla*$_{NDM-5,}$ *bla*$_{OXA-181}$, IncFII replicon, for both clinical strains and transformants (Figure 3-3); and *I-Ceu-I* PFGE followed by southern hybridization with *bla*$_{OXA-181}$ and 16S probes for clinical strains (Figure 3-4) suggested that *bla*$_{NDM-5}$ was located on an IncFII plasmid of ≈90Kb, while *bla*$_{OXA-181}$ was chromosomally encoded (200, 201).

Draft whole genome sequences, were obtained from Illumina pair-end reads. ResFinder (203) using assemblies as input confirmed that CR *Kp*-2

possesses a smaller resistome as compared to CR *Kp*-1 (Table 3-6). Likewise, BLAST revealed that for CR *Kp*-1, *bla*$_{NDM-5}$ was localized on an IncFII plasmid of approximately 90 Kb (99% similarity with pCC1409, a *bla*$_{NDM-5}$-harboring IncFII plasmid from a *K. pneumoniae* ST 147 isolated in Poland; KT725789.1) (Figure 3-5 A). Interestingly, this same plasmid was also present in CR *Kp*-2, however it is slightly smaller due to the loss of a ≈ 25Kb region including not only *bla*$_{NDM-5}$, but also several other resistance determinants (e.g. *bla*$_{TEM-1}$, *mphA, erm*B, *dfrA12, aadA2, rmt*B) (Figure 3-5A). ISFinder (246) showed the presence of multiple transposable elements, including IS26, distributed along the above-mentioned region (Figure 3-5B). Of note, the finding of rmtB is manifested by the difference in susceptibility to aminoglycosides (Table 3-5 : 256 vs 2 mg/L). In addition, the presence of numerous resistant determinants may indicate that this is a "hotspot region". Given the replicative transposition mechanism of IS26 and its previously shown critical role in the mobilization and reorganization of antibiotic resistance genes in Gram-negative bacteria, we hypothesize that the excision of this region might have been aided by IS26 (220, 221).

ISEcp1 was identified upstream of *bla*$_{OXA-181}$, as previously described (39); *I-Ceu-I* PFGE and probe hybridization suggest that this carbapenemase is chromosomally located in both clinical strains. Interestingly, transformation experiments revealed that on the CR *Kp*-2 *E.coli* transformant, *bla*$_{OXA-181}$ was also carried by a plasmid, which based on

size and IncFII probe hybridization is the same plasmid that lost the above mentioned ≈ 25Kb *bla*$_{NDM-5}$ harboring region. This might indicate a transposition event aided by the flanking ISEcp1 accompanying this gene, highlighting the plasticity of this plasmid to incorporate or lose genes (Figure 3-3 and Figure 3-4).

In the US, *bla*$_{NDM}$ has been circulating since 2010 (247) whereas *bla*$_{OXA-48}$ was imported in 2012 by patients who were initially hospitalized in Saudi Arabia and India (248). The rapid and widespread dissemination of *bla*$_{OXA-181}$ throughout Southeast Asia, the recent finding of *bla*$_{OXA-181}$ (associated with the ISEcp1 mobile genetic element) in the chromosome of a wastewater S*hewanella xiamenensis* isolate (50), and the recognition that most cases outside this area of the world are from patients that had a recent travel history to Southeast Asia and Asian Pacific region, suggest this as the likely place of origin for *bla*$_{OXA-181}$ and an important reservoir of this carbapenemase gene. Multi-locus sequence typing (MLST) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) revealed that both CR *Kp*-1 and CR *Kp*-2 belong to ST147. A *K. pneumoniae* isolate belonging to the same ST type (ST147) and carrying an IncFII plasmid with *bla*$_{OXA-181}$ and *bla*$_{NDM-5}$ was previously reported in South Korea, from a patient transferred from a tertiary care hospital in Abu Dhabi, United Arab Emirates (UAE) (249). However, the very first report of a *K. pneumoniae* isolate harboring *bla*$_{OXA-181}$ and *bla*$_{NDM-5}$ occurred in Singapore in 2013, and was isolated from the urine of a patient that

had been transferred from Bangladesh (243). Another isolate harboring both carbapenemase genes has also been described in Egypt, however it is an *E.coli* carrying each gene on a different plasmid (250). In the US, A *K. pneumoniae* isolate carrying $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-232}}$ was reported in another patient transferred from India (251). However, $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$ have only been reported in the same patient, but in different *E. coli* isolates (252).

In summary we describe the occurrence of two clinically important carbapenemases (the "evil twins"), $bla_{\text{OXA-181}}$ (chromosomally encoded) and $bla_{\text{NDM-5}}$ (plasmid encoded) in a CR *Klebsiella pneumoniae* clinical isolate in the US. Our results also underscore the ability of ceftazidime/avibactam (CAZ/AVI) to indicate the presence of metallo-β-lactamases when carbapenem resistant isolates show resistance to this combination. These findings strongly underscore the importance of molecular surveillance programs like CRACKLE that characterize resistant strains, and highlights the emergence of novel genotypes in the United States.

Conclusion

These two studies underscore the complex genetic pathways of bla_{NDM} dissemination, where the rapid and widespread propagation has been aided by medical repatriation of hospitalized patients or patients returning to their home countries after a period of foreign travel. At the genomic level, bla_{NDM} dissemination is driven by transposition of the bla_{NDM} containing transposable element into different plasmids, usually of broad host range,

as well as further transmission as a result of plasmid conjugation and clonal spread. This genetic plasticity often results in strains co-harboring multiple plasmids with different resistance determinants, rendering them multidrug resistant and sometimes even untreatable. Even though we describe in detail the dynamics of dissemination in two different geographic regions, it is increasingly evident that globalization plays a major role in the rapid dissemination of antibiotic resistance. This highlights that the clinical and public health threat posed by antibiotic resistance has an international dimension and as such, surveillance programs to monitor and control the problem need to be international in scope.

Table 3-1. Antimicrobial susceptibility (MICs in mg/L) of NDM-producing clinical isolates

		MIC														
Isolate	**Species**	CSL	PTZ	FEP	FOT	TAZ	AXO	DOR	ERT	IMI	MER	AZT	AMK	CIP	POLB	TGY
6200	*Aba*	>128/64	>128/4	>32	ND	>16	ND	>64	ND	64	>64	ND	16	≤0,5	1	≤0.5
6234	*Kpn*	64/32	>128/4	>32	>16	>16	>16	1	4	2	1	32	64	>8	0,5	≤0.5
6409	*Eco*	>128/64	>128/4	>32	>16	>16	>32	16	>64	16	16	32	>64	>8	8	>8
6411	*Ans*	>128/64	>128/4	>32	ND	>16	ND	>64	ND	>64	>64	32	16	8	4	4

CSL: cefoperazone-sulbactam; PTZ: piperacillin/Tazobactam; FEP: cefepime; FOT: cefotaxime; TAZ: ceftazidime; AXO:ceftriaxione; DORI: doripenem; ERT:ertapenem; IMI: imipenem; MERO: meropenem; AZT:aztreonam; AMK:amikacin; CIP: ciprofloxacin; POLB:polymyxin B, TGY: tigecycline. ND: not determined

Table 3-2. Accession number and resistome of *bla*$_{NDM-1}$-harboring isolates

Species		Chromosome	Plasmids		
A. baumannii	Accession No. Size	NZ_CP010397.1 3,902,527 bp	CP010398.1 114,848 bp	CP010399.1 47,274 bp	CP010400.1 9,327 bp
	Resistance determinants	*bla*$_{ADC-80}$ *bla*$_{OXA-94}$	none	*aph(3')VIIa* *bla*$_{NDM-1}$	none
A. nosocomialis	Accession No. Size	CP010368.1 3,858,956 bp	NZ_CP010369.1 89,111 bp	CP010903.1 66,409 bp	CP010370.2 47,274 bp
	Resistance determinants	*bla*$_{ADC-80}$	None	None	*aph(3')VIIa*, *bla*$_{NDM-1}$
K.pneumoniae	Accession No. Size	NZ_JWRK01000001.1 5,329,244**	CP010390.1 198,371 bp	CP010391.1 178,193 bp	
	Resistance determinants	*bla*$_{CTXM-15}$ *bla*$_{SHV-11}$ *oqxA, oqxB* *fosA*	*strA, strB, aac(3')IIa, aac(6')lb-a, qnrB66, sul2, tetA, dfrA14, catB3, bla*$_{TEM-1}$, *bla*$_{CTXM-15}$, *bla*$_{OXA-1}$	*aph3'VIa, aacA29, aadA2, mph(E), msr(E), catB3, cmlA1, sul2, sul1, bla*$_{NDM-1}$, *bla*$_{CARB-2}$	
E.coli	Accession No. Size	NZ_CP010371.1 4,761,012 bp	NZ_CP010373.2 193,908 bp	NZ_CP010372.1 151,583 bp	
	Resistance determinants	*sul1*	*strA, strB, catA1, sul2, sul1, tetB, dfrA1, aadA16, bla*$_{NDM-1}$ (3X)	*catA1, sul1, tetB, dfrA7, bla*$_{TEM-1}$	

Table 3-3. Representative IncA/C plasmids giving significant similarity to plasmid NZ_CP010373.2

Species	% Similarity	Comment
Salmonella enterica Serovar Corvallis	83	IncA/C Plasmid Carrying *bla*$_{NDM-1}$, *bla*$_{CMY-16}$, and *fosA3* in a *Salmonella enterica* Serovar Corvallis Strain Isolated from a Migratory Wild Bird in Germany (KR091911)
E.coli	83	This study
E.coli	80	*bla*$_{KPC}$-harboring IncFIA plasmid pBK32533, from *E. coli*
Enterobacter spp.	80	*bla*$_{KPC}$, *bla*$_{TEM}$ and Metallo-β-lactamase
Citrobacter freundii	79	*bla*$_{VIM-4}$, *bla*$_{CTX-M}$
Providencia stuartii	77	Novel 178-Kb Plasmid Carrying *bla*$_{NDM-1}$ in a *Providencia stuartii* Strain Isolated in Afghanistan

Table 3-4. Clinical features of patients infected with *bla*$_{\text{NDM-1}}$ isolates

Isolate	Spp.	City of collec-tion	Patient Origin	Age (yr)	Sex	Infec-tion type [a]	Comor-bidity[b]	LOS (days)	Days prior to positive culture	Source	Invasive inter-ventions [c]	Previous hospital-llzations [d]	Clinical Out-come	Travel histor y [e]
6200	*Aba*	Neiva	Caquetá	60	F	IAB-PNEU 1-LCBI	DM, RA	48	34	Blood	SURG	Yes	Dead	NO
6234	*Kpn*	Neiva	Huila	53	M	ND	None	9	6	Blood	None	Yes	Alive	NO
6409	*Eco*	Pasto	Putumayo	74	F	PNEU 1	DM	28	15	Urine	SURG, FC	Yes	Dead	NO
6411	*Ans*	Pasto	Putumayo	76	M	Sepsis	None	13	7	Urine	SURG, FC, CL, INT	Yes	Dead	NO

[a] IAB: intra-abdominal infection, not specified elsewhere.; PNEU-1: Clinically-defined pneumonia; LCBI: Laboratory-confirmed bloodstream infection

[b] DM: diabetes mellitus; RA : Rheumatoid arthritis

[c] SURG: Surgery ; CL : Central line ; INT: Intubation; FC: Foley catheter

d Healthcare exposures in past year

e Travels in the past 12 months/ Close personal contact with people (e.g. relatives, co-workers) who have traveled in the past 12 months

Table 3-5. Minimum inhibitory concentrations (mg/L) for CR Kp-1 and CR Kp-2 as evaluated using E-test ®.

Antibiotic	CR *Kp*-1	CR *Kp*-2
Tigecycline	0.5	0.5
Amikacin	>256	2
Gentamicin	>256	2
Ampicillin/sulbactam	>256	>256
Piperacilin/Tazobactam	256	>256
Ceftazidime/Avibactam	>256	0.5
Ceftazidime	>256	48
Cefepime	>256	96
Ceftriaxone	>32	>32
Cefotaxime	>32	>32
Ertapenem	>32	>32
Imipenem	>32	4
Meropenem	>32	24
Doripenem	>32	8
Polymyxin B*	≤0.5	1

*Polymyxin B susceptibility was evaluated by broth microdilution

Table 3-6. Resistome of CR Kp-1 and CR Kp-2

	Genome size (bp)	No. of contigs	Resistance determinants
CR *Kp*-1	5608317	87	*oqxA, oqxB,* $bla_{\text{SHV-11}}$*, fosA, rmtF, aacA4, aac(6')Ib-cr, qnrB12, dfrA14,* $bla_{\text{OXA-181}}$*, ARR-2,* $bla_{\text{CTX-M-15}}$*, mph(A), sul1, erm(B), dfrA12, aadA2, rmtB,* $bla_{\text{TEM-1}}$*,* $bla_{\text{NDM-5}}$
CR *Kp*-2	5587222	79	*oqxA, oqxB,* $bla_{\text{SHV-11}}$*, fosA, rmtF, aacA4, aac(6')Ib-cr, qnrB12, dfrA14,* $bla_{\text{OXA-181}}$*, ARR-2,* $bla_{\text{CTX-M-15}}$

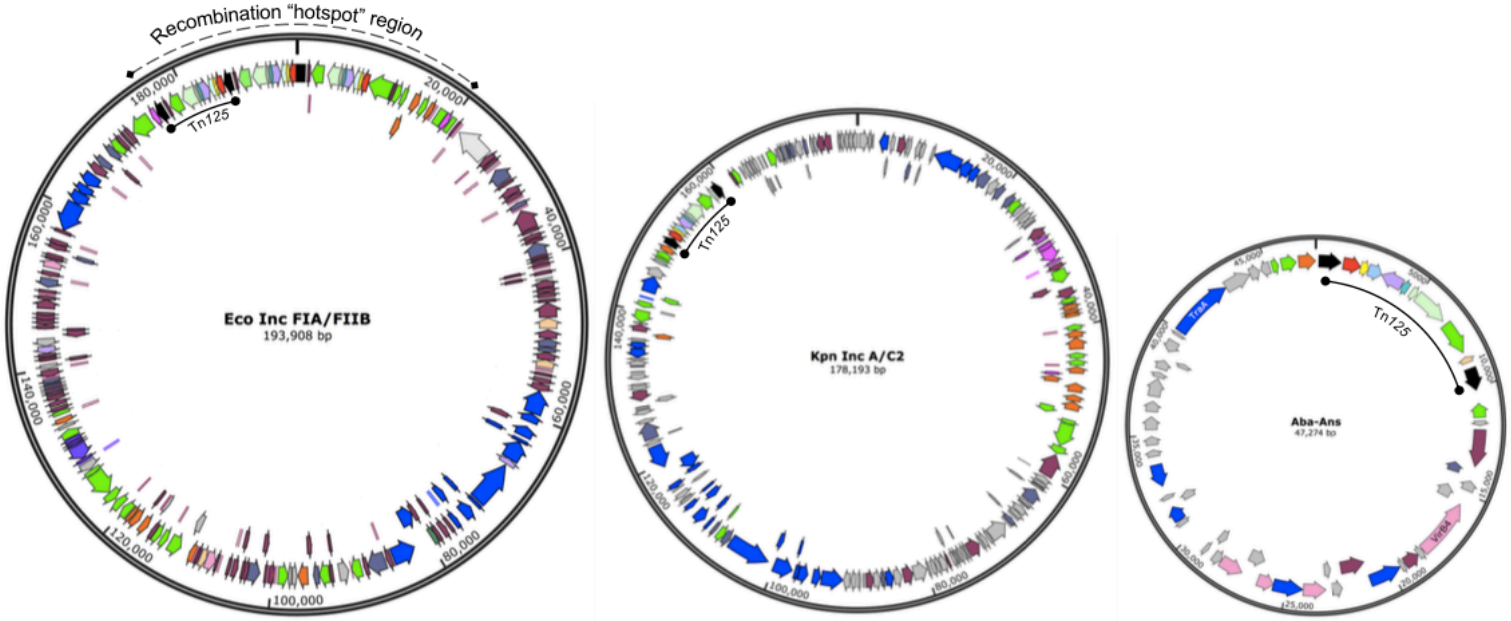


Figure 3-1. *bla*$_{\text{NDM-1}}$ carrying plasmids

Selected genes are highlighted. Green, mobile genetic element-related genes; blue, genes involved in plasmid mobilization; pink, type IV secretion system; black, *IS*Aba125; yellow, *bla*$_{\text{NDM-1}}$; red: *ble*$_{\text{MBL}}$

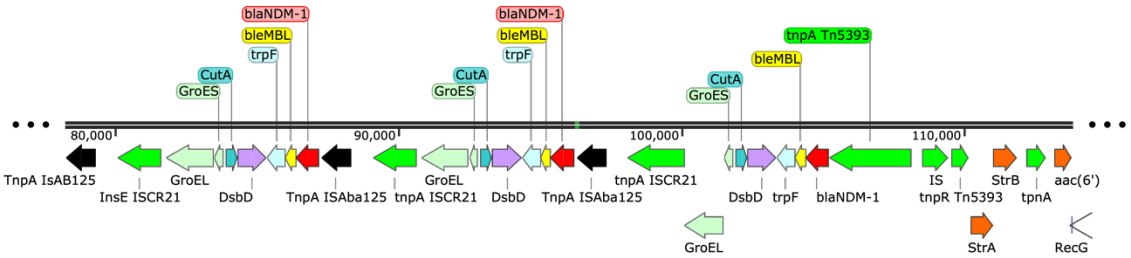


Figure 3-2. Organization of the *bla*$_{\text{NDM-1}}$-containing element in *E. coli*.

The first two copies of *bla*$_{\text{NDM-1}}$ are contained within the Tn*125* transposon (flanked by IS*Aba125* [shown in black]). The third copy lacks the right-flanking IS*Aba125* and is followed instead by a Tn*5393*-like element. Multiple transposable elements are found within this region, indicating that it may serve as a "hot spot" for the incorporation of new resistance determinants through homologous recombination via IS elements, site-specific recombination, or transposition.

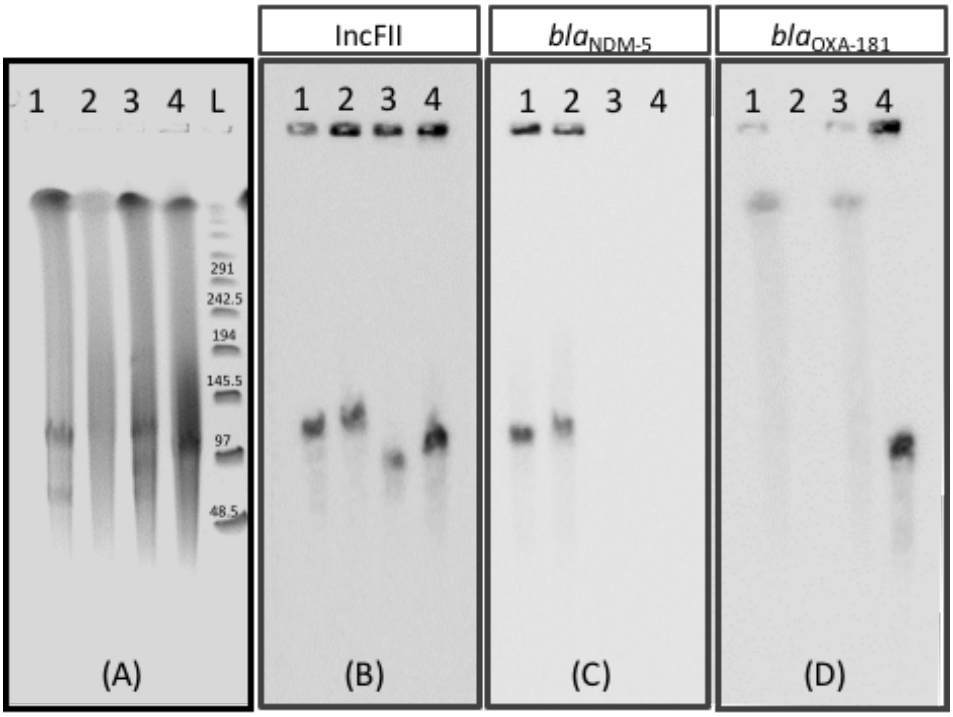


Figure 3-3. IncFII Plasmid location of bla_{NDM-5} as confirmed by S1 pulsed-field gel electrophoresis (PFGE)

Clinical strains (CR *Kp*-1- lane 1, CR *Kp*-2, lane 3) as well as transformants (lanes 2 and 4, respectively) were embedded in agarose and lysed. Resulting DNA containing plugs were incubated with S1 nuclease to convert supercoiled plasmids into full-length linear molecules and subsequently separated by PFGE (A). Finally, a Southern hybridization was performed using Inc FII replicon probe (B), bla_{NDM-5} probe (C), $bla_{OXA-181}$ probe (D).

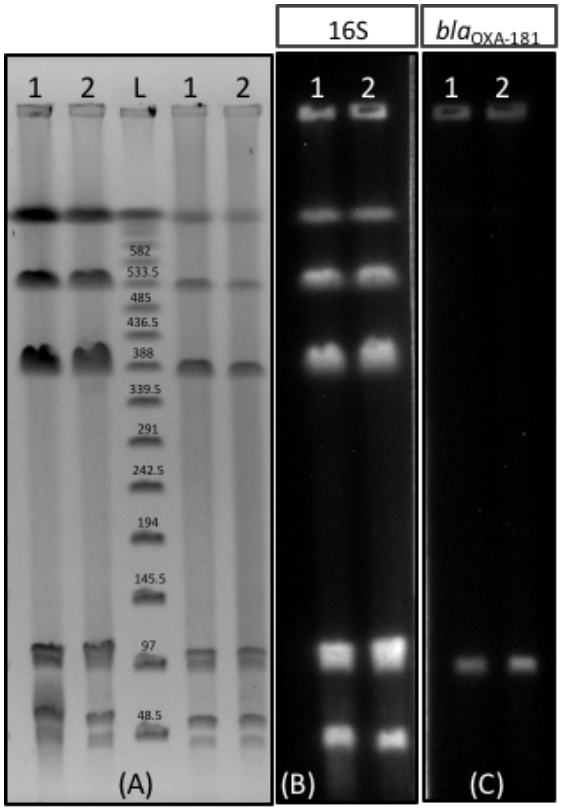


Figure 3-4. Chromosomal location of *bla*$_{\text{OXA-181}}$ as confirmed by I-Ceu-I pulsed-field gel electrophoresis (PFGE)

Clinical strains (CR Kp-1- lane 1, CR *Kp*-2, lane 2) were embedded in agarose and lysed. Resulting DNA containing plugs were incubated with *I-Ceu-I* endonuclease which cuts at a 26-bp site in the *rrl* gene of the *rrn* operons. Fragments were separated by PFGE (A) and Southern hybridization was performed using 16S rRNA probe (B) and *bla*$_{\text{OXA-181}}$ probe (C).

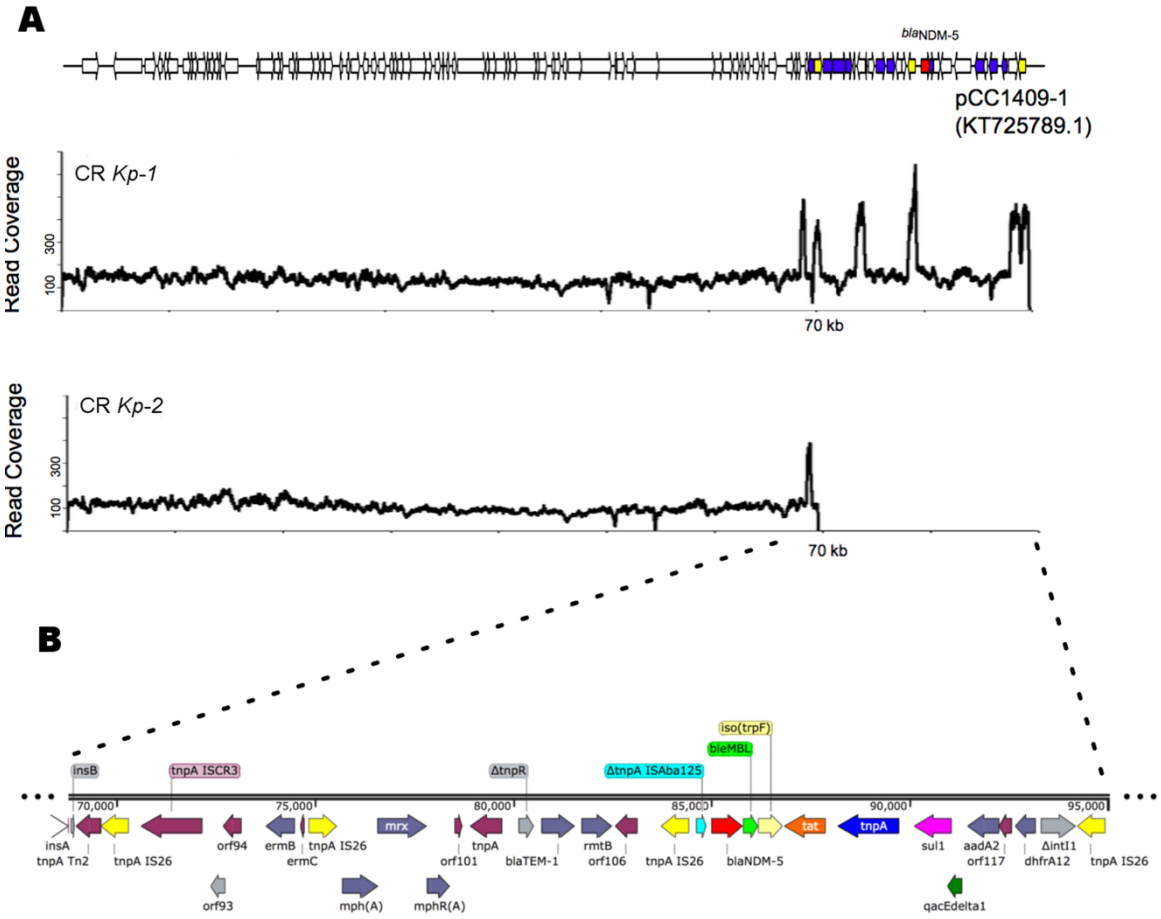


Figure 3-5. Coverage of Illumina reads from *K. pneumoniae* isolates CR Kp-1 and CR Kp-2 mapped using bowtie2 across the reference Inc FII plasmid pCC1409 (KT725789.1) (A); Organization of the 25 Kb bla_{NDM-5} containing region of the IncFII plasmid present only in CR *Kp*-1. This entire region has excised out of the IncFII plasmid present in CR *Kp*-2 (B).

CHAPTER 4 - AN ANALYSIS OF THE EPIDEMIC OF KPC-PRODUCING *Klebsiella pneumoniae*: CONVERGENCE OF TWO EVOLUTIONARY MECHANISMS CREATES THE "PERFECT STORM"

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Introduction

The spread of Gram-negative bacteria resistant to carbapenems is an urgent public health threat and a critical priority according to the World Health Organization (WHO) (253). The speed of dissemination of these pathogens, and the lack of new antibiotics active against them, threaten the medical care of hospitalized patients, especially those who are critically ill (24, 254). Several classes of carbapenemases have emerged in members of the *Enterobacteriaceae* family, including metallo-β-lactamases (e.g. NDM; New Delhi β-lactamase), class D carbapenemases (e.g. OXA-48), and class A carbapenemase *Klebsiella pneumoniae* carbapenemase (KPC) (255). KPC was identified for the first time in 1996 in a *Klebsiella pneumoniae* (Kpn) isolate from North Carolina, USA (19). Since then, 24 variants have been described and KPC-producing Gram-negative organisms with endemic levels described in several countries, including the United States, Greece, Italy, Israel and Colombia (21, 163).

KPC is commonly associated with transposable elements (e.g. Tn*4401a-g*) and plasmids (21, 256). The rapid dissemination of *K. pneumoniae* carrying *bla*$_{KPC}$ has been primarily associated with the spread of a single clonal group (CG) designated CG258, a large cluster containing 43 different sequence types (STs), with ST258 and ST512 being the two predominant STs. For instance, KPC-Kpn ST258 causes 90% of all infections in Israel and is responsible for >80% of outbreaks in the United States (18, 21, 257, 258). Colombia was the first country in South America where KPC was reported in 2005; the *bla*$_{KPC}$ gene was initially identified in *K. pneumoniae* isolates (232). Since then, it has been discovered in other species of *Enterobacteriaceae* and other Gram-negative bacteria (including *Pseudomonas aeruginosa*), becoming endemic in Colombia (163). Preliminary molecular studies suggested that clonal spread of CG258 is not the primary mechanism of dissemination of *bla*$_{KPC}$ in Colombia, as in other parts of the world. (259).

We postulated that the mechanisms of dissemination of *bla*$_{KPC,}$ are more complex within Colombia. In order to gain insights into the evolutionary events related to the epidemic occurrence of KPC-Kpn in Colombia, we performed a detailed genomic analyses of 133 KPC-Kpn isolates collected before and after the identification of the first KPC-Kpn in 2005 (232). Isolates included were recovered from 2002 to 2014, in 24 hospitals from 10 of the most densely populated Colombian cities (Figure 1-1).

Methods

Strain selection

Strains were collected between 2002 and 2014 from Colombian hospitals belonging to a bacterial resistance surveillance network coordinated by CIDEIM (Centro Internacional de Entrenamiento e Investigaciones Medicas, Cali-Colombia). The network is composed of tertiary-care hospitals located in ten cities throughout the country (Figure 4-1). Once isolates were collected at each hospital, they were sent to CIDEIM and kept in a repository. From this collection, a set of isolates were selected based on *i*) year of isolation, focusing before and after the identification of the first KPC-carrying *Klebsiella pneumoniae* isolate in Colombia (2005), in order to achieve the best temporary spread, *ii*) geographical diversity encompassing a wide area of Colombia (Figure 4-1), *iii*) isolates recovered from a fluid that likely represented an infection in an attempt to avoid colonizing isolates, and *iv*) representative isolates from previously characterized outbreaks (260)) (Supplementary Table 1). All isolates were re-identified using a MALDI Biotyper (RAB Lab, Bruker Daltonics, Bremen, Germany) and prepared for sequencing.

DNA preparation, library construction, sequencing, assembly and annotation

DNA was isolated with the MasterPure Gram-positive DNA purification kit (Epicenter Biosciences). Illumina sequencing libraries were prepared using the TruSeq kit with Illumina indexed-encoded adapters. Libraries were

pooled for whole genome sequencing (WGS) on Illumina MiSeq, NextSeq or HiSeq 2500, and paired-end sequence reads were obtained representing at least 100-fold genome coverage. Using CLC Bio Workbench (CLC Bio Qiagen), reads were trimmed for quality (score limit = 0.03, maximum 2 ambiguous nucleotides, 45 minimum nucleotides in reads) after removing adapters. Trimmed reads were *de-novo* assembled with automatic bubble and word size, 1000 bp as the minimum contig size, auto-detecting paired distances and mapping reads back to contigs. Genes were annotated in each genome assembly using the RAST server (http://rast.nmpdr.org). The sequence data for the isolates included in this study have been submitted to Genbank under Bioproject number PRJNA378654.

In silico MLST analysis, identification of antimicrobial resistance determinants, virulence factors plasmid typing and capsular typing. We determined the sequence types of all isolates *in silico* using the MLST 1.8 server (https://cge.cbs.dtu.dk/services/MLST/). Using assemblies as input, resistance and plasmid replicon genes were identified using Resfinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and Plasmidfinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/), respectively, using a coverage cutoff of 90%. Virulence genes and capsular typing (*wzi* sequencing) were identified using the Institut Pasteur's Klebsiella BigsDB site (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The presence of

Tn*4401* was confirmed via BLASTn, and its isoform was determined as described previously (261)

Phylogenetic Analyses.

To assess phylogenetic relationship among the 133 Colombian isolates a core genome was generated for each isolate by excluding all regions annotated by RAST as horizontally transferred elements in the reference strain *K. pneumoniae* 30660/NJST258_1 (Accession NZ_CP006923) (Supplementary table 3), in addition to the recombination region as previously defined (21). Additionally, 34 other publicly available genome sequences from NCBI were included (Supplementary table 2), as geographical references. The nucleotide sequences of each one of the orthogroups defined in the core genome were aligned and concatenated to obtain a phylogenetic matrix. The matrix was used to reconstruct the phylogeny of these strains with RAxML (262), using a General Time Reversible (GTR) evolution model and a GAMMA model of rate heterogeneity selecting the best from 20 different runs and 1,000 bootstrap resampling. For the CG258 phylogenetic reconstruction, 33 isolates sequenced in this study and 298 publicly available genome sequences from NCBI were included (Supplementary table 1). SNPs matrices were generated using pairwise whole genome alignment to the NZ_CP006923 reference with Mummer (263), after masking the recombination region as defined by Chen et al. (21). Maximum Likelihood reconstructions were generated using RAxML with a GTR evolution model, a GAMMA model of

rate heterogeneity, Lewis's ascertainment bias and a 100 bootstrap re-sampling. The trees were edited and plotted using iTOL v3.2.4 (264)

Results

In the period before the initial detection and emergence of *bla*$_{KPC}$ in Colombia ("pre-KPC" period, 2002-2005), a variety of *K. pneumoniae* strains of different sequence types (STs) (n=41; including 9 newly described STs) were circulating in Colombia. Most isolates carried genes conferring resistance to several antibiotics including aminoglycosides, quinolones, fosfomycin trimetroprim, tetracyclines, sulfonamides and β-lactams (mostly genes encoding SHV-*like* and CTX-M extended spectrum β-lactamases [ESBLs]). Additionally, isolates were found to carry a variety of plasmid types (Figure 4-2). In 2005, the first KPC-Kpn isolates were collected from two different hospitals in Medellin, the second largest city in Colombia (Figure 4-1) (232). These isolates belonged to ST338 and ST337 (Figure 4-4, indicated in red), not related to CG258. In addition to carrying different resistance determinants and plasmids, typing of *wzi* (the locus of capsular polysaccharide synthesis) demonstrated that these isolates had different capsular types (*wzi* 442 and 108, respectively) (Figure 4-2 and Figure 4-3). Both isolates carried *bla*$_{KPC-2}$ within a Tn*3*-like Tn*4401*b structure located on an IncFIB(K) plasmid (258). Phylogenetic analyses demonstrated that these two isolates were distantly related to the original North Carolina isolate from 1996 (ST37) (Figure 4-4). Of note, none of the *bla*$_{KPC}$-harboring isolates to that date belonged to CG258; however,

there was one isolate, also from 2005, that belonged to ST258 but did not carry *bla*$_{KPC}$, an occurrence previously reported in Israel (265) (Figure 4-2). Between 2005 and 2007, *bla*$_{KPC-2}$ was identified in several cities among *K. pneumoniae* isolates with heterogeneous genetic backgrounds, as evidenced by 8 different STs: 338, 339, 353, 337, 789, 101 and 14 (ST14 being the most frequent ST, representing 30% of the isolates). These *bla*$_{KPC-2}$-carrying isolates contained a variety of capsular types, virulence-associated genes, and plasmid replicons. However, only Tn*4401*b was identified in all of them (Figure 4-2 and Figure 4-3).

The first reported *bla*$_{KPC-3}$ producing Kpn isolate belonging to CG258 in Colombia was isolated in 2008; this discovery was initially described as an apparent introduction from Israel in an outbreak setting in a hospital in Medellin (260). Despite this event being considered the principal introduction of CG258 and *bla*$_{KPC-3}$ in Colombia, our results indicate that KPC-3 producing *K. pneumoniae* belonging to this genetic lineage were already circulating undetected in the country. In fact, we identified an isolate carrying *bla*$_{KPC-3}$ recovered in 2007 (a year before the apparent introduction of the Israeli clone) (Figure 4-3 and Figure 4-4, indicated in blue) from a patient that was hospitalized in a different city (Ibague, 127 miles from Medellin, see (Figure 4-1). The *bla*$_{KPC-3}$ gene in this isolate (KPC_48) was located on a Tn*4401*b transposon within a ≈80 Kb plasmid (Figure 4-6). Four plasmid replicons were identified for this isolate, including IncI2. BLAST analyses revealed that the plasmid contained in

this strain was highly similar to the completely sequenced IncI2 plasmid pBK15692 (NC_022520.1) (Figure 4-7), the most widely found in the NY/NJ area since 2005 (266). Additionally, KPC_48 shares the same virulence factors as the majority of ST258 isolates collected in Colombia in subsequent years, namely type 3 fimbriae *mrk*ABCDF, yersiniabactin *irp*, *ybt*, *fyu* and colibactin *clb*A-I/L-R gene clusters. In contrast, ST512 strains associated with the Israeli outbreak only contain the type 3 fimbriae (*mrk*ABCDF) gene cluster, carry bla_{KPC-3} within a Tn*4401*a structure, and share the same set of plasmid replicons, including pQIL (Figure 4-2 and Figure 4-3).

Figure 4-5 illustrates the comparative genomic analyses of Colombian *K. pneumoniae* belonging to CG258. Our results clearly indicate that the *K. pneumoniae* ST512 harboring bla_{KPC-3} collected in Medellin in 2008 (identified as the "Israeli outbreak" index strain, KPC_63) and two other KPC-3 positive isolates from 2009 isolated in the same hospital (KPC_68 & 69), were more closely related to each other than to the rest of the ST258 bla_{KPC-3}-harbouring isolates. Moreover, these isolates clustered together with a 2006, KPC-3 producing isolate from Israel (Figure 5, highlighted in pink). In contrast, all the remaining 21 bla_{KPC-3} - containing *K. pneumoniae,* recovered in all ten cities country-wide, are closely related to the first KPC positive ST258 isolate recovered in 2007 (KPC-48), and

grouped together with isolates recovered in the New York/New Jersey area from 2006 to 2012 (Figure 4-5, highlighted in blue).

To further characterize the KPC-carrying *K. pneumoniae* Colombian isolates, we analyzed the *cps* locus (capsule polysaccharide bio-synthesis operon) by *wzi* typing, and the plasmid replicon types. CPS is one of the primary determinants of antigenicity associated with *K. pneumoniae* and capsule switching and is a species-specific mechanism used to escape the host immune response. Additionally, DNA exchange in-and-around the *cps* region has been suggested as an important mechanism used by *K. pneumoniae* to rapidly diversify and evolve (267). Interestingly, typing of the *wzi* locus revealed that, consistent with our genomic analyses, a remarkable degree of *cps* diversity is present in our collection (Figure 4-3). Indeed, isolates harboring bla_{KPC-2} exhibited 10 different *cps* variants (some of them not even associated with a particular K- type), whereas all isolates belonging to CG258 and harboring bla_{KPC-3} only belonged to *wzi* type 154. Moreover, the plasmids found in bla_{KPC}-containing isolates (Figure 4-2 and Figure 4-8) were diverse; as observed before (259). Indeed, a variety of plasmid replicons were found in bla_{KPC-2} harboring isolates (including IncQ1, IncHI1B, IncP6, IncU, IncN2, IncFIA[HI1], IncA/C2, IncFII and IncN), most of them associated with broad host range transmission. In contrast, 3 incompatibility groups were exclusively found in CG258 bla_{KPC-3} carrying isolates. Plasmids IncX3 and pQIL were only

detected in ST512 isolates, whereas IncI2 were exclusive of the ST258 isolates.

Discussion

Our detailed genomic studies reconstruct a unique and complex pattern of dissemination of KPC-Kpn in Colombia, contributing to the high endemic levels of carbapenem resistance that threaten the healthcare system in this developing country. These observations are in contrast with the data from other endemic countries including Israel, Italy, Greece and the US, where CG258 has been the main driver of *bla*$_{KPC}$ dissemination (163, 258, 268-270). Our results indicate that before the emergence of KPC ("pre-KPC period", 2002-2005), extended spectrum β-lactamases (ESBLs) were prevalent amongst *K. pneumoniae* circulating in Colombia, a finding that is consistent with previous surveillance studies conducted during this period (211). Of note, there was a complete absence of carbapenem resistance at that time (271), suggesting that the acquisition of Tn*4401b* harboring *bla*$_{KPC-2}$ by a clinical strain represented a sentinel event that influenced the subsequent spread of these organisms in the Colombian healthcare system. Remarkably, after this sentinel event, circulation of *bla*$_{KPC-2}$ among *K. pneumoniae* isolates with heterogeneous genetic backgrounds between 2005 and 2007 was rapidly documented and also coincided with the spread of *bla*$_{CTX-M-15}$ in the country (Figure 4-2) (272, 273). The high prevalence of ESBLs (up to 71% (211)) in *K. pneumoniae* from Colombian hospitals might have led to an increased use of

carbapenems, thus providing selective pressure that favored the emergence of carbapenem resistance, mediated by the horizontal transfer and promiscuity of *bla*$_{KPC-2}$-carrying plasmids among Gram-negative bacteria in Colombia. Indeed, during that and subsequent periods, other species carrying *bla*$_{KPC-2}$ were identified, including *Enterobacter cloacae* complex, *Enterobacter aerogenes*, *Citrobacter freundii, Serratia marcescens, Klebsiella oxytoca*, *Salmonella enterica sv. Typhimurium* and even *Pseudomonas aeruginosa* (an unusual pathogen to harbor *bla*$_{KPC}$) (122, 259, 274). Other factors contributing to the spread may have related with travel within the country and sharing of patients between institutions. However, the exact driving force for this remarkable spread is unknown.

Taken together, our results suggest that the initial dissemination of *bla*$_{KPC}$ in Colombia was independent of the presence of CG258. Instead, the major factor that influenced the initial spread was high rates of horizontal plasmid transfer and/or transposition of *bla*$_{KPC-2}$ on Tn*4401*b. Of note, a BLASTn analysis showed that the first *bla*$_{KPC-2}$-carrying plasmid of *P. aeruginosa* (pCOL-1) was highly similar to a plasmid found in one of the *K. pneumoniae* isolates recovered in 2009 (Figure 4-2 and Figure 4-9; 99% query coverage, 100% identity by BLAST), supporting inter-species transfer (275).

Upon deeper analysis we discovered that the Israeli clone was not responsible for the introduction and clonal expansion of CG258. Instead,

it appears that the index ST258 isolate harbors IncI2 plasmids that have been circulating in the US (New York/New Jersey area) since 2005 (266). Interestingly, the *bla*$_{KPC-3}$-harboring plasmid carried by isolate KPC_48 is very similar to pBK15692, a 77 Kb plasmid that has a characteristic IncI2 backbone which includes genes encoding type IV pili and shufflon regions. In addition to *bla*$_{KPC-3}$ (contained within a Tn*4401*b inserted into a *Tn*1331 element forming a nested transposon), it also carries *bla*$_{OXA-9}$, *bla*$_{TEM-1,}$ *aac(6')-Ib-cr* and *aadA1* (Figure 4-7). Although the index isolate for the "Israeli clone" (obtained from a patient who traveled to Medellin to undergo a liver transplant (260)) was initially assigned to ST258, our sequencing indicates that it truly belonged to ST512 and carried *bla*$_{KPC-3}$ within a Tn*4401*a structure associated with the characteristic pKpQIL plasmid (Figure 4-2 and Figure 4-3) (276). At the time, Israel was dealing with a countrywide outbreak of carbapenem-resistant *K. pneumoniae* CG258 (277). Epidemiological studies indicated that the prevalent clone identified by PFGE and designated as "Q" grouped isolates belonging to ST258 (258). However, in 2006 isolates belonging to ST512 (single nucleotide variant of ST258) started to appear in Israel, and in some cases, even became the prevalent ST (278). Since isolates belonging to ST258 and ST512 have closely related PFGE patterns, it is possible that some of the isolates classified at the time as belonging to clone "Q", were actually ST512.

Two additional pieces of evidence support the possible US-Colombia link. Firstly, we discovered that the apparent genetic platform of dissemination

(Tn*4401b*) is within an IncI2 plasmid (very similar to pBK15692) which is associated with the dissemination of *bla*$_{KPC-3}$ (21, 266). Indeed, the majority of ST258 isolates characterized in this work carried *bla*$_{KPC-3}$ within a Tn*4401*b. In contrast, only three ST512 isolates (the index case, and two other strains isolated in Medellin from the same hospital where the Israeli outbreak occurred) carried *bla*$_{KPC-3}$ on a *Tn*4401a transposon variant. Secondly, our 2007 CG258 isolate (KPC_48) shares the same virulence factors with the majority of ST258 isolates collected in Colombia after its emergence, namely type 3 fimbriae *mrk*ABCDF, yersiniabactin *irp*/*ybt*/*fyu* and colibactin *clb*A-I/L-R gene clusters. In contrast, ST512 strains associated with the Israeli outbreak only contain the type 3 fimbriae (*mrk*ABCDF) gene cluster (Figure 4-3).

Results from this work, and another recent study conducted in Medellin (279), suggest that since its introduction from Israel in 2008, ST512 is confined to that city, co-circulating with other *K. pneumoniae* carrying *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ from different STs. After 2008, our molecular and epidemiological data support the observation that up to 68% of nosocomial *K. pneumoniae* isolates harbored *bla*$_{KPC}$ and these organisms became endemic in Colombia and thus both KPC-2 and KPC-3 variants disseminated (280). Our phylogenetic data shown in Figure 4 strongly suggests compartmentalization of the genes encoding these two variants. Indeed, *bla*$_{KPC-2}$ continues to disseminate via horizontal gene transfer among *K. pneumoniae* isolates from different genetic backgrounds and

capsule types, representing the most common *bla*$_{KPC}$ gene in our collection (62% of *K. pneumoniae* isolates). Of note, only *bla*$_{KPC-2}$ has disseminated into other *Enterobacteriaceae* and *P. aeruginosa* isolates in Colombia (122, 259, 274). On the other hand, a monophyletic clade of the "high risk clone" CG258 *K. pneumoniae* carrying *bla*$_{KPC-3}$ is also expanding simultaneously. These results are in sharp contrast to the epidemiology described in the majority of studies of other endemic countries like United States, Israel, Italy or Greece, where the main culprit for dissemination has been *K. pneumoniae* CG258 (21). In 2009 the Centers for Disease Control and Prevention (CDC) reported that up to 70% of the *K. pneumoniae bla*$_{KPC}$ strains in their collection belong to CG258 (258). This number is even greater in Israel, where 90% of the strains were part of CG258 at the peak of the epidemic. Interestingly, the expansion of a single clone in that country may also explain why the strict infection control measures implemented were successful to combat the epidemic (268). However, more recent observations point out that the dynamics within single institutions or smaller regions have unique characteristics (281-284) and suggest a change in the trend of dominance of CG258 for a somewhat concomitant spread of CG258 and non-CG258 in other endemic countries like Israel, United States, Italy, Brazil and Argentina (279, 281, 285-288).

Certain limitations of the study should be mentioned. As a non-governmental sentinel surveillance system, CIDEIM's antimicrobial resistance network relies on the voluntary collaboration of hospitals

around the country. This limits our pool of samples and naturally, introduces a bias in the strains received per city and per year included in the study. Also, given its retrospective nature, this work does not delineate the true incidence of Kpn-KPC infections in the population nor it discriminates between community-acquired or nosocomial infection.

Conclusion

In conclusion, the combination of two evolutionary mechanisms in a challenged health system from a developing country have created the "perfect storm" for a massive epidemic of carbapenemase-producing Gram-negative bacteria. This stochastic strategy employed by *K. pneumoniae* in Colombia may pose a significant public health threat in other developing countries with similar infrastructures and widespread use of carbapenems, where traditional infection control measures (useful in parts of the world where clonal expansion of CG258 plays a major role in KPC dissemination) would be insufficient. In these countries, the challenge of KPC-Kpn may demand novel approaches combining comprehensive molecular surveillance, innovative infection control procedures and antibiotic stewardship strategies across different settings.

Table 4-1. *K. pneumoniae* isolates used for the CC258 phylogeny shown in Figure 4-5.

Isolate	Year of isolation	ST	KPC	Geographical location	Accession	DOI
KPC_20	2005	258	None	Colombia	BioSample:SAMN06629597	This publication
KPC_48	2007	258	KPC-3	Colombia	BioSample:SAMN06629625	This publication
KPC_63	2008	512	KPC-3	Colombia	BioSample:SAMN06629640	This publication
KPC_65	2009	258	KPC-3	Colombia	BioSample:SAMN06629642	This publication
KPC_66	2009	258	KPC-2	Colombia	BioSample:SAMN06629643	This publication
KPC_68	2009	512	KPC-3	Colombia	BioSample:SAMN06629645	This publication
KPC_69	2009	512	KPC-3	Colombia	BioSample:SAMN06629646	This publication
KPC_70	2009	258	KPC-3	Colombia	BioSample:SAMN06629647	This publication
KPC_71	2009	258	KPC-3	Colombia	BioSample:SAMN06629648	This publication
KPC_72	2009	258	KPC-3	Colombia	BioSample:SAMN06629649	This publication
KPC_74	2010	258	KPC-3	Colombia	BioSample:SAMN06629651	This publication
KPC_75	2010	258	KPC-3	Colombia	BioSample:SAMN06629652	This publication
KPC_77	2010	258	KPC-3	Colombia	BioSample:SAMN06629654	This publication
KPC_78	2010	258	KPC-3	Colombia	BioSample:SAMN06629655	This publication
KPC_79	2010	258	KPC-3	Colombia	BioSample:SAMN06629656	This publication
KPC_82	2011	258	KPC-3	Colombia	BioSample:SAMN06629659	This publication
KPC_83	2011	258	KPC-3	Colombia	BioSample:SAMN06629660	This publication
KPC_84	2011	258	KPC-3	Colombia	BioSample:SAMN06629661	This publication
KPC_86	2011	258	KPC-3	Colombia	BioSample:SAMN06629663	This publication
KPC_88	2011	258	KPC-3	Colombia	BioSample:SAMN06629665	This publication
KPC_89	2011	258	KPC-3	Colombia	BioSample:SAMN06629666	This publication
KPC_91	2011	258	KPC-3	Colombia	BioSample:SAMN06629668	This publication
KPC_92	2012	258	KPC-3	Colombia	BioSample:SAMN06629669	This publication
KPC_96	2012	258	KPC-3	Colombia	BioSample:SAMN06629673	This publication
KPC_101	2013	258	KPC-3	Colombia	BioSample:SAMN06629678	This publication
KPC_103	2013	258	KPC-2	Colombia	BioSample:SAMN06629680	This publication
Australia 10	2010	1199	KPC-2	Australia	SRS692083	10.1371/journal.pone.0133727
Australia 12	2012	258	KPC-2	Australia	AMWO00000000	10.1093/gbe/evv062
Brazil 07 A	2007	258	KPC-2	Brazil	SRS692085	10.1371/journal.pone.0133727
Brazil 07 B	2007	258	KPC-2	Brazil	SRS692086	10.1371/journal.pone.0133727
Brazil 09	2009	442	KPC-2	Brazil	CP003999	10.1186/1471-2164-15-54
Canada 07	2007	258	KPC-3	Canada	SRS556481	10.1073/pnas.1321364111
Canada 09 A	2009	258	KPC-3	Canada	SRS556482	10.1073/pnas.1321364111
Canada 09 B	2009	258	KPC-2	Canada	SRS556483	10.1073/pnas.1321364111
Canada 12 A	2012	258	KPC-3	Canada	SRS556528	10.1073/pnas.1321364111
Canada 12 B	2012	258	KPC-2	Canada	SRS556530	10.1073/pnas.1321364111
Colombia 09 A	2009	258	KPC-3	Colombia	SRS692100	10.1371/journal.pone.0133727
Colombia 09 B	2009	258	KPC-3	Colombia	SRS692101	10.1371/journal.pone.0133727
Colombia 10 A	2010	512	KPC-3	Colombia	SRS556499	10.1073/pnas.1321364111
Colombia 10 B	2010	512	KPC-3	Colombia	SRS556500	10.1073/pnas.1321364111
Colombia 12 A	2012	512	KPC-3	Colombia	SRS556532	10.1073/pnas.1321364111
Colombia 12 B	2012	512	KPC-3	Colombia	SRS556533	10.1073/pnas.1321364111
Denmark 09 A	2009	258	KPC-2	Denmark	SRS692102	10.1371/journal.pone.0133727
Denmark 09 B	2009	258	KPC-2	Denmark	SRS692103	10.1371/journal.pone.0133727
Finland 09 A	2009	258	KPC-2	Finland	SRS692104	10.1371/journal.pone.0133727
Finland 09 B	2009	258	KPC-2	Finland	SRS692105	10.1371/journal.pone.0133727
Greece 07	2007	258	KPC-2	Greece	SRS692107	10.1371/journal.pone.0133727
Greece 08	2008	258	KPC-2	Greece	SRS692108	10.1371/journal.pone.0133727
Greece 09	2009	258	KPC-2	Greece	SRS692109	10.1371/journal.pone.0133727
Greece 10 A	2010	258	KPC-2	Greece	ERS822446	10.1093/jac/dkv467
Greece 10 B	2010	258	KPC-2	Greece	ERS822447	10.1093/jac/dkv467
Greece 10 C	2010	258	KPC-2	Greece	ERS822449	10.1093/jac/dkv467
Greece 10 D	2010	258	KPC-2	Greece	ERS822450	10.1093/jac/dkv467
Greece 10 E	2010	258	KPC-2	Greece	ERS822451	10.1093/jac/dkv467
Greece 10 F	2010	258	KPC-2	Greece	ERS822452	10.1093/jac/dkv467
Greece 10 G	2010	258	KPC-2	Greece	SRS692110	10.1371/journal.pone.0133727
Greece 11	2011	258	KPC-2	Greece	SRS692111	10.1371/journal.pone.0133727
Greece 13 A	2013	258	KPC-2	Greece	ERS822453	10.1093/jac/dkv467
Greece 13 B	2013	258	KPC-2	Greece	ERS822455	10.1093/jac/dkv467
Greece 13 C	2013	258	KPC-2	Greece	ERS822461	10.1093/jac/dkv467
Greece 13 D	2013	258	KPC-2	Greece	ERS822465	10.1093/jac/dkv467
Greece 13 E	2013	258	KPC-2	Greece	ERS822467	10.1093/jac/dkv467
Greece 13 F	2013	258	KPC-2	Greece	ERS822469	10.1093/jac/dkv467
Greece 14 A	2014	258	KPC-2	Greece	ERS822454	10.1093/jac/dkv467
Greece 14 B	2014	258	KPC-2	Greece	ERS822456	10.1093/jac/dkv467
Greece 14 C	2014	258	KPC-2	Greece	ERS822457	10.1093/jac/dkv467
Greece 14 D	2014	258	KPC-2	Greece	ERS822458	10.1093/jac/dkv467
Greece 14 E	2014	258	KPC-2	Greece	ERS822459	10.1093/jac/dkv467
Greece 14 F	2014	258	KPC-2	Greece	ERS822460	10.1093/jac/dkv467
Greece 14 G	2014	258	KPC-2	Greece	ERS822462	10.1093/jac/dkv467
Greece 14 H	2014	258	KPC-2	Greece	ERS822463	10.1093/jac/dkv467
Greece 14 I	2014	258	KPC-2	Greece	ERS822464	10.1093/jac/dkv467
Greece 14 J	2014	258	KPC-2	Greece	ERS822466	10.1093/jac/dkv467
Greece 14 K	2014	258	KPC-2	Greece	ERS822468	10.1093/jac/dkv467
Greece 14 L	2014	258	KPC-2	Greece	ERS822470	10.1093/jac/dkv467
Greece 14 M	2014	258	KPC-2	Greece	ERS822471	10.1093/jac/dkv467
Greece 14 N	2014	258	KPC-2	Greece	ERS822472	10.1093/jac/dkv467
Greece 14 O	2014	258	KPC-2	Greece	ERS822473	10.1093/jac/dkv467
Israel 06	2006	258	KPC-3	Israel	ALIS00000000	10.1128/JB.01546

Italy A	NA	258	KPC-3	Bologna, Italy	CANR00000000	10.1128/genomeA.00113-12
Italy B	NA	258	KPC-3	Bologna, Italy	CANS00000000	10.1128/genomeA.00113-12
Italy 08 A	2008	258	KPC-3	Italy	SRS692124	10.1371/journal.pone.0133727
Italy 08 B	2008	258	KPC-3	Palermo, Italy	SRS692129	10.1371/journal.pone.0133727
Italy 09 A	2009	258	KPC-2	Italy	SRS556503	10.1073/pnas.1321364111
Italy 09 B	2009	258	KPC-3	Palermo, Italy	SRS692130	10.1371/journal.pone.0133727
Italy 09 C	2009	258	KPC-3	Palermo, Italy	SRS692131	10.1371/journal.pone.0133727
Italy 09 D	2009	258	KPC-3	Palermo, Italy	SRS692132	10.1371/journal.pone.0133727
Italy 09 E	2009	258	KPC-3	Palermo, Italy	SRS692136	10.1371/journal.pone.0133727
Italy 09 F	2009	258	KPC-3	Palermo, Italy	SRS692139	10.1371/journal.pone.0133727
Italy 09 G	2009	258	KPC-3	Palermo, Italy	SRS692141	10.1371/journal.pone.0133727
Italy 10 A	2010	258	KPC-2	Genova, Italy	NZ_LJOO00000000	10.1371/journal.pone.0096827
Italy 10 B	2010	258	KPC-3	Palermo, Italy	SRS692142	10.1371/journal.pone.0133727
Italy 10 C	2010	258	KPC-3	Palermo, Italy	SRS692143	10.1371/journal.pone.0133727
Italy 10 D	2010	258	KPC-3	Palermo, Italy	SRS692144	10.1371/journal.pone.0133727
Italy 10 E	2010	258	KPC-3	Palermo, Italy	SRS692145	10.1371/journal.pone.0133727
Italy 11 A	2011	258	KPC-2	Bergamo, Italy	ERS480638	10.1128/AAC.04224-14
Italy 11 B	2011	258	KPC-2	Bergamo, Italy	ERS480642	10.1128/AAC.04224-14
Italy 11 C	2011	258	KPC-2	Bergamo, Italy	ERS480644	10.1128/AAC.04224-14
Italy 11 D	2011	512	KPC-3	Bergamo, Italy	ERS480646	10.1128/AAC.04224-14
Italy 11 E	2011	258	KPC-2	Reggio Emilia, Italy	ERS480656	10.1128/AAC.04224-14
Italy 11 F	2011	258	KPC-2	Reggio Emilia, Italy	ERS480657	10.1128/AAC.04224-14
Italy 11 G	2011	258	KPC-2	Reggio Emilia, Italy	ERS480658	10.1128/AAC.04224-14
Italy 11 H	2011	512	KPC-3	Reggio Emilia, Italy	ERS480659	10.1128/AAC.04224-14
Italy 11 I	2011	258	KPC-2	Reggio Emilia, Italy	ERS480660	10.1128/AAC.04224-14
Italy 11 J	2011	512	KPC-3	San Giovanni Rotondo, Italy	ERS480670	10.1128/AAC.04224-14
Italy 11 K	2011	512	KPC-3	San Giovanni Rotondo, Italy	ERS480671	10.1128/AAC.04224-14
Italy 11 L	2011	512	KPC-3	San Giovanni Rotondo, Italy	ERS480672	10.1128/AAC.04224-14
Italy 11 M	2011	512	KPC-3	San Giovanni Rotondo, Italy	ERS480673	10.1128/AAC.04224-14
Italy 11 N	2011	512	KPC-3	San Giovanni Rotondo, Italy	ERS480674	10.1128/AAC.04224-14
Italy 11 O	2011	258	KPC-2	Italy	SRS692126	10.1371/journal.pone.0133727
Italy 11 P	2011	258	KPC-2	Italy	SRS692127	10.1371/journal.pone.0133727
Italy 11 Q	2011	512	KPC-3	Italy	SRS692128	10.1371/journal.pone.0133727
Italy 11 R	2011	258	KPC-3	Palermo, Italy	SRS692148	10.1371/journal.pone.0133727
Italy 11 S	2011	258	KPC-3	Palermo, Italy	SRS692149	10.1371/journal.pone.0133727
Italy 11 T	2011	258	KPC-3	Palermo, Italy	SRS692150	10.1371/journal.pone.0133727
Italy 11 U	2011	258	KPC-3	Palermo, Italy	SRS692151	10.1371/journal.pone.0133727
Italy 11 V	2011	258	KPC-3	Palermo, Italy	SRS692152	10.1371/journal.pone.0133727
Italy 11 W	2011	258	KPC-3	Palermo, Italy	SRS692153	10.1371/journal.pone.0133727
Italy 11 X	2011	258	KPC-3	Palermo, Italy	SRS692154	10.1371/journal.pone.0133727
Italy 12 A	2012	512	KPC-3	Bologna, Italy	ERS480597	10.1128/AAC.04224-14
Italy 12 B	2012	512	KPC-3	Bologna, Italy	ERS480598	10.1128/AAC.04224-14
Italy 12 C	2012	512	KPC-3	Bologna, Italy	ERS480599	10.1128/AAC.04224-14
Italy 12 D	2012	512	KPC-3	Bologna, Italy	ERS480600	10.1128/AAC.04224-14
Italy 12 E	2012	512	KPC-3	Bologna, Italy	ERS480620	10.1128/AAC.04224-14
Italy 12 F	2012	512	KPC-3	Bologna, Italy	ERS480621	10.1128/AAC.04224-14
Italy 12 G	2012	512	None	Cesena, Italy	ERS480681	10.1128/AAC.04224-14
Italy 13 A	2013	258	KPC-2	Pavia, Italy	ERS480602	10.1128/AAC.04224-14
Italy 13 B	2013	258	KPC-2	Pavia, Italy	ERS480603	10.1128/AAC.04224-14
Italy 13 C	2013	258	KPC-2	Pavia, Italy	ERS480604	10.1128/AAC.04224-14
Italy 13 D	2013	258	KPC-2	Pavia, Italy	ERS480614	10.1128/AAC.04224-14
Italy 13 E	2013	258	KPC-2	Pavia, Italy	ERS480615	10.1128/AAC.04224-14
Italy 13 F	2013	512	KPC-3	Cesena, Italy	ERS480622	10.1128/AAC.04224-14
Italy 13 G	2013	512	KPC-3	Cesena, Italy	ERS480630	10.1128/AAC.04224-14
Italy 13 H	2013	512	KPC-3	Cesena, Italy	ERS480631	10.1128/AAC.04224-14
Italy 13 I	2013	512	None	Cesena, Italy	ERS480632	10.1128/AAC.04224-14
Italy 13 J	2013	512	KPC-3	Cesena, Italy	ERS480636	10.1128/AAC.04224-14
Poland 08	2008	258	KPC-3	Poland	SRS692161	10.1371/journal.pone.0133727
Poland 09 A	2009	258	KPC-2	Poland	SRS692162	10.1371/journal.pone.0133727
Poland 09 B	2009	258	KPC-2	Poland	SRS692163	10.1371/journal.pone.0133727
Poland 09 C	2009	258	KPC-2	Poland	SRS692166	10.1371/journal.pone.0133727
USA 03 A	2003	258	None	NJ, USA	SRS556455	10.1073/pnas.1321364111
USA 03 B	2003	258	KPC-3	NJ, USA	SRS692212	10.1371/journal.pone.0133727
USA 04 A	2004	258	KPC-3	NJ, USA	SRS556456	10.1073/pnas.1321364111
USA 04 B	2004	258	None	NYC, USA	SRS556457	10.1073/pnas.1321364111
USA 04 C	2004	258	KPC-3	NJ, USA	SRS556458	10.1073/pnas.1321364111
USA 04 D	2004	258	KPC-3	NJ, USA	SRS692213	10.1371/journal.pone.0133727
USA 04 E	2004	258	KPC-3	NJ, USA	SRS692214	10.1371/journal.pone.0133727
USA 04 F	2004	258	KPC-3	NY, USA	SRS692221	10.1371/journal.pone.0133727
USA 05 A	2005	258	KPC-3	NJ, USA	SRS556459	10.1073/pnas.1321364111
USA 05 B	2005	258	KPC-2	NJ, USA	SRS556460	10.1073/pnas.1321364111
USA 05 C	2005	258	KPC-3	NJ, USA	SRS556489	10.1073/pnas.1321364111
USA 05 D	2005	258	KPC-3	NJ, USA	SRS692215	10.1371/journal.pone.0133727
USA 05 E	2005	258	KPC-2	NY, USA	SRS692224	10.1371/journal.pone.0133727
USA 05 F	2005	258	KPC-2	NY, USA	SRS692226	10.1371/journal.pone.0133727
USA 05 G	2005	258	KPC-2	NY, USA	SRS692227	10.1371/journal.pone.0133727
USA 05 H	2005	258	KPC-3	PA, USA	SRS692234	10.1371/journal.pone.0133727
USA 05 I	2005	258	KPC-3	PA, USA	SRS692235	10.1371/journal.pone.0133727
USA 06 A	2006	258	KPC-3	NJ, USA	SRS556461	10.1073/pnas.1321364111
USA 06 B	2006	258	KPC-3	NJ, USA	SRS556490	10.1073/pnas.1321364111
USA 06 C	2006	258	None	AZ, USA	SRS692178	10.1371/journal.pone.0133727
USA 06 D	2006	258	KPC-3	AZ, USA	SRS692179	10.1371/journal.pone.0133727
USA 06 E	2006	258	KPC-3	DE, USA	SRS692187	10.1371/journal.pone.0133727
USA 06 F	2006	258	KPC-2	GA, USA	SRS692191	10.1371/journal.pone.0133727
USA 06 G	2006	258	KPC-3	NJ, USA	SRS692216	10.1371/journal.pone.0133727

USA 07 A	2007	258	KPC-2	OH, USA	ANGI02000000	10.1128/genomeA.00168
USA 07 B	2007	258	KPC-2	Cleveland_OH_USA	APVO00000000	10.1128/AAC.00125-14
USA 07 C	2007	258	KPC-2	Cleveland_OH_USA	APVP00000000	10.1128/AAC.00125-14
USA 07 D	2007	258	KPC-2	Cleveland_OH_USA	APVV00000000	10.1128/AAC.00125-14
USA 07 E	2007	258	KPC-2	Cleveland_OH_USA	ARSJ00000000	10.1128/AAC.00125-14
USA 07 F	2007	258	KPC-3	Cleveland_OH_USA	ARSK00000000	10.1128/AAC.00125-14
USA 07 G	2007	258	KPC-2	Cleveland_OH_USA	CP011985	10.1128/AAC.00125-14
USA 07 H	2007	258	KPC-3	NYC, USA	SRS556462	10.1073/pnas.1321364111
USA 07 I	2007	258	KPC-2	NYC, USA	SRS556463	10.1073/pnas.1321364111
USA 07 J	2007	418	KPC-3	NJ	SRS556464	10.1073/pnas.1321364111
USA 07 K	2007	258	KPC-3	NJ, USA	SRS556465	10.1073/pnas.1321364111
USA 07 L	2007	258	KPC-3	NYC, USA	SRS556466	10.1073/pnas.1321364111
USA 07 M	2007	258	KPC-3	NJ, USA	SRS556491	10.1073/pnas.1321364111
USA 07 N	2007	258	KPC-3	CO, USA	SRS692186	10.1371/journal.pone.0133727
USA 07 O	2007	258	KPC-2	MA, USA	SRS692202	10.1371/journal.pone.0133727
USA 07 P	2007	258	None	NJ, USA	SRS692217	10.1371/journal.pone.0133727
USA 07 Q	2007	258	KPC-3	NM, USA	SRS692219	10.1371/journal.pone.0133727
USA 07 R	2007	258	None	PA, USA	SRS692236	10.1371/journal.pone.0133727
USA 08 A	2008	258	KPC-2	Cleveland_OH_USA	APVR00000000	10.1128/AAC.00125-14
USA 08 B	2008	258	KPC-2	Cleveland_OH_USA	APVS00000000	10.1128/AAC.00125-14
USA 08 C	2008	258	KPC-2	Cleveland_OH_USA	APVT00000000	10.1128/AAC.00125-14
USA 08 D	2008	258	KPC-7	Cleveland_OH_USA	APVU00000000	10.1128/AAC.00125-14
USA 08 E	2008	258	KPC-3	Cleveland_OH_USA	AQOT00000000	10.1128/AAC.00125-14
USA 08 F	2008	258	KPC-2	Cleveland_OH_USA	AQQA00000000	10.1128/AAC.00125-14
USA 08 G	2008	258	KPC-3	Cleveland_OH_USA	ARPP00000000	10.1128/AAC.00125-14
USA 08 H	2008	258	KPC-3	Cleveland_OH_USA	ARPQ00000000	10.1128/AAC.00125-14
USA 08 I	2008	258	KPC-2	Cleveland_OH_USA	ARRU00000000	10.1128/AAC.00125-14
USA 08 J	2008	258	KPC-2	Cleveland_OH_USA	ARRV00000000	10.1128/AAC.00125-14
USA 08 K	2008	258	KPC-3	Cleveland_OH_USA	ARRW00000000	10.1128/AAC.00125-14
USA 08 L	2008	258	KPC-3	Cleveland_OH_USA	ARRX00000000	10.1128/AAC.00125-14
USA 08 M	2008	258	KPC-3	Cleveland_OH_USA	ARRY00000000	10.1128/AAC.00125-14
USA 08 N	2008	258	KPC-2	Cleveland_OH_USA	ARSC00000000	10.1128/AAC.00125-14
USA 08 O	2008	258	KPC-3	Cleveland_OH_USA	ARSE00000000	10.1128/AAC.00125-14
USA 08 P	2008	258	KPC-3	Cleveland_OH_USA	ARSG00000000	10.1128/AAC.00125-14
USA 08 Q	2008	258	KPC-3	Cleveland_OH_USA	ARSH00000000	10.1128/AAC.00125-14
USA 08 R	2008	258	KPC-2	Cleveland_OH_USA	ARSI00000000	10.1128/AAC.00125-14
USA 08 S	2008	258	KPC-3	Cleveland_OH_USA	ARSL00000000	10.1128/AAC.00125-14
USA 08 T	2008	258	KPC-2	Cleveland_OH_USA	ARVM00000000	10.1128/AAC.00125-14
USA 08 U	2008	258	None	Cleveland_OH_USA	ARVN00000000	10.1128/AAC.00125-14
USA 08 V	2008	258	KPC-3	Cleveland_OH_USA	ARVO00000000	10.1128/AAC.00125-14
USA 08 W	2008	258	KPC-2	Cleveland_OH_USA	ARVQ00000000	10.1128/AAC.00125-14
USA 08 X	2008	258	KPC-2	Cleveland_OH_USA	CP011989	10.1128/AAC.00125-14
USA 08 Y	2008	258	KPC-3	Florida, USA	NZ_LAKK01000012	10.1128/genomeA.00659-15
USA 08 Z	2008	258	KPC-2	NJ, USA	SRS556492	10.1073/pnas.1321364111
USA 08 AA	2008	258	KPC-3	IL, USA	SRS692198	10.1371/journal.pone.0133727
USA 08 AB	2008	258	None	MA, USA	SRS692203	10.1371/journal.pone.0133727
USA 08 AC	2008	258	KPC-3	NH, USA	SRS692211	10.1371/journal.pone.0133727
USA 08 AD	2008	258	None	RI, USA	SRS692240	10.1371/journal.pone.0133727
USA 08 AE	2008	258	KPC-2	VA, USA	SRS692245	10.1371/journal.pone.0133727
USA 09 A	2009	258	KPC-3	NJ, USA	SRS556467	10.1073/pnas.1321364111
USA 09 B	2009	379	KPC-3	NJ, USA	SRS556468	10.1073/pnas.1321364111
USA 09 C	2009	258	KPC-2	NJ, USA	SRS556469	10.1073/pnas.1321364111
USA 09 D	2009	258	KPC-3	NJ, USA	SRS556470	10.1073/pnas.1321364111
USA 09 E	2009	379	KPC-3	NJ, USA	SRS556471	10.1073/pnas.1321364111
USA 09 F	2009	258	KPC-3	NYC, USA	SRS556472	10.1073/pnas.1321364111
USA 09 G	2009	258	KPC-3	NYC, USA	SRS556473	10.1073/pnas.1321364111
USA 09 H	2009	258	KPC-2	NY, USA	SRS556474	10.1073/pnas.1321364111
USA 09 I	2009	379	KPC-3	NJ, USA	SRS556475	10.1073/pnas.1321364111
USA 09 J	2009	379	KPC-3	NJ, USA	SRS556476	10.1073/pnas.1321364111
USA 09 K	2009	258	KPC-3	NYC, USA	SRS556477	10.1073/pnas.1321364111
USA 09 L	2009	258	KPC-2	TX, USA	SRS556484	10.1073/pnas.1321364111
USA 09 M	2009	258	None	NJ, USA	SRS556493	10.1073/pnas.1321364111
USA 09 N	2009	258	KPC-3	GA, USA	SRS692197	10.1371/journal.pone.0133727
USA 09 O	2009	258	KPC-3	IL, USA	SRS692200	10.1371/journal.pone.0133727
USA 09 P	2009	258	KPC-2	NY, USA	SRS692228	10.1371/journal.pone.0133727
USA 09 Q	2009	258	KPC-2	NY, USA	SRS692229	10.1371/journal.pone.0133727
USA 09 R	2009	258	KPC-3	NY, USA	SRS692230	10.1371/journal.pone.0133727
USA 09 S	2009	258	None	Mid Atlantic	SRS556501	10.1073/pnas.1321364111
USA 09 T	2009	258	KPC-3	Mid Atlantic	SRS556502	10.1073/pnas.1321364111
USA 10 A	2010	258	KPC-3	Cleveland_OH_USA	APVW00000000	10.1128/AAC.00125-14
USA 10 B	2010	258	KPC-3	Cleveland_OH_USA	APVX00000000	10.1128/AAC.00125-14
USA 10 C	2010	258	KPC-3	Detroit_MI_USA	ARPO00000000	10.1128/AAC.00125-14
USA 10 D	2010	258	KPC-3	NJ, USA	CP006918	10.1073/pnas.1321364111
USA 10 E	2010	258	KPC-2	USA	CP010361	Direct submission
USA 10 F	2010	258	KPC-3	Detroit_MI_USA	CP011976	10.1128/AAC.00125-14
USA 10 G	2010	258	KPC-3	NJ, USA	NZ_CP006923	10.1073/pnas.1321364111
USA 10 H	2010	258	KPC-3	NJ, USA	SRS556478	10.1073/pnas.1321364111
USA 10 I	2010	258	KPC-2	NJ, USA	SRS556479	10.1073/pnas.1321364111
USA 10 J	2010	258	KPC-2	NY, USA	SRS556480	10.1073/pnas.1321364111
USA 10 K	2010	258	KPC-3	NJ, USA	SRS556486	10.1073/pnas.1321364111
USA 10 L	2010	258	KPC-2	NYC, USA	SRS556487	10.1073/pnas.1321364111
USA 10 M	2010	258	KPC-3	NYC, USA	SRS556488	10.1073/pnas.1321364111
USA 10 N	2010	258	KPC-3	NJ, USA	SRS556494	10.1073/pnas.1321364111
USA 10 O	2010	258	KPC-2	NYC, USA	SRS556495	10.1073/pnas.1321364111
USA 10 P	2010	258	KPC-2	PA, USA	SRS556511	10.1073/pnas.1321364111
USA 10 Q	2010	258	KPC-2	PA, USA	SRS556512	10.1073/pnas.1321364111
USA 10 R	2010	258	KPC-2	PA, USA	SRS556513	10.1073/pnas.1321364111
USA 10 S	2010	258	KPC-2	PA, USA	SRS556514	10.1073/pnas.1321364111

USA 10 T	2010	258	KPC-3	PA, USA	SRS556515	10.1073/pnas.1321364111
USA 10 U	2010	258	KPC-3	PA, USA	SRS556516	10.1073/pnas.1321364111
USA 10 V	2010	258	KPC-3	PA, USA	SRS556518	10.1073/pnas.1321364111
USA 10 W	2010	258	KPC-3	PA, USA	SRS556523	10.1073/pnas.1321364111
USA 10 X	2010	258	KPC-3	PA, USA	SRS556524	10.1073/pnas.1321364111
USA 10 Y	2010	258	KPC-3	PA, USA	SRS556525	10.1073/pnas.1321364111
USA 10 Z	2010	258	KPC-3	NY, USA	SRS692231	10.1371/journal.pone.0133727
USA 10 AA	2010	258	KPC-2	Mid Atlantic	SRS556504	10.1073/pnas.1321364111
USA 10 AB	2010	258	KPC-3	Mid Atlantic	SRS556505	10.1073/pnas.1321364111
USA 10 AC	2010	258	KPC-2	Mid Atlantic	SRS556506	10.1073/pnas.1321364111
USA 10 AD	2010	258	KPC-2	Mid Atlantic	SRS556507	10.1073/pnas.1321364111
USA 11 A	2011	258	KPC-3	NHI, USA	AJZV01	10.1126/scitranslmed.3004129
USA 11 B	2011	258	KPC-3	NHI, USA	AJZW01	10.1126/scitranslmed.3004129
USA 11 C	2011	258	KPC-3	NHI, USA	AJZX01	10.1126/scitranslmed.3004129
USA 11 D	2011	258	KPC-3	NHI, USA	AJZY01	10.1126/scitranslmed.3004129
USA 11 E	2011	258	KPC-3	NHI, USA	AJZZ01	10.1126/scitranslmed.3004129
USA 11 F	2011	258	KPC-3	NHI, USA	AKAA01	10.1126/scitranslmed.3004129
USA 11 G	2011	258	KPC-3	NHI, USA	AKAB01	10.1126/scitranslmed.3004129
USA 11 H	2011	258	KPC-3	NHI, USA	AKAD01	10.1126/scitranslmed.3004129
USA 11 I	2011	258	KPC-3	NHI, USA	AKAE01	10.1126/scitranslmed.3004129
USA 11 J	2011	258	KPC-3	NHI, USA	AKAF01	10.1126/scitranslmed.3004129
USA 11 K	2011	258	KPC-3	NHI, USA	AKAG01	10.1126/scitranslmed.3004129
USA 11 L	2011	258	KPC-3	NHI, USA	AKAH01	10.1126/scitranslmed.3004129
USA 11 M	2011	258	KPC-3	NHI, USA	AKAI01	10.1126/scitranslmed.3004129
USA 11 N	2011	258	KPC-3	NHI, USA	AKAJ01	10.1126/scitranslmed.3004129
USA 11 O	2011	258	KPC-3	NHI, USA	AKAK01	10.1126/scitranslmed.3004129
USA 11 P	2011	258	KPC-3	NHI, USA	AKAL01	10.1126/scitranslmed.3004129
USA 11 Q	2011	258	KPC-3	NHI, USA	AKAM01	10.1126/scitranslmed.3004129
USA 11 R	2011	258	KPC-3	NHI, USA	AKAN01	10.1126/scitranslmed.3004129
USA 11 S	2011	258	KPC-2	Cleveland_OH_USA	APVY00000000	10.1128/AAC.00125-14
USA 11 T	2011	258	KPC-2	Cleveland_OH_USA	APVZ00000000	10.1128/AAC.00125-14
USA 11 U	2011	258	KPC-2	Cleveland_OH_USA	APWA00000000	10.1128/AAC.00125-14
USA 11 V	2011	258	KPC-2	Cleveland_OH_USA	APWB00000000	10.1128/AAC.00125-14
USA 11 W	2011	258	KPC-3	Cleveland_OH_USA	APWC00000000	10.1128/AAC.00125-14
USA 11 X	2011	258	KPC-2	Cleveland_OH_USA	APWD00000000	10.1128/AAC.00125-14
USA 11 Y	2011	258	KPC-2	North_East_OH_USA	ARSP00000000	10.1128/AAC.00125-14
USA 11 Z	2011	258	KPC-2	Cleveland_OH_USA	ARVP00000000	10.1128/AAC.00125-14
USA 11 AA	2011	258	KPC-3	NHI, USA	CP008827	10.1126/scitranslmed.3004129
USA 11 AB	2011	11	KPC-3	VA, USA	CP011578	Direct submission
USA 11 AC	2011	258	KPC-3	NHI, USA	NZ_CP007727	10.1126/scitranslmed.3004129
USA 11 AD	2011	258	KPC-3	NYC, USA	SRS556496	10.1073/pnas.1321364111
USA 11 AE	2011	258	KPC-3	NYC, USA	SRS556497	10.1073/pnas.1321364111
USA 11 AF	2011	258	KPC-3	NYC, USA	SRS556498	10.1073/pnas.1321364111
USA 11 AG	2011	258	KPC-3	NYC, USA	SRS556508	10.1073/pnas.1321364111
USA 11 AH	2011	258	KPC-2	NYC, USA	SRS556509	10.1073/pnas.1321364111
USA 11 AI	2011	258	KPC-3	IL, USA	SRS556510	10.1073/pnas.1321364111
USA 11 AJ	2011	258	KPC-2	PA, USA	SRS556517	10.1073/pnas.1321364111
USA 11 AK	2011	258	KPC-2	PA, USA	SRS556519	10.1073/pnas.1321364111
USA 11 AL	2011	258	KPC-2	PA, USA	SRS556520	10.1073/pnas.1321364111
USA 11 AM	2011	258	KPC-3	NYC, USA	SRS556521	10.1073/pnas.1321364111
USA 11 AN	2011	258	None	NYC, USA	SRS556522	10.1073/pnas.1321364111
USA 11 AO	2011	258	None	PA, USA	SRS556526	10.1073/pnas.1321364111
USA 11 AP	2011	258	KPC-2	NYC, USA	SRS556527	10.1073/pnas.1321364111
USA 11 AQ	2011	258	KPC-2	NYC, USA	SRS556529	10.1073/pnas.1321364111
USA 11 AR	2011	258	KPC-2	FL, USA	SRS692190	10.1371/journal.pone.0133727
USA 11 AS	2011	258	KPC-3	ND, USA	SRS692210	10.1371/journal.pone.0133727
USA 11 AT	2011	258	KPC-3	PA, USA	SRS692239	10.1371/journal.pone.0133727
USA 11 AU	2011	258	KPC-3	WV, USA	SRS692248	10.1371/journal.pone.0133727
USA 12 A	2012	258	KPC-2	North_East_OH_USA	APWF00000000	10.1128/AAC.00125-14
USA 12 B	2012	258	KPC-2	North_East_OH_USA	APWG00000000	10.1128/AAC.00125-14
USA 12 C	2012	258	KPC-3	North_East_OH_USA	APWI00000000	10.1128/AAC.00125-14
USA 12 D	2012	258	KPC-2	North_East_OH_USA	APWJ00000000	10.1128/AAC.00125-14
USA 12 E	2012	258	KPC-2	North_East_OH_USA	ARSN00000000	10.1128/AAC.00125-14
USA 12 F	2012	258	KPC-2	North_East_OH_USA	ARSO00000000	10.1128/AAC.00125-14
USA 12 G	2012	258	KPC-2	North_East_OH_USA	ARSQ00000000	10.1128/AAC.00125-14
USA 12 H	2012	258	KPC-3	VA, USA	CP011647	Direct submission
USA 12 I	2012	258	KPC-2	NHI, USA	NZ_CP008797	Direct submission
USA 12 J	2012	258	KPC-2	NHI, USA	NZ_CP008831	Direct submission
USA 12 K	2012	258	KPC-2	USA	NZ_CP011980	10.1128/AAC.00125-14
USA 12 L	2012	258	KPC-3	NYC, USA	SRS556531	10.1073/pnas.1321364111
USA 12 M	2012	258	KPC-2	TX, USA	SRS556534	10.1073/pnas.1321364111
USA 12 N	2012	258	KPC-2	TX, USA	SRS556535	10.1073/pnas.1321364111
USA 12 O	2012	258	KPC-3	FL, USA	SRS556537	10.1073/pnas.1321364111
USA 12 P	2012	258	KPC-3	FL, USA	SRS556538	10.1073/pnas.1321364111
USA 13 A	2013	258	None	NIH, USA	CP009872	10.1126/scitranslmed.3009845
USA 13 B	2013	258	KPC-3	NHI, USA	NZ_CP009771	10.1126/scitranslmed.3009845

Table 4-2. *K. pneumoniae* isolates used for phylogeny shown in Figure 4-4.

Isolate	Name on tree	Isolation year	Country	City of isolation	Hospital	ST	KPC	Accession	DOI
KPC_1	2005_ST338	2005	Colombia	Medellin	H13	338	KPC-2	SAMN06629578	This study
KPC_2	2002_NEW ST1	2002	Colombia	Medellin	H16	NEW ST1	NEG	SAMN06629579	This study
KPC_3	2002_NEW ST2	2002	Colombia	Bogota	H6	NEW ST2	NEG	SAMN06629580	This study
KPC_4	Not on tree	2002	Colombia	Bogota	H14	NEW ST3	NEG	*not available	This study
KPC_5	2002_999	2002	Colombia	Cali	H11	999	NEG	SAMN06629582	This study
KPC_6	2003_NEW ST4	2003	Colombia	Barranquilla	H2	NEW ST4	NEG	SAMN06629583	This study
KPC_7	2003_ST1191	2003	Colombia	Barranquilla	H2	1191	NEG	SAMN06629584	This study
KPC_8	2003_ST552	2003	Colombia	Barranquilla	H2	552	NEG	SAMN06629585	This study
KPC_9	2003_ST252	2003	Colombia	Bucaramanga	H7	252	NEG	SAMN06629586	This study
KPC_10	2003_ST2310	2003	Colombia	Bucaramanga	H9	2310	NEG	SAMN06629587	This study
KPC_11	2003_ST1584	2003	Colombia	Medellin	H1	1584	NEG	SAMN06629588	This study
KPC_12	2004_ST2411	2004	Colombia	Bogota	H22	2411	NEG	SAMN06629589	This study
KPC_13	2004_ST13	2004	Colombia	Cartagena	H19	13	NEG	SAMN06629590	This study
KPC_14	2004_ST833	2004	Colombia	Medellin	H23	833	NEG	SAMN06629591	This study
KPC_15	2005_ST442	2005	Colombia	Medellin	H1	442	NEG	SAMN06629592	This study
KPC_16	2005_ST353	2005	Colombia	Bogota	H22	353	KPC-2	SAMN06629593	This study
KPC_17	2005_ST337	2005	Colombia	Medellin	H16	337	KPC-2	SAMN06629594	This study
KPC_18	2005_NEW ST6	2005	Colombia	Medellin	H16	NEW ST6	NEG	SAMN06629595	This study
KPC_19	2006_ST339	2006	Colombia	Medellin	H16	339	KPC-2	SAMN06629596	This study
KPC_20	2005_ST258	2005	Colombia	Bucaramanga	H9	258	NEG	SAMN06629597	This study
KPC_21	2005_ST14	2005	Colombia	Bogota	H6	14	NEG	SAMN06629598	This study
KPC_22	2005_ST292	2005	Colombia	Cali	H21	292	NEG	SAMN06629599	This study
KPC_23	2005_NEW ST7	2005	Colombia	Pereira	H24	NEW ST7	NEG	SAMN06629600	This study
KPC_24	2005_ST77	2005	Colombia	Barranquilla	H2	77	NEG	SAMN06629601	This study
KPC_25	2006_ST1271	2006	Colombia	Ibague	H8	1271	NEG	SAMN06629602	This study
KPC_26	2006_ST789	2006	Colombia	Ibague	H8	789	NEG	SAMN06629603	This study
KPC_27	2006_ST15	2006	Colombia	Medellin	H13	15	NEG	SAMN06629604	This study
KPC_28	2006_ST299	2006	Colombia	Medellin	H13	299	NEG	SAMN06629605	This study
KPC_29	2006_ST48	2006	Colombia	Medellin	H13	48	NEG	SAMN06629606	This study
KPC_30	2006_ST14	2006	Colombia	Medellin	H13	14	NEG	SAMN06629607	This study
KPC_31	2006_ST17	2006	Colombia	Medellin	H1	17	NEG	SAMN06629608	This study
KPC_32	2006_ST39	2006	Colombia	Bogota	H12	39	NEG	SAMN06629609	This study
KPC_33	2006_ST14	2006	Colombia	Bogota	H12	14	NEG	SAMN06629610	This study
KPC_34	2006_ST14	2006	Colombia	Bogota	H12	14	NEG	SAMN06629611	This study
KPC_35	2006_ST14	2006	Colombia	Bogota	H22	14	KPC-2	SAMN06629612	This study
KPC_36	2006_ST533	2006	Colombia	Bogota	H22	533	NEG	SAMN06629613	This study
KPC_37	2006_ST39	2006	Colombia	Bogota	H22	39	NEG	SAMN06629614	This study
KPC_38	2006_ST11	2006	Colombia	Bucaramanga	H9	11	NEG	SAMN06629615	This study
KPC_39	2006_ST1440	2006	Colombia	Cali	H21	1440	NEG	SAMN06629616	This study
KPC_40	2006_ST34	2006	Colombia	Pereira	H24	34	NEG	SAMN06629617	This study
KPC_41	2006_ST14	2006	Colombia	Medellin	H16	14	KPC-2	SAMN06629618	This study
KPC_42	2006_ST1303	2006	Colombia	Barranquilla	H2	1303	NEG	SAMN06629619	This study
KPC_43	2006_ST101	2006	Colombia	Barranquilla	H2	101	NEG	SAMN06629620	This study
KPC_44	2006_ST339	2006	Colombia	Medellin	H16	339	KPC-2	SAMN06629621	This study
KPC_45	Not on tree	2006	Colombia	Medellin	H16	789	KPC-2	*not available	This study
KPC_46	2006_ST495	2010	Colombia	Medellin	H16	495	NEG	SAMN06629623	This study
KPC_47	2010_ST12	2007	Colombia	Medellin	H16	12	NEG	SAMN06629624	This study
KPC_48	2007_ST258	2007	Colombia	Ibague	H8	258	KPC-3	SAMN06629625	This study

KPC_49	2007_NEW ST8	2007	Colombia	Medellin	H13	NEW ST8	NEG	SAMN06629626	This study
KPC_50	2007_ST273	2007	Colombia	Bogota	H12	273	NEG	SAMN06629627	This study
KPC_51	2007_ST101	2007	Colombia	Bucaramanga	H20	101	NEG	SAMN06629628	This study
KPC_52	2007_ST17	2007	Colombia	Bucaramanga	H20	17	NEG	SAMN06629629	This study
KPC_53	2007_ST17	2007	Colombia	Bucaramanga	H20	17	NEG	SAMN06629630	This study
KPC_54	2007_ST45	2007	Colombia	Cali	H21	45	NEG	SAMN06629631	This study
KPC_55	2007_ST101	2007	Colombia	Cali	H21	101	KPC-2	SAMN06629632	This study
KPC_56	2007_ST101	2007	Colombia	Barranquilla	H2	101	NEG	SAMN06629633	This study
KPC_57	2007_ST45	2007	Colombia	Medellin	H16	45	NEG	SAMN06629634	This study
KPC_58	2007_ST14	2007	Colombia	Medellin	H16	14	KPC-2	SAMN06629635	This study
KPC_59	2007_ST14	2007	Colombia	Medellin	H16	14	KPC-2	SAMN06629636	This study
KPC_60	2007_NEW ST9	2007	Colombia	Cali	H11	NEW ST9	NEG	SAMN06629637	This study
KPC_61	2007_ST1681	2008	Colombia	Cali	H11	1681	NEG	SAMN06629638	This study
KPC_62	2008_ST14	2008	Colombia	Medellin	H16	14	KPC-2	SAMN06629639	This study
KPC_63	2008_ST512	2009	Colombia	Medellin	H16	512	KPC-3	SAMN06629640	This study
KPC_64	2009_ST147	2009	Colombia	Barranquilla	H2	147	KPC-2	SAMN06629641	This study
KPC_65	2009_ST258	2009	Colombia	Bogota	H12	258	KPC-3	SAMN06629642	This study
KPC_66	2009_ST258	2009	Colombia	Bogota	H14	258	KPC-2	SAMN06629643	This study
KPC_67	2009_ST622	2009	Colombia	Cali	H11	622	KPC-2	SAMN06629644	This study
KPC_68	2009_ST512	2009	Colombia	Medellin	H16	512	KPC-3	SAMN06629645	This study
KPC_69	2009_ST512	2009	Colombia	Medellin	H16	512	KPC-3	SAMN06629646	This study
KPC_70	2009_ST258	2009	Colombia	Bucaramanga	H9	258	KPC-3	SAMN06629647	This study
KPC_71	2009_ST258	2009	Colombia	Bucaramanga	H20	258	KPC-3	SAMN06629648	This study
KPC_72	2009_ST258	2010	Colombia	Ibague	H8	258	KPC-3	SAMN06629649	This study
KPC_73	2010_ST20	2010	Colombia	Barranquilla	H2	20	KPC-2	SAMN06629650	This study
KPC_74	2010_ST258	2010	Colombia	Bogota	H12	258	KPC-3	SAMN06629651	This study
KPC_75	2010_ST258	2010	Colombia	Cali	H11	258	KPC-3	SAMN06629652	This study
KPC_76	2010_ST12	2010	Colombia	Medellin	H16	12	NEG	SAMN06629653	This study
KPC_77	2010_ST258	2010	Colombia	Bucaramanga	H7	258	KPC-3	SAMN06629654	This study
KPC_78	2010_ST258	2010	Colombia	Bucaramanga	H20	258	KPC-3	SAMN06629655	This study
KPC_79	2010_ST258	2010	Colombia	Ibague	H8	258	KPC-3	SAMN06629656	This study
KPC_80	2010_ST101	2011	Colombia	Pereira	H24	101	KPC-2	SAMN06629657	This study
KPC_81	2011_ST668	2011	Colombia	Barranquilla	H2	668	KPC-2	SAMN06629658	This study
KPC_82	2011_ST258	2011	Colombia	Bogota	H5	258	KPC-3	SAMN06629659	This study
KPC_83	2011_ST258	2011	Colombia	Bogota	H10	258	KPC-3	SAMN06629660	This study
KPC_84	2011_ST258	2011	Colombia	Bogota	H17	258	KPC-3	SAMN06629661	This study
KPC_85	2011_ST1440	2011	Colombia	Cali	H11	1440	KPC-2	SAMN06629662	This study
KPC_86	2011_ST258	2011	Colombia	Medellin	H13	258	KPC-3	SAMN06629663	This study
KPC_87	Not on tree	2011	Colombia	Bucaramanga	H7	258	KPC-3	*not available	This study
KPC_88	2011_ST258	2011	Colombia	Bucaramanga	H20	258	KPC-3	SAMN06629665	This study
KPC_89	2011_ST258	2011	Colombia	Ibague	H8	258	KPC-3	SAMN06629666	This study
KPC_90	2011_ST505	2011	Colombia	Neiva	H15	505	KPC-2	SAMN06629667	This study
KPC_91	2011_ST258	2012	Colombia	Pereira	H24	258	KPC-3	SAMN06629668	This study
KPC_92	2012_ST258	2012	Colombia	Bogota	H5	258	KPC-3	SAMN06629669	This study
KPC_93	2012_ST36	2012	Colombia	Medellin	H1	36	KPC-2	SAMN06629670	This study
KPC_94	2012_ST15	2012	Colombia	Cali	H4	15	KPC-2	SAMN06629671	This study
KPC_95	2012_ST147	2012	Colombia	Bucaramanga	H7	147	KPC-2	SAMN06629672	This study
KPC_96	2012_ST258	2007	Colombia	Neiva	H15	258	KPC-3	SAMN06629673	This study
KPC_97	2007_ST101	2013	Colombia	Ibague	H8	101	KPC-2	SAMN06629674	This study
KPC_98	2013_ST560	2013	Colombia	Bogota	H14	560	KPC-2	SAMN06629675	This study
KPC_99	2013_ST13	2013	Colombia	Bogota	H18	13	KPC-2	SAMN06629676	This study
KPC_100	2013_ST348	2013	Colombia	Cali	H3	348	KPC-2	SAMN06629677	This study

KPC_101	2013_ST258	2013	Colombia	Cali	H4	258	KPC-3	SAMN06629678	This study
KPC_102	2013_ST22	2013	Colombia	Medellin	H13	22	KPC-2	SAMN06629679	This study
KPC_103	2013_ST258	2013	Colombia	Bucaramanga	H9	258	KPC-2	SAMN06629680	This study
KPC_104	2013_ST528	2013	Colombia	Ibague	H8	528	NEG	SAMN06629681	This study
KPC_105	2013_ST528	2013	Colombia	Neiva	H15	528	KPC-2	SAMN06629682	This study
KPC_106	2014_ST36	2014	Colombia	Medellin	H13	36	KPC-2	SAMN06629683	This study
KPC_107	2014_ST629	2014	Colombia	Cali	H4	629	KPC-2	SAMN06629684	This study
KPC_108	2014_ST13	2014	Colombia	Bogota	H18	13	KPC-2	SAMN06629685	This study
KPC_109	2010_ST979	2010	Colombia	Medellin	H16	979	KPC-2	SAMN06629686	This study
KPC_110	2012_ST14	2012	Colombia	Medellin	H16	14	KPC-2	SAMN06629687	This study
KPC_111	2013_ST560	2013	Colombia	Neiva	H15	560	KPC-2	SAMN06629688	This study
KPC_112	2005_ST378	2005	Colombia	Barranquilla	H2	378	NEG	SAMN06629689	This study
KPC_113	2004_ST273	2004	Colombia	Medellin	H23	273	NEG	SAMN06629690	This study
KPC_114	2004_ST151	2004	Colombia	Cartagena	H19	151	NEG	SAMN06629691	This study
KPC_115	2004_ST584	2004	Colombia	Cartagena	H19	584	NEG	SAMN06629692	This study
KPC_116	2003_ST1584	2003	Colombia	Medellin	H1	1584	NEG	SAMN06629693	This study
KPC_117	2003_ST834	2003	Colombia	Barranquilla	H2	834	NEG	SAMN06629694	This study
KPC_118	2003_ST14	2003	Colombia	Barranquilla	H2	14	NEG	SAMN06629695	This study
KPC_119	2003_ST252	2003	Colombia	Barranquilla	H2	252	NEG	SAMN06629696	This study
KPC_120	2002_NEW ST10	2002	Colombia	Cali	H11	NEW ST10	NEG	SAMN06629697	This study
KPC_121	2002_ST20	2002	Colombia	Cali	H11	20	NEG	SAMN06629698	This study
KPC_122	2002_ST17	2002	Colombia	Cali	H11	17	NEG	SAMN06629699	This study
KPC_123	2002_ST30	2002	Colombia	Bogota	H6	30	NEG	SAMN06629700	This study
KPC_124	2002_ST138	2002	Colombia	Medellin	H16	138	NEG	SAMN06629701	This study
KPC_125	2002_ST35	2002	Colombia	Medellin	H16	35	NEG	SAMN06629702	This study
KPC_126	2002_ST8	2002	Colombia	Medellin	H16	8	NEG	SAMN06629703	This study
KPC_127	2002_ST477	2002	Colombia	Medellin	H16	477	NEG	SAMN06629704	This study
KPC_128	2005_NEW ST11	2005	Colombia	Medellin	H16	NEW ST11	NEG	SAMN06629705	This study
KPC_129	2005_ST1681	2005	Colombia	Bucaramanga	H9	1681	NEG	SAMN06629706	This study
KPC_130	2003_ST1681	2003	Colombia	Bucaramanga	H7	1681	NEG	SAMN06629707	This study
KPC_131	2003_ST105	2003	Colombia	Bucaramanga	H9	105	NEG	SAMN06629708	This study
KPC_132	2002_NEW ST12	2002	Colombia	Bogota	H22	NEW ST12	NEG	SAMN06629709	This study
KPC_133	2002_NEW ST9	2002	Colombia	Cali	H11	NEW ST9	NEG	SAMN06629710	This study
AUS 02		2002	Australia	N/S	N/A	14	ND	ERS005788	10.1073/pnas.1501049112
BRA 09		2009	Brazil	N/S	N/A	442	KPC-2	CP003999	10.1186/1471-2164-15-54
CAN 09 A		2009	Canada	N/S	N/A	512	KPC-3	SRS692096	10.1371/journal.pone.0133727
CAN 09 B		2009	Canada	N/S	N/A	20	KPC-3	SRS692097	10.1371/journal.pone.0133727
CHN 11		2011	China	N/S	N/A	11	KPC-2	CP003200	10.1128/JB.00043-12
E.cloacae 11		2011	USA	N/S	N/A	97	POS	CP008897	10.1126/scitranslmed.3009845
FRA 09		2009	France	N/S	N/A	340	NEG	ERS503310	10.1093/jac/dkv467
GRC 10 A		2010	Greece	N/S	N/A	258	KPC-2	ERS822446	10.1093/jac/dkv467
GRC 10 B		2010	Greece	N/S	N/A	147	KPC-2	ERS822448	10.1093/jac/dkv467
GRC 13 A		2013	Greece	N/S	N/A	258	KPC-2	ERS822465	10.1093/jac/dkv467
GRC 13 B		2013	Greece	N/S	N/A	258	KPC-2	ERS822467	10.1093/jac/dkv467
GRC 14 A		2014	Greece	N/S	N/A	258	KPC-2	ERS822456	10.1093/jac/dkv467
GRC 14 B		2014	Greece	N/S	N/A	258	KPC-2	ERS822460	10.1093/jac/dkv467
GRC 14 C		2014	Greece	N/S	N/A	258	KPC-2	ERS822464	10.1093/jac/dkv467
GRC 14 D		2014	Greece	N/S	N/A	258	KPC-2	ERS822466	10.1093/jac/dkv467
GRC 14 E		2014	Greece	N/S	N/A	258	KPC-2	ERS822472	10.1093/jac/dkv467
GRC 14 F		2014	Greece	N/S	N/A	258	KPC-2	ERS822473	10.1093/jac/dkv467
IND 07		2007	India	N/S	N/A	101	KPC-2	SRS692114	10.1371/journal.pone.0133727
ISR 07 A		2007	Israel	N/S	N/A	277	KPC-2	SRS692121	10.1371/journal.pone.0133727

ISR 07 B	2007	Israel	N/S	N/A	340	KPC-2	SRS692122	10.1371/journal.pone.0133727
ISR 07 C	2007	Israel	N/S	N/A	376	KPC-2	SRS692123	10.1371/journal.pone.0133727
***K.oxytoca* 12**	2012	USA	N/S	N/A	151	POS	CP008788	10.1126/scitranslmed.3009845
***K.pneumoniae* KPI 73**	1973	Indonesia	N/S	N/A	67	ND	ACZD01	10.1073/pnas.1501049112
***K.pneumoniae* KPI0 04**	2004	Vietnam	N/S	N/A	755*	ND	ERS011815/ERR025476	10.1073/pnas.1501049112
K.pneumoniae KPIV 06	2006	USA	N/S	N/A	DLV-761	ND	ERS012010/ERR025631	10.1073/pnas.1501049112
***K.variicola* KPIII 06**	2003	South America	N/S	N/A	745*	ND	NC_013850	10.1073/pnas.1501049112
SGP 96	1996	Singapore	N/S	N/A	42	ND	ERS011903	10.1073/pnas.1501049112
USA 01	2001	USA	N/S	N/A	334	KPC-3	SRS692233	10.1371/journal.pone.0133727
USA 04	2004	USA	N/S	N/A	42	KPC-2	SRS692223	10.1371/journal.pone.0133727
USA 10 A	2010	NJ, USA	N/S	N/A	258	KPC-3	CP006918	10.1073/pnas.1321364111
USA 10 B	2010	NJ, USA	N/S	N/A	258	KPC-3	NZ_CP006923.1	10.1073/pnas.1321364111
VNM 04	2004	Vietnam	N/S	N/A	42	ND	ERS011813	10.1073/pnas.1501049112
VNM 05	2005	Vietnam	N/S	N/A	147	ND	ERS011837	10.1073/pnas.1501049112

N/S: Not specified; *Low quality sequences were not including in the tree or uploaded to Genbank.

Table 4-3. Masked genomic regions for the Colombian *K.pneumoniae* isolates phylogeny

Excluded mobile element	Position in reference genome
CPS locus	1793933..1815027
Transposase	complement(676658..677662)
Transposase	739991..740143
Integrating conjugative element, PFGI_1 class, parb	743425..745047
Transposase	749878..751077
Conjugative coupling factor trad, PFGI-1 class	759723..761822
Integrating conjugative element membrane protein	762082..762840
Integrating conjugative element membrane protein, PFL 4702 family	765366..765719
Conjugative transfer region protein, TIGR03750 family	765729..766100
Conjugative transfer atpase, PFL_4706 family	769488..772262
Transposase	921325..922319
Transposase	2094036..2094959
Transposase	2102396..2103376
Transposase	complement(2537482..2538462)
TIGR00156 family protein	2538618..2539022
Transposase	complement(2636592..2637515)
Transposase iskpn26	27510416..2752031
Transposase iskpn18	2979492..2979734
Transposase iskpn18	2979731..2980610
Transposase iskpn18	4384273..4384515
Transposase iskpn18	4384512..4385390
Transposase	complement(4690518..4691498)
Transposase	complement(4693764..4694243)
Transposase	complement(4694609..4694875)
Transposase	complement(4861155..4862653)
Transposase	4865007..4866206
Transposase	4884691..4885890
Transposase	complement(5085191..5086171)
Integrase	219732..220518
Integrase	462993..464219
Integrase	796125..797072
Integrase	complement(909549..910154)
Integrase	complement(1237948..1238973)
Integrase	1392298..1393548
Integrase	complement(1850216..1851001)
Integrase	complement(1901222..1902496)
Integrase	complement(3004617..3005798)
Integrase iskpn1	complement(3135291..3136076)
Integrase iskpn1	3512862..3513914
Integrase	complement(3841398..3842183)
Integrase	complement(3985299..3986345)
Integrase	4001080..4002243
Integrase	4217459..4218649
Integrase	complement(4704820..4706082)
Integrase iskpn1	complement(4881099..4881900)
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS02285; phage(gi824479609)	462993..464219
PHAGE_Entero_P4_NC_001609: transcriptional regulator; KPNJ1_RS02295; phage(gi9627517)	465435..465719
PHAGE_Escher_HK75_NC_016160: kila; KPNJ1_RS02300; phage(gi356870724)	465730..466509
PHAGE_Thermo_THSA_485A_NC_018264: protein of unknown function DUF927; KPNJ1_RS02330; phage(gi397912648)	468445..470580
PHAGE_Entero_sfi_NC_027339: N/A; KPNJ1_RS02345; phage(gi849250290)	472083..473249
PHAGE_Clostr_phi3626_NC_003524: putative prohead protease; KPNJ1_RS02350; phage(gi20065968)	473301..473861
PHAGE_Shigel_sfiv_NC_022749: portal protein; KPNJ1_RS02355; phage(gi557307529)	473863..475104
PHAGE_Entero_mepx1_NC_019709: head-tail connector II; KPNJ1_RS02360; phage(gi428781879)	475101..475436
PHAGE_Shigel_sfii_NC_021857: head-tail connector protein; KPNJ1_RS02365; phage(gi526244642)	475433..475732
PHAGE_Burkho_phie125_NC_003309: putative class I holin; KPNJ1_RS02370; phage(gi17975232)	475732..476175
PHAGE_Psychr_Psymv2_NC_023734: terminase small subunit; KPNJ1_RS02380; phage(gi593779760)	476451..476807
PHAGE_Geobac_virus_E2_NC_009552: putative terminase large subunit; KPNJ1_RS02385; phage(gi148747729)	476791..478452
PHAGE_Aeromo_44RR2_8t_NC_005135: hypothetical protein ST44RRORF234c; KPNJ1_RS02410; phage(gi37651715)	complement(481337..482788)
PHAGE_Burkho_phi1026b_NC_005284: gp58; KPNJ1_RS06300; phage(gi38707948)	1234156..1235040
PHAGE_Salmon_RE_2010_NC_019488: integrase; KPNJ1_RS06315; phage(gi418489683)	complement(1237948..1238973)
PHAGE_Salmon_RE_2010_NC_019488: repressor; KPNJ1_RS06320; phage(gi418489684)	complement(1238976..1239605)
PHAGE_Salmon_RE_2010_NC_019488: excisionase; KPNJ1_RS06325; phage(gi418489685)	1239728..1239970
PHAGE_Salmon_RE_2010_NC_019488: regulatory protein; KPNJ1_RS06330; phage(gi418489686)	1240003..1240512
PHAGE_Salmon_RE_2010_NC_019488: hypothetical protein; KPNJ1_RS06335; phage(gi418489688)	1240684..1241022
PHAGE_Salmon_RE_2010_NC_019488: hypothetical protein; KPNJ1_RS06340; phage(gi418489689)	1241090..1241323
PHAGE_Salmon_RE_2010_NC_019488: zinc-finger containing protein; KPNJ1_RS06345; phage(gi418489690)	1241323..1241550
PHAGE_Salmon_RE_2010_NC_019488: DNA adenine methyltransferase; KPNJ1_RS06350; phage(gi418489691)	1241547..1242398
PHAGE_Salmon_RE_2010_NC_019488: replication protein; KPNJ1_RS06355; phage(gi418489692)	1242395..1244779
PHAGE_Salmon_RE_2010_NC_019488: hypothetical protein; KPNJ1_RS06360; phage(gi418489693)	1244942..1245130
PHAGE_Salmon_RE_2010_NC_019488: SOS induction modulator; KPNJ1_RS06365; phage(gi418489694)	1245142..1245375
PHAGE_Entero_phifl3a_NC_013648: hypothetical protein; KPNJ1_RS06385; phage(gi281416218)	1248743..1249012
PHAGE_Salmon_RE_2010_NC_019488: capsid packaging protein; KPNJ1_RS06390; phage(gi418489696)	complement(1249069..1250112)
PHAGE_Salmon_RE_2010_NC_019488: terminase atpase subunit; KPNJ1_RS06395; phage(gi418489697)	complement(1250112..1251875)
PHAGE_Salmon_RE_2010_NC_019488: capsid scaffolding protein; KPNJ1_RS06400; phage(gi418489698)	1252016..1252849

PHAGE_Salmon_RE_2010_NC_019488: major capsid protein; KPNJ1_RS06405; phage(gi418489699)	1252866..1253918
PHAGE_Salmon_RE_2010_NC_019488: terminase endonuclease subunit; KPNJ1_RS06410; phage(gi418489700)	1253922..1254575
PHAGE_Salmon_RE_2010_NC_019488: head completion-stabilization protein; KPNJ1_RS06415; phage(gi418489701)	1254671..1255135
PHAGE_Salmon_RE_2010_NC_019488: tail component protein; KPNJ1_RS06420; phage(gi418489702)	1255135..1255338
PHAGE_Salmon_RE_2010_NC_019488: lysis protein S; KPNJ1_RS06425; phage(gi418489703)	1255339..1255557
PHAGE_Salmon_RE_2010_NC_019488: lysozyme; KPNJ1_RS06430; phage(gi418489704)	1255538..1256047
PHAGE_Salmon_RE_2010_NC_019488: lysozyme subunit B; KPNJ1_RS06440; phage(gi418489705)	1256432..1256860
PHAGE_Salmon_RE_2010_NC_019488: tail protein; KPNJ1_RS06445; phage(gi418489706)	1256956..1257378
PHAGE_Salmon_RE_2010_NC_019488: tail completion protein; KPNJ1_RS06450; phage(gi418489707)	1257371..1257817
PHAGE_Salmon_RE_2010_NC_019488: hypothetical protein; KPNJ1_RS06455; phage(gi418489708)	complement(1257840..1258706)
PHAGE_Salmon_RE_2010_NC_019488: baseplate assembly protein V; KPNJ1_RS06460; phage(gi418489709)	1258801..1259373
PHAGE_Salmon_RE_2010_NC_019488: baseplate wedge subunit; KPNJ1_RS06465; phage(gi418489710)	1259370..1259732
PHAGE_Salmon_RE_2010_NC_019488: baseplate assembly protein J; KPNJ1_RS06470; phage(gi418489711)	1259719..1260627
PHAGE_Salmon_RE_2010_NC_019488: tail protein I; KPNJ1_RS06475; phage(gi418489712)	1260620..1261291
PHAGE_Bacill_G_NC_023719: gp353; KPNJ1_RS06480; phage(gi593777809)	1261293..1263242
PHAGE_Salico_cgphi29_NC_020844: hypothetical protein; KPNJ1_RS06485; phage(gi472340194)	1263252..1264370
PHAGE_Salmon_RE_2010_NC_019488: tail fiber protein; KPNJ1_RS06490; phage(gi418489713)	1264422..1265495
PHAGE_Salmon_RE_2010_NC_019488: major tail sheath protein; KPNJ1_RS06495; phage(gi418489720)	1265644..1266816
PHAGE_Salmon_RE_2010_NC_019488: major tail tube protein; KPNJ1_RS06500; phage(gi418489721)	1266826..1267341
PHAGE_Salmon_RE_2010_NC_019488: tail protein E; KPNJ1_RS06505; phage(gi418489722)	1267394..1267693
PHAGE_Salmon_RE_2010_NC_019488: tail protein E'; KPNJ1_RS06510; phage(gi418489723)	1267708..1267827
PHAGE_Salmon_RE_2010_NC_019488: tail protein; KPNJ1_RS06520; phage(gi418489725)	1270448..1270933
PHAGE_Acanth_moumouvirus_NC_020104: exodeoxyribonuclease VII large subuni; KPNJ1_RS07080; phage(gi441432687)	complement(1387444..1388835)
PHAGE_Phaeoc_virus_NC_021312: GMP reductase; KPNJ1_RS07085; phage(gi508181884)	1388994..1390460
PHAGE_Halovi_HGTV_1_NC_021328: carbamoyl phosphate synthase small subunit; KPNJ1_RS07090; phage(gi509140368)	1390528..1392105
PHAGE_Entero_phiv10_NC_007804: putative integrase; KPNJ1_RS07095; phage(gi89152450)	1392298..1393548
PHAGE_Entero_phiv10_NC_007804: putative adenine methylase; KPNJ1_RS07110; phage(gi89152451)	complement(1393932..1394525)
PHAGE_Salmon_SPN1S_NC_016761: hypothetical protein; KPNJ1_RS07115; phage(gi374531225)	complement(1394522..1394680)
PHAGE_Entero_phiv10_NC_007804: putative transcriptional activator; KPNJ1_RS07120; phage(gi155370095)	complement(1394673..1394966)
PHAGE_Entero_phiv10_NC_007804: putative transcriptional regulator; KPNJ1_RS07125; phage(gi89152453)	complement(1395076..1395324)
PHAGE_Entero_epsilon15_NC_004775: rect; KPNJ1_RS07130; phage(gi30387413)	complement(1395376..1396398)
PHAGE_Entero_epsilon15_NC_004775: hypothetical protein epsilon15p35; KPNJ1_RS07135; phage(gi30387414)	complement(1396408..1397307)
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p38; KPNJ1_RS07140; phage(gi89152455)	complement(1397304..1397603)
PHAGE_Entero_phiv10_NC_007804: putative transcriptional repressor; KPNJ1_RS07145; phage(gi89152457)	complement(1397970..1398551)
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p41; KPNJ1_RS07150; phage(gi89152458)	1398705..1398938
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p42; KPNJ1_RS07155; phage(gi89152475)	1399085..1399294
PHAGE_Entero_phiv10_NC_007804: putative primosomal protein; KPNJ1_RS07160; phage(gi89152459)	1399294..1400061
PHAGE_Entero_phiv10_NC_007804: putative replication protein p; KPNJ1_RS07165; phage(gi155370096)	1400058..1400843
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p45; KPNJ1_RS07170; phage(gi89152461)	1400963..1401310
PHAGE_Vibrio_pyd38_A_NC_021534: hypothetical protein; KPNJ1_RS07175; phage(gi514051016)	1401503..1401913
PHAGE_Burkho_bcepny3_NC_009604: bcepny3gp60; KPNJ1_RS07190; phage(gi149882961)	1402507..1403250
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p52; KPNJ1_RS07195; phage(gi89152466)	1403421..1403633
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p53; KPNJ1_RS07200; phage(gi89152467)	1403630..1404298
PHAGE_Escher_HK639_NC_016158: hypothetical protein; KPNJ1_RS07205; phage(gi356870632)	1404291..1404530
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p55; KPNJ1_RS07210; phage(gi89152469)	1404530..1404868
PHAGE_Salmon_SSU5_JQ965645: hypothetical protein; KPNJ1_RS07215; phage(gi390013938)	1404943..1405200
PHAGE_Entero_phiv10_NC_007804: putative terminase small subunit; KPNJ1_RS07220; phage(gi155370097)	1405278..1405862
PHAGE_Entero_phiv10_NC_007804: putative terminase large subunit; KPNJ1_RS07225; phage(gi89152423)	1405859..1407334
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p03; KPNJ1_RS07230; phage(gi89152424)	complement(1407401..1407712)
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p06; KPNJ1_RS07235; phage(gi89152427)	1408514..1408720
PHAGE_Entero_phiv10_NC_007804: putative head-to-tail-joining protein; KPNJ1_RS07240; phage(gi89152428)	1408735..1410417
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p08; KPNJ1_RS07245; phage(gi89152429)	1410414..1410710
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p09; KPNJ1_RS07250; phage(gi89152430)	1410713..1411393
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p10; KPNJ1_RS07255; phage(gi89152431)	1411408..1412394
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p11; KPNJ1_RS07260; phage(gi89152432)	1412448..1412885
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p12; KPNJ1_RS07265; phage(gi89152433)	1412896..1413237
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p13; KPNJ1_RS07270; phage(gi89152434)	1413288..1413611
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p14; KPNJ1_RS07275; phage(gi89152435)	1413611..1414216
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p15; KPNJ1_RS07280; phage(gi89152436)	1414216..1416714
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p16; KPNJ1_RS07285; phage(gi89152437)	1416714..1417178
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p17; KPNJ1_RS07290; phage(gi89152438)	1417178..1417717
PHAGE_Entero_phiv10_NC_007804: hypothetical structural protein; KPNJ1_RS07295; phage(gi89152439)	1417728..1420559
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p19; KPNJ1_RS07300; phage(gi89152440)	1420559..1422469
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p20; KPNJ1_RS07305; phage(gi89152441)	1422469..1425234
PHAGE_Entero_phiv10_NC_007804: putative anti-immunity protein; KPNJ1_RS07310; phage(gi89152443)	complement(1425846..1426535)
PHAGE_Entero_phiv10_NC_007804: hypothetical protein phiv10p24; KPNJ1_RS07315; phage(gi89152444)	complement(1426850..1427146)
PHAGE_Entero_phiv10_NC_007804: putative tail fiber; KPNJ1_RS07320; phage(gi89152445)	1427303..1428253
PHAGE_Entero_phiv10_NC_007804: putative integral membrane protein; KPNJ1_RS07330; phage(gi89152446)	1429954..1430358
PHAGE_Entero_phiv10_NC_007804: putative holin; KPNJ1_RS07335; phage(gi89152447)	1430345..1430650
PHAGE_Entero_phiv10_NC_007804: putative endolysin; KPNJ1_RS07340; phage(gi89152448)	1430640..1431269
PHAGE_Entero_phiv10_NC_007804: putative lysis accessory protein; KPNJ1_RS07345; phage(gi89152449)	1431266..1431748
PHAGE_Aggreg_S1249_NC_013597: hypothetical protein; KPNJ1_RS15010; phage(gi273809558)	complement(2980851..2981996)

PHAGE_Rhizob_rheph06_NC_027296: N/A; KPNJ1_RS15015; phage(gi848469507)	complement(2982000..2982440)
PHAGE_Psychr_pow20_A_NC_020841: hypothetical protein; KPNJ1_RS15025; phage(gi472339833)	complement(2982921..2983427)
PHAGE_Pseudo_SPM_1_NC_023596: hypothetical protein; KPNJ1_RS15030; phage(gi589892610)	complement(2983424..2983843)
PHAGE_Vibrio_vb_vchm_138_NC_019518: putative major capsid protein; KPNJ1_RS15040; phage(gi422936575)	complement(2984133..2985074)
PHAGE_Pectob_ZF40_NC_019522: putative head protein; KPNJ1_RS15050; phage(gi422936684)	complement(2985584..2986618)
PHAGE_Bdello_phi1422_NC_019525: putative head protein; KPNJ1_RS15055; phage(gi422936860)	complement(2986838..2987386)
PHAGE_Vibrio_CP_T1_NC_019457: putative portal protein; KPNJ1_RS15060; phage(gi418489212)	complement(2987442..2988893)
PHAGE_Edward_FW_3_NC_026611: N/A; KPNJ1_RS15065; phage(gi764162249)	complement(2989131..2990531)
PHAGE_Shigel_POCJ13_NC_025434: N/A; KPNJ1_RS15070; phage(gi725949747)	complement(2990482..2991234)
PHAGE_Shigel_sfii_NC_021857: i-spanin; KPNJ1_RS15080; phage(gi526244687)	complement(2991891..2992280)
PHAGE_Entero_mep460_NC_019716: endolysin; KPNJ1_RS15085; phage(gi428782372)	complement(2992277..2992807)
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS15090; phage(gi824479635)	complement(2992810..2993058)
PHAGE_Salmon_Fels_1_NC_010391: bacteriophage antitermination protein Q; KPNJ1_RS15105; phage(gi169257178)	complement(2993464..2994246)
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0902.Fels1; KPNJ1_RS15110; phage(gi169257177)	complement(2994243..2994719)
PHAGE_Salmon_Fels_1_NC_010391: putative bacteriophage DNA primase; KPNJ1_RS15115; phage(gi169257176)	complement(2994716..2995492)
PHAGE_Salmon_Fels_1_NC_010391: putative helicase; KPNJ1_RS15120; phage(gi169257175)	complement(2995680..2997293)
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0899.1n.Fels1; KPNJ1_RS15125; phage(gi169257173)	complement(2997647..2997940)
PHAGE_Entero_IME10_NC_019501: repressor protein; KPNJ1_RS15130; phage(gi422934295)	2998234..2998902
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0898.7n.Fels1; KPNJ1_RS15135; phage(gi169257170)	2999073..2999387
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0898.6n.Fels1; KPNJ1_RS15140; phage(gi169257169)	2999380..2999568
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0898.3n.Fels1; KPNJ1_RS15145; phage(gi169257166)	2999738..3000103
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0898.2n.Fels1; KPNJ1_RS15150; phage(gi169257165)	3000096..3000350
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0898.1n.Fels1; KPNJ1_RS15160; phage(gi169257164)	3000537..3000962
PHAGE_Entero_phip27_NC_003356: putative serine protease; KPNJ1_RS15170; phage(gi18249869)	3001150..3001977
PHAGE_Entero_phip27_NC_003356: hypothetical protein P27p06; KPNJ1_RS15175; phage(gi18249870)	3002082..3002600
PHAGE_Entero_phip27_NC_003356: hypothetical protein P27p14; KPNJ1_RS15180; phage(gi18249878)	3002606..3003316
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0896.1n.Fels1; KPNJ1_RS15185; phage(gi169257161)	3003306..3003530
PHAGE_Salmon_Fels_1_NC_010391: hypothetical protein STM0895.1n.Fels1; KPNJ1_RS15200; phage(gi169257158)	3004288..3004434
PHAGE_Entero_Sf101_NC_027398: N/A; KPNJ1_RS15205; phage(gi849122281)	3004445..3004636
PHAGE_Entero_HK544_NC_019767: integrase; KPNJ1_RS15210; phage(gi428783241)	complement(3004617..3005798)
PHAGE_Cafete_BV_PW1_NC_014637: putative HD superfamily phosphohydrolase; KPNJ1_RS15220; phage(gi310831117)	complement(3006742..3008274)
PHAGE_Tricho_ni_ascovirus_2c_NC_008518: hypothetical protein TNAV2c_gp071; KPNJ1_RS15225; phage(gi116326757)	complement(3008491..3009252)
PHAGE_Entero_Fels_2_NC_010463: P2 gpd-like tail protein; KPNJ1_RS17550; phage(gi169936019)	complement(3476889..3477989)
PHAGE_Entero_Fels_2_NC_010463: P2 gpu-like tail protein; KPNJ1_RS17555; phage(gi169936020)	complement(3477986..3478471)
PHAGE_Entero_Fels_2_NC_010463: P2 gpt-like tail protein; KPNJ1_RS17560; phage(gi169936021)	complement(3478468..3481095)
PHAGE_Entero_Fels_2_NC_010463: P2 gpe-like tail protein; KPNJ1_RS17565; phage(gi169936023)	complement(3481222..3481521)
PHAGE_Entero_Fels_2_NC_010463: P2 gpfii-like protein; KPNJ1_RS17570; phage(gi169936024)	complement(3481574..3482089)
PHAGE_Entero_Fels_2_NC_010463: P2 gpfi-like protein; KPNJ1_RS17575; phage(gi169936025)	complement(3482099..3483271)
PHAGE_Entero_Fels_2_NC_010463: P2 gph-like protein; KPNJ1_RS17580; phage(gi169936030)	complement(3483410..3484486)
PHAGE_Burkho_bcepmu_NC_005882: gp52; KPNJ1_RS17595; phage(gi48696962)	complement(3485451..3488402)
PHAGE_Entero_Fels_2_NC_010463: P2 gpi-like baseplate assembly protein; KPNJ1_RS17600; phage(gi169936031)	complement(3488404..3489003)
PHAGE_Entero_Fels_2_NC_010463: P2 gpj-like baseplate assembly protein; KPNJ1_RS17605; phage(gi169936032)	complement(3488996..3489904)
PHAGE_Entero_Fels_2_NC_010463: P2 gpw-like baseplate protein; KPNJ1_RS17610; phage(gi169936033)	complement(3489891..3490253)
PHAGE_Entero_Fels_2_NC_010463: P2 gpv-like protein; KPNJ1_RS17615; phage(gi169936034)	complement(3490250..3490822)
PHAGE_Entero_Fels_2_NC_010463: P2 gps-like tail completion protein; KPNJ1_RS17625; phage(gi169936035)	complement(3491606..3492052)
PHAGE_Entero_Fels_2_NC_010463: P2 gpr-like tail completion protein; KPNJ1_RS17630; phage(gi169936036)	complement(3492045..3492476)
PHAGE_Entero_Fels_2_NC_010463: P2 lysc-like protein; KPNJ1_RS17635; phage(gi169936037)	complement(3492439..3492585)
PHAGE_Entero_Fels_2_NC_010463: P2 lysb-like protein; KPNJ1_RS17640; phage(gi169936038)	complement(3492572..3493000)
PHAGE_Entero_Fels_2_NC_010463: endolysin; KPNJ1_RS17650; phage(gi169936041)	complement(3493385..3493894)
PHAGE_Entero_Fels_2_NC_010463: lysis protein; KPNJ1_RS17655; phage(gi169936042)	complement(3493875..3494090)
PHAGE_Entero_Fels_2_NC_010463: P2 gpx-like tail protein; KPNJ1_RS17660; phage(gi169936043)	complement(3494094..3494297)
PHAGE_Entero_Fels_2_NC_010463: P2 gpl-like protein; KPNJ1_RS17665; phage(gi169936044)	complement(3494297..3494761)
PHAGE_Entero_Fels_2_NC_010463: P2 gpm-like protein; KPNJ1_RS17670; phage(gi169936045)	complement(3494857..3495507)
PHAGE_Entero_Fels_2_NC_010463: P2 gpn-like major capsid protein; KPNJ1_RS17675; phage(gi169936046)	complement(3495511..3496569)
PHAGE_Entero_Fels_2_NC_010463: P2 gpo-like protein; KPNJ1_RS17680; phage(gi169936047)	complement(3496586..3497419)
PHAGE_Entero_Fels_2_NC_010463: P2 gpp-like protein; KPNJ1_RS17685; phage(gi169936048)	3497562..3499328
PHAGE_Entero_Fels_2_NC_010463: P2 gpq-like protein; KPNJ1_RS17690; phage(gi169936049)	3499328..3500353
PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2723.1.Fels2; KPNJ1_RS17695; phage(gi169936050)	complement(3500415..3502157)
PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2726.Fels2; KPNJ1_RS17700; phage(gi169936051)	complement(3502433..3502621)
PHAGE_Entero_Fels_2_NC_010463: tumb protein; KPNJ1_RS17705; phage(gi169936052)	complement(3503225..3503458)
PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2728.Fels2; KPNJ1_RS17710; phage(gi169936053)	complement(3503469..3503657)
PHAGE_Entero_Fels_2_NC_010463: P2 gpa-like protein; KPNJ1_RS17715; phage(gi169936054)	complement(3503811..3506225)
PHAGE_Entero_Fels_2_NC_010463: DNA adenine methylase-like protein; KPNJ1_RS17720; phage(gi169936055)	complement(3506222..3507079)
PHAGE_Entero_Fels_2_NC_010463: P2 gporf82-like protein; KPNJ1_RS17725; phage(gi169936056)	complement(3507076..3507303)

PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2732.Fels2; KPNJ1_RS17730; phage(gi169936057)	complement(3507303..3507536)
PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2733.Fels2; KPNJ1_RS17735; phage(gi169936058)	complement(3507604..3507945)
PHAGE_Entero_Fels_2_NC_010463: hypothetical protein STM2735.Fels2; KPNJ1_RS17740; phage(gi169936060)	complement(3507909..3508109)
PHAGE_Entero_Fels_2_NC_010463: bacteriophage regulatory protein CII; KPNJ1_RS17745; phage(gi169936061)	complement(3508117..3508626)
PHAGE_Entero_Fels_2_NC_010463: P2 CI-like protein; KPNJ1_RS17750; phage(gi169936063)	3509026..3509904
PHAGE_Bordet_BPP_1_NC_005357: reverse transcriptase; KPNJ1_RS17760; phage(gi41179367)	complement(3510959..3512443)
PHAGE_Entero_Fels_2_NC_010463: P2 Int-like protein; KPNJ1_RS17765; phage(gi169936064)	3512862..3513914
PHAGE_Cronob_ENT47670_NC_019927: putative tail protein; KPNJ1_RS19930; phage(gi431810495)	complement(3958810..3961287)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS19935; phage(gi431810526)	complement(3961274..3961669)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS19940; phage(gi431810518)	complement(3961666..3962136)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS19945; phage(gi431810519)	complement(3962136..3962612)
PHAGE_Cronob_ENT47670_NC_019927: putative tail protein; KPNJ1_RS19950; phage(gi431810494)	complement(3962655..3966101)
PHAGE_Vibrio_ICP1_NC_015157: putative antirepressor protein; KPNJ1_RS19960; phage(gi325171055)	complement(3966825..3967610)
PHAGE_Salmon_SPN3UB_NC_019545: hypothetical protein; KPNJ1_RS19965; phage(gi423262398)	complement(3967676..3968389)
PHAGE_Salmon_vb_soss_Oslo_NC_018279: Arc-like DNA binding domain protein; KPNJ1_RS19970; phage(gi399528776)	complement(3968379..3968549)
PHAGE_Salmon_SPN3UB_NC_019545: Arc-like DNA binding domain protein; KPNJ1_RS19975; phage(gi423262396)	3968649..3969008
PHAGE_Entero_mep235_NC_019708: hypothetical protein; KPNJ1_RS19985; phage(gi428781870)	complement(3969789..3970043)
PHAGE_Entero_mepx1_NC_019709: hypothetical protein; KPNJ1_RS19990; phage(gi428781933)	complement(3970046..3970801)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS19995; phage(gi431810508)	complement(3970977..3971654)
PHAGE_Cronob_ENT47670_NC_019927: major tail subunit; KPNJ1_RS20000; phage(gi431810520)	complement(3971707..3972459)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20005; phage(gi431810530)	complement(3972528..3972920)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20010; phage(gi431810532)	complement(3972917..3973342)
PHAGE_Vibrio_pyd38_A_NC_021534: hypothetical protein; KPNJ1_RS20015; phage(gi514050970)	complement(3973345..3973707)
PHAGE_Salmon_vb_soss_Oslo_NC_018279: hypothetical protein; KPNJ1_RS20020; phage(gi399528768)	complement(3973707..3973880)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20025; phage(gi431810531)	complement(3973880..3974260)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20035; phage(gi431810503)	complement(3974513..3975607)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20040; phage(gi431810525)	complement(3975619..3976047)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20045; phage(gi431810499)	complement(3976051..3977436)
PHAGE_Cronob_ENT47670_NC_019927: phage head morphogenesis; KPNJ1_RS20055; phage(gi431810507)	complement(3978027..3979031)
PHAGE_Cronob_ENT47670_NC_019927: hypothetical protein; KPNJ1_RS20060; phage(gi431810500)	complement(3979006..3980427)
PHAGE_Cronob_phies15_NC_018454: terminase large subunit; KPNJ1_RS20065; phage(gi401817592)	complement(3980440..3981912)
PHAGE_Escher_HK639_NC_016158: terminase small subunit; KPNJ1_RS20070; phage(gi356870601)	complement(3981912..3982514)
PHAGE_Salmon_SE1_NC_011802: Gp15; KPNJ1_RS20080; phage(gi219681237)	complement(3983320..3983784)
PHAGE_Escher_TL_2011c_NC_019442: lysozyme; KPNJ1_RS20085; phage(gi418487070)	complement(3983781..3984311)
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS20090; phage(gi824479635)	complement(3984314..3984562)
PHAGE_Spirop_1_C74_NC_003793: putative transposase; KPNJ1_RS20105; phage(gi20514680)	complement(3985299..3986345)
PHAGE_Gifsy_1_NC_010392: bacteriophage antiterminator protein Q; KPNJ1_RS20110; phage(gi169257244)	complement(3986573..3987262)
PHAGE_Xantho_OP1_NC_007709: HNH endonuclease family protein; KPNJ1_RS20115; phage(gi84662624)	complement(3987259..3987789)
PHAGE_Gifsy_1_NC_010392: conserved hypothetical bacteriophage protein; KPNJ1_RS20120; phage(gi169257245)	complement(3987782..3987919)
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS20125; phage(gi824479632)	complement(3987916..3988551)
PHAGE_Escher_HK639_NC_016158: nine; KPNJ1_RS20130; phage(gi356870663)	complement(3988544..3988714)
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS20135; phage(gi824479629)	complement(3988714..3989169)
PHAGE_Stx2_converting_1717_NC_011357: hypothetical protein Stx2-1717_gp30; KPNJ1_RS20140; phage(gi209447155)	complement(3989422..3989670)
PHAGE_Entero_cdti_NC_009514: Valyl-trna synthetase; KPNJ1_RS20145; phage(gi148609417)	complement(3989670..3990317)
PHAGE_Entero_ES18_NC_006949: gp41; KPNJ1_RS20150; phage(gi62362254)	complement(3990490..3991332)
PHAGE_Vibrio_pyd38_A_NC_021534: hypothetical protein; KPNJ1_RS20155; phage(gi514051016)	complement(3991439..3991945)
PHAGE_Entero_933W_NC_000924: Ren protein; KPNJ1_RS20160; phage(gi9632496)	complement(3991942..3992235)
PHAGE_Entero_HK022_NC_002166: gene P protein; KPNJ1_RS20165; phage(gi9634151)	complement(3992235..3993665)
PHAGE_Cronob_ENT47670_NC_019927: replication protein; KPNJ1_RS20170; phage(gi431810506)	complement(3993655..3994554)
PHAGE_Entero_sfi_NC_027339: N/A; KPNJ1_RS20180; phage(gi849250270)	complement(3994779..3995000)
PHAGE_Escher_HK639_NC_016158: cro; KPNJ1_RS20185; phage(gi356870651)	complement(3995041..3995274)
PHAGE_Escher_HK639_NC_016158: ci; KPNJ1_RS20190; phage(gi356870650)	3995402..3996091
PHAGE_Pseudo_PAJU2_NC_011373: hypothetical protein PAJU2_gp35; KPNJ1_RS20195; phage(gi209552454)	3996442..3996657
PHAGE_Entero_phi80_NC_021190: N/A; KPNJ1_RS20210; phage(gi824479615)	3997040..3997324
PHAGE_Ralsto_RSK1_NC_022915: putative phage recombination protein; KPNJ1_RS20215; phage(gi560186064)	3997340..3998185
PHAGE_Cronob_ENT47670_NC_019927: exonuclease; KPNJ1_RS20220; phage(gi431810511)	3998182..3998862
PHAGE_Escher_HK639_NC_016158: adenine specific methyltransferase; KPNJ1_RS20225; phage(gi356870635)	3999014..3999670
PHAGE_Azospi_Cd_NC_010355: hypothetical protein apcd_gp13; KPNJ1_RS20230; phage(gi168495116)	3999667..4000434
PHAGE_Stx2_converting_I_NC_003525: hypothetical protein Stx2Ip098; KPNJ1_RS20235; phage(gi20065893)	4000431..4000649
PHAGE_Stx2_converting_I_NC_003525: hypothetical protein Stx2Ip097; KPNJ1_RS20240; phage(gi20065892)	4000651..4000866
PHAGE_Escher_HK639_NC_016158: integrase; KPNJ1_RS20245; phage(gi356870629)	4001080..4002243
PHAGE_Acanth_mimivirus_NC_014649: cysteinyl-trna synthetase; KPNJ1_RS20265; phage(gi311977536)	complement(4003800..4005185)
PROPHAGE_Escher_CFT073: putative prophage integrase; KPNJ1_RS21340; phage(gi26250313)	4217459..4218649
PHAGE_Lactob_LF1_NC_019486: chromosome partitioning atpase; KPNJ1_RS21345; phage(gi418489414)	4218637..4219587
PHAGE_Entero_P4_NC_001609: amber mutation-suppressing protein; KPNJ1_RS21355; phage(gi9627520)	complement(4220581..4221147)
PHAGE_Erwini_ENT90_NC_019932: transcriptional regulator; KPNJ1_RS21360; phage(gi431810990)	complement(4221165..4221410)
PHAGE_Entero_P4_NC_001609: head size determination protein sid; KPNJ1_RS21365; phage(gi9627518)	complement(4221407..4222144)
PHAGE_Entero_P4_NC_001609: transcriptional regulator; KPNJ1_RS21375; phage(gi9627517)	4222686..4222952
PHAGE_Entero_P4_NC_001609: putative CI repressor; KPNJ1_RS21380; phage(gi9627516)	4223312..4223506

PHAGE_Entero_P4_NC_001609: hypothetical protein P4p07; KPNJ1_RS21390; phage(gi9627513)	4223727..4224047
PHAGE_Entero_P4_NC_001609: DNA primase; KPNJ1_RS21395; phage(gi9627512)	4224059..4226392
PROPHAGE_Xantho_33913: isxcc1 transposase; KPNJ1_RS23675; phage(gi21230085)	complement(4693764..4694243)
PROPHAGE_Salmon_LT2: transposase; KPNJ1_RS23685; phage(gi16766077)	complement(4694609..4694875)
PHAGE_Entero_P4_NC_001609: DNA primase; KPNJ1_RS23690; phage(gi9627512)	complement(4695620..4697953)
PHAGE_Entero_P4_NC_001609: hypothetical protein P4p07; KPNJ1_RS23695; phage(gi9627513)	complement(4697968..4698288)
PHAGE_Entero_P4_NC_001609: putative CI repressor; KPNJ1_RS23705; phage(gi9627516)	complement(4698509..4699057)
PHAGE_Entero_P4_NC_001609: transcriptional regulator; KPNJ1_RS23710; phage(gi9627517)	complement(4699054..4699320)
PHAGE_Entero_P4_NC_001609: head size determination protein sid; KPNJ1_RS23715; phage(gi9627518)	4699881..4700618
PHAGE_Entero_psp3_NC_005340: Pag; KPNJ1_RS23720; phage(gi41057379)	4700615..4700860
PHAGE_Entero_P4_NC_001609: amber mutation-suppressing protein; KPNJ1_RS23725; phage(gi9627520)	4700878..4701444
PHAGE_Entero_P4_NC_001609: integrase; KPNJ1_RS23745; phage(gi9627511)	complement(4704820..4706082)

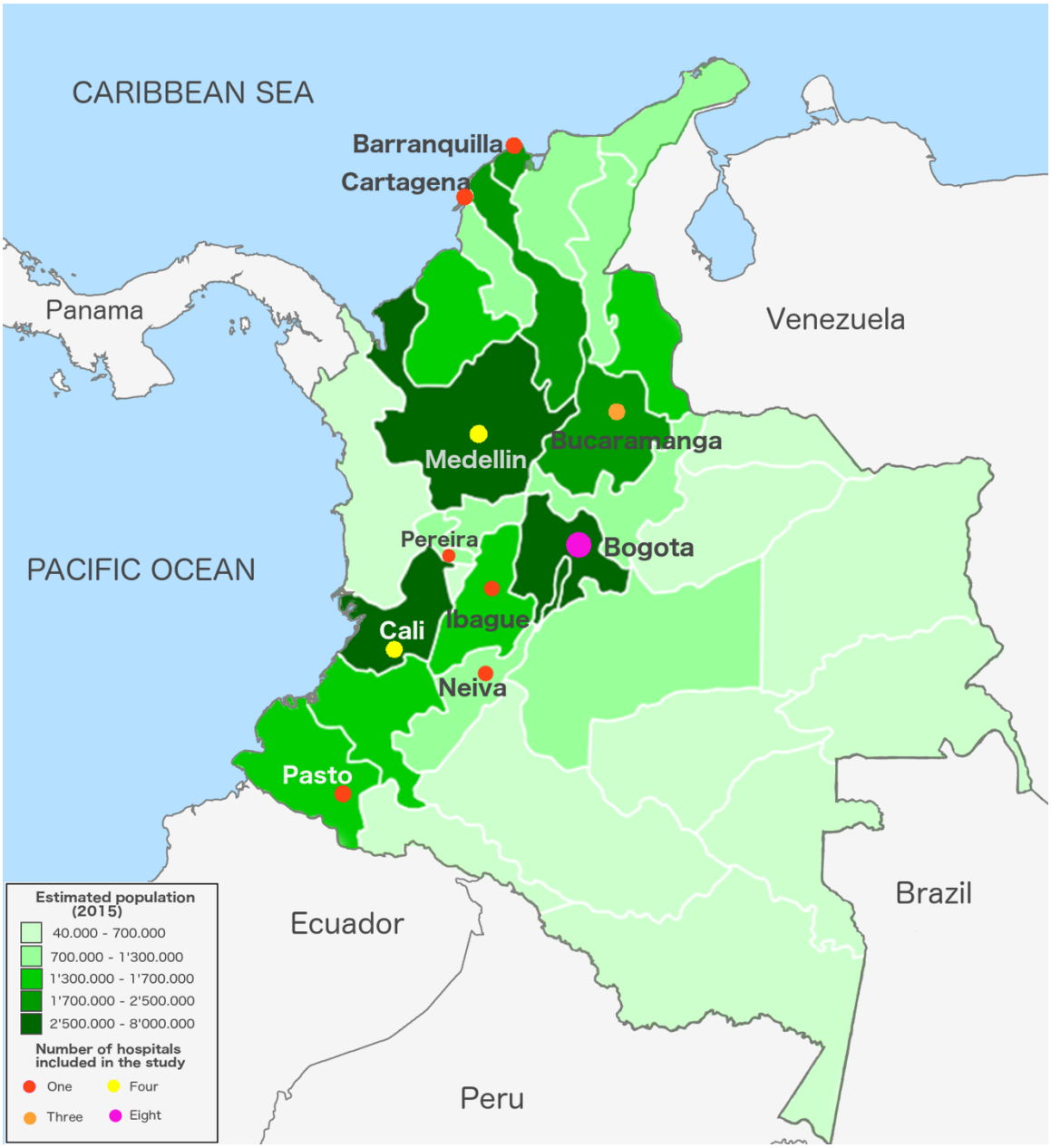


Figure 4-1. Map of Colombia showing the locations where isolates were collected.

The 10 cities included in the study are located in the most densely populated areas of the country (as indicated by darker hues). The number of hospitals per city is indicated by colored circles.

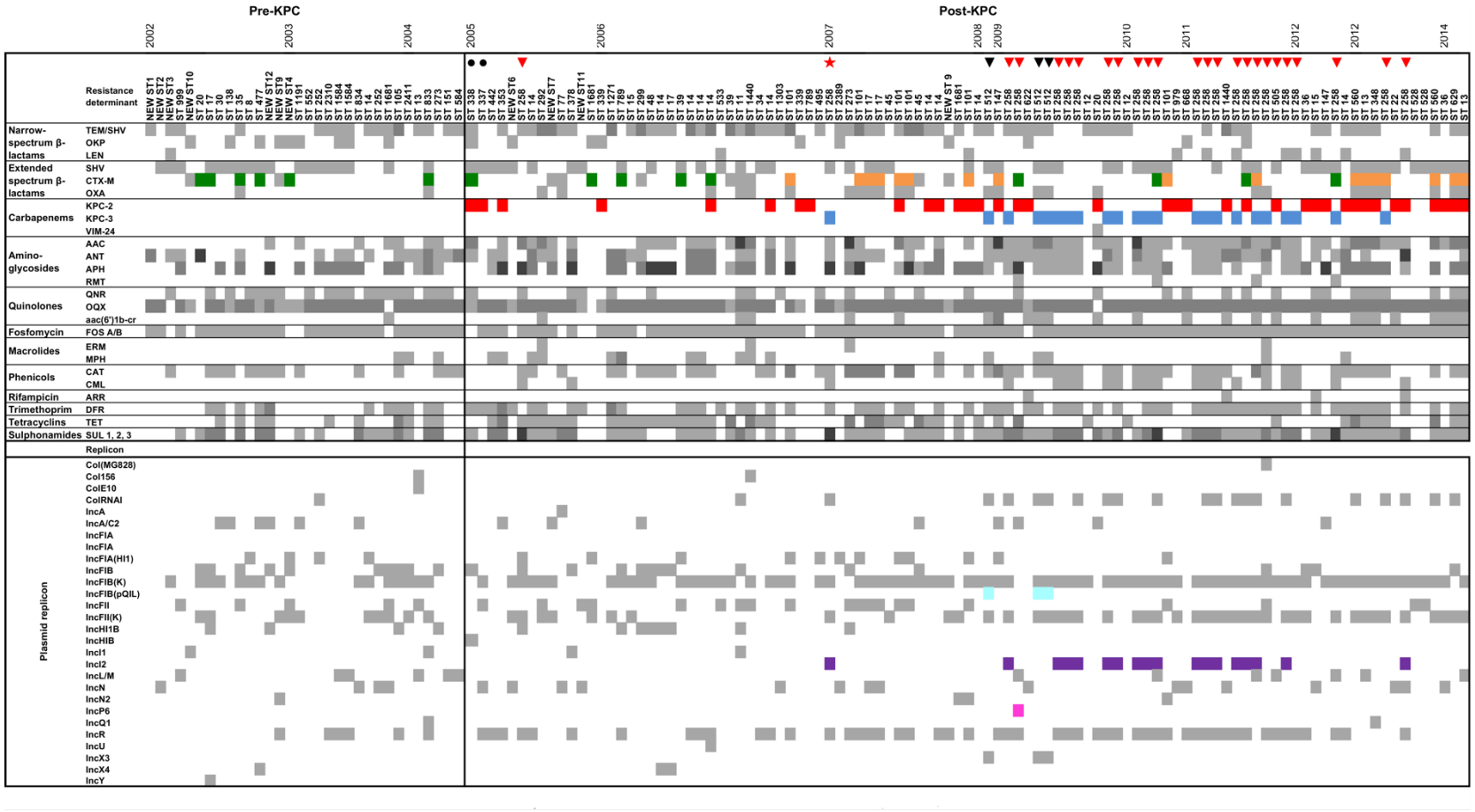


Figure 4-2. Resistance determinants by antibiotic class and plasmid replicons identified in the 133 *K. pneumoniae* isolates included in the study.

Figure 4-2. (Continued) Isolates are chronologically organized by year of isolation (2002 to 2014). *K. pneumoniae* isolates belonging to ST258 are identified by a red triangle and isolates belonging to ST512 are identified by a black triangle; first bla_{KPC-2} harboring isolates recovered in 2005 are denoted by black circles. For all classes of resistance determinants, white means absence and any color means presence. Gray scale indicates the number of copies of the same gene, regardless of the variant found (light, 1 copy; medium, 2 copies; dark, 3 copies). β-lactamases of special interest are highlighted in color: $bla_{CTX-M-12}$ (green), $bla_{CTX-M-15}$ (orange), bla_{KPC-2} (red), and bla_{KPC-3}(blue). Resistance determinants detected in the isolates are narrow spectrum β-lactamases (TEM-1, SHV-1 SHV-11, OKP-1, LEN-1/12/16,); extended spectrum β-lactamases (SHV-5/11/12/25/27/31/33/101/108/129, CTX-M 2/12/15/96, OXA-1/2/9/47), carbapenemases (KPC-2/3, VIM-24); aminoglycoside modifying enzymes [aminoglycoside N-acetyltransferases (AAC), aminoglycoside O-nucleotidyltranferases (ANT), aminoglycoside O-phosphotransferases (APH)]; 16S rRNA modifying enzymes [methyltransferases (RMT)]; quinolone conferring resistance enzymes [plasmid-mediated quinolone-resistance (QNR), OqxA-B efflux pump, and N-acetyltransferase Aac(6′)-Ib-cr]; fosfomycin resistance proteins (FosA, FosB); macrolides, lincosamides and streptogramins (MLS) resistance determinants [ErmB rRNA methylase (ERM) and macrolide phosphorylases (MphA/B)]; chloramphenicol resistance determinants [chloramphenicol acetyltransferase (CatA1) and chloramphenicol resistance efflux protein, CmlA]; rifampin ADP-ribosyltransferase (Arr); trimethoprim dihydrofolate reductase (DfrA); tetracycline efflux pump (TetA - C); *sul1*, *sul2* and *sul3* genes encoding dihydropteroate synthetase (DHPS). For plasmid replicons, an 80% similarity cut-off in PlasmidFinder was used; gray indicates presence and color indicates plasmid types of interest: IncFIB(pQIL), cyan; IncI2, purple; IncP6, magenta

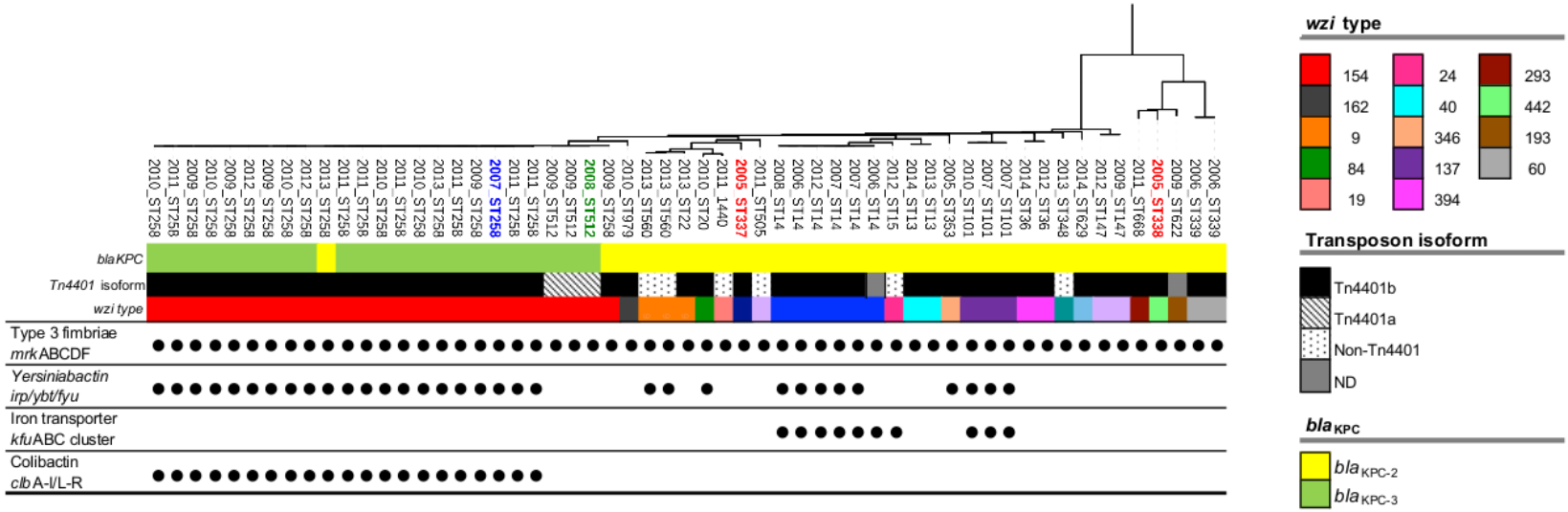


Figure 4-3. Transposable element carrying *bla*$_{KPC}$, virulence factors and capsular diversity as revealed by *wzi* typing in the *bla*$_{KPC}$ harboring *K. pneumoniae* isolates, identified by year of isolation and ST.

Indicated in red are isolates corresponding to the first *bla*$_{KPC}$-harboring isolates reported in Colombia; in blue is the oldest *bla*$_{KPC}$-harboring CG258 isolate found in our collection; in green is the index isolate from the "Israeli clone". Genes encoding for the main virulence factors described in *K. pneumoniae* are grouped (type 3 fimbriae, yerisiniabactin, iron transporter, colibactin); a dot indicates presence of at least one gene from the group, blank indicates absence. Each *wzi* type is indicated by a different color. For the transposable element, ND indicates not determined

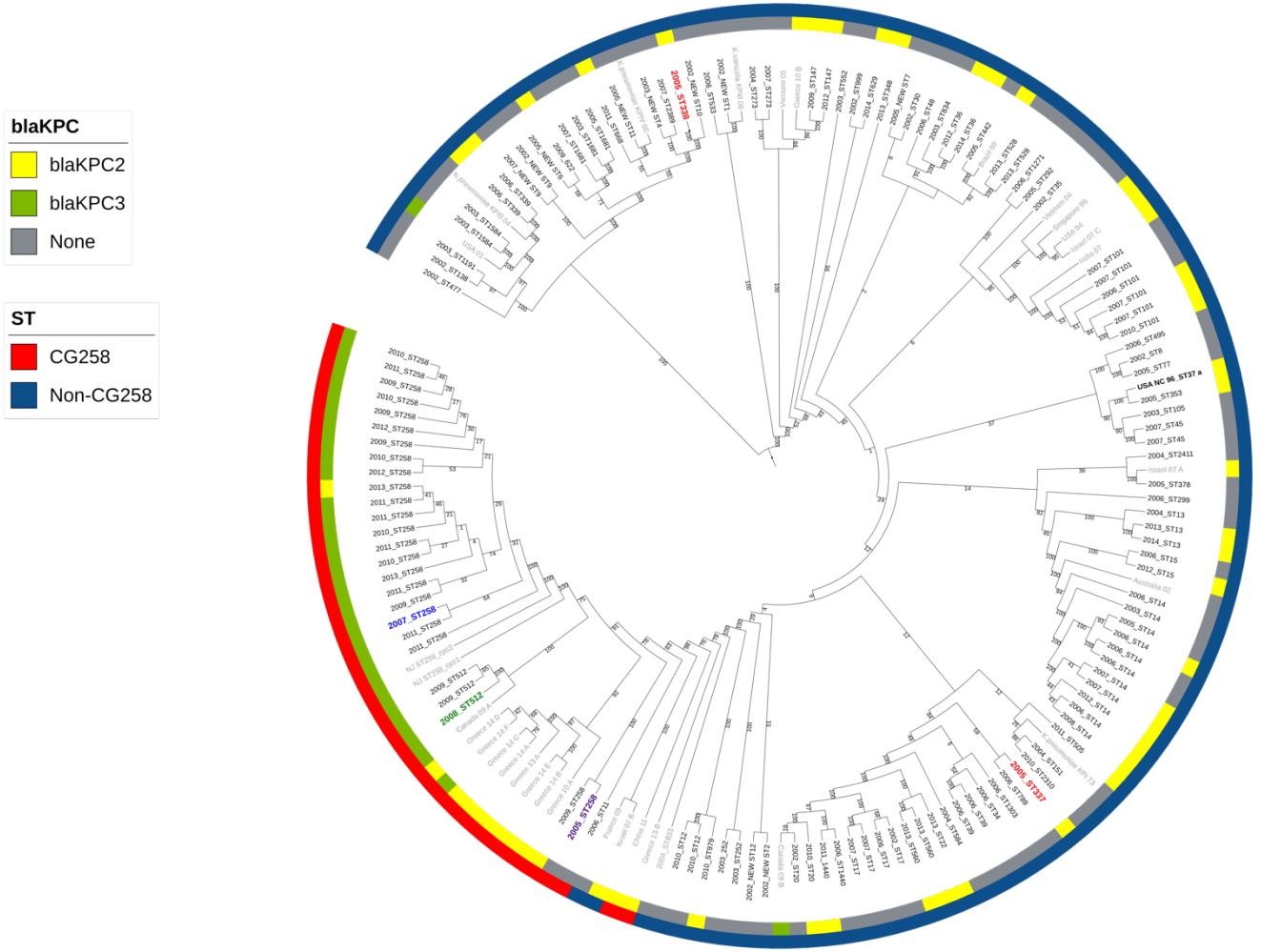


Figure 4-4. Circular representation of the transformed phylogenetic tree (ignoring branch lengths) generated by RAxML and drawn using the online iTOL program, showing the genetic relationships among 133 Colombian *K. pneumoniae* isolates.

Figure 4-4. (continued) Isolates in gray correspond to *K. pneumoniae* isolates from different parts of the world added as reference. Isolates in red correspond to the first two Colombian *K. pneumoniae* isolates described, carrying bla_{KPC-2}. Isolate in purple, corresponds to the oldest ST258 isolate in our collection. Isolate in blue, corresponds to the oldest ST258 KPC-Kpn isolate in our collection. Isolate in green corresponds to the index isolate from the "Israeli outbreak" in Medellin. [a] First KPC-Kpn reported in the world (North Carolina, USA)**.**

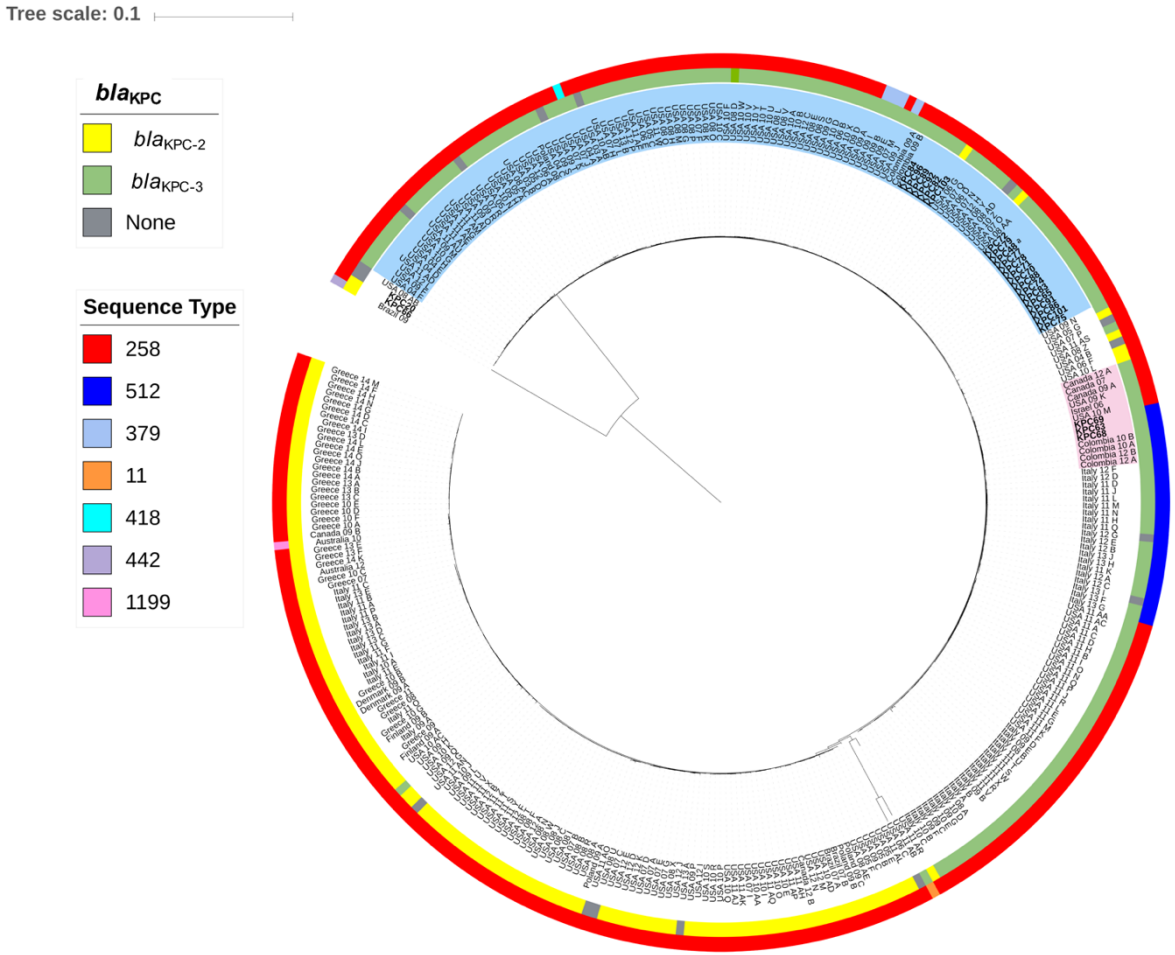


Figure 4-5. Circular representation of the transformed phylogenetic tree (ignoring branch lengths), showing the genetic relationships among 331 CG-258 *K. pneumoniae* isolates.

Figure 4-5. (continued) Phylogenetic tree was generated by RAxML and drawn using the online iTOL program. Isolates shown in bold correspond to Colombian isolates collected in this study, all other isolates were obtained from Genbank and are named with the respective country where they were collected followed by two digits indicating the year of isolation. Outer ring indicates the ST; inner ring indicates the KPC variant carried by each isolate. Cluster highlighted in pink include the "Israeli outbreak"-related Colombian isolates from our collection (ST512), as well as others found on Genbank. Cluster highlighted in blue include the oldest ST258 isolate found in our collection (a), as well as most of the other ST258 Colombian isolates found in thus study

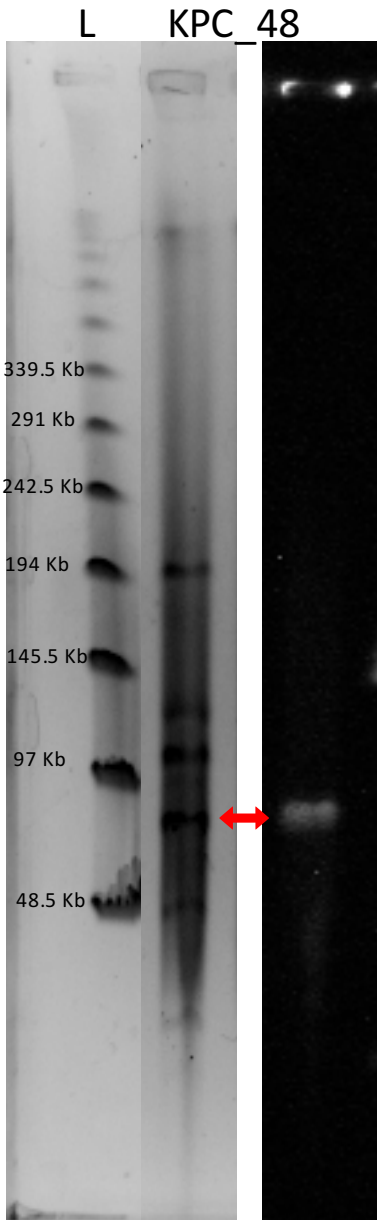


Figure 4-6. Plasmid location of *bla*$_{KPC-2}$ on isolate KPC_48 confirmed by S1 pulsed-field gel electrophoresis (PFGE).

Bacterial cells were embedded in agarose and lysed. Resulting DNA containing plugs were incubated with S1 nuclease to convert supercoiled plasmids into full-length linear molecules and subsequently separated by PFGE (left panel) including lambda ladder as reference (L). Finally, a Southern hybridization was performed using *bla*$_{KPC-2}$ probe (right panel). Red arrow indicates the plasmid where *bla*$_{KPC-2}$ is located.

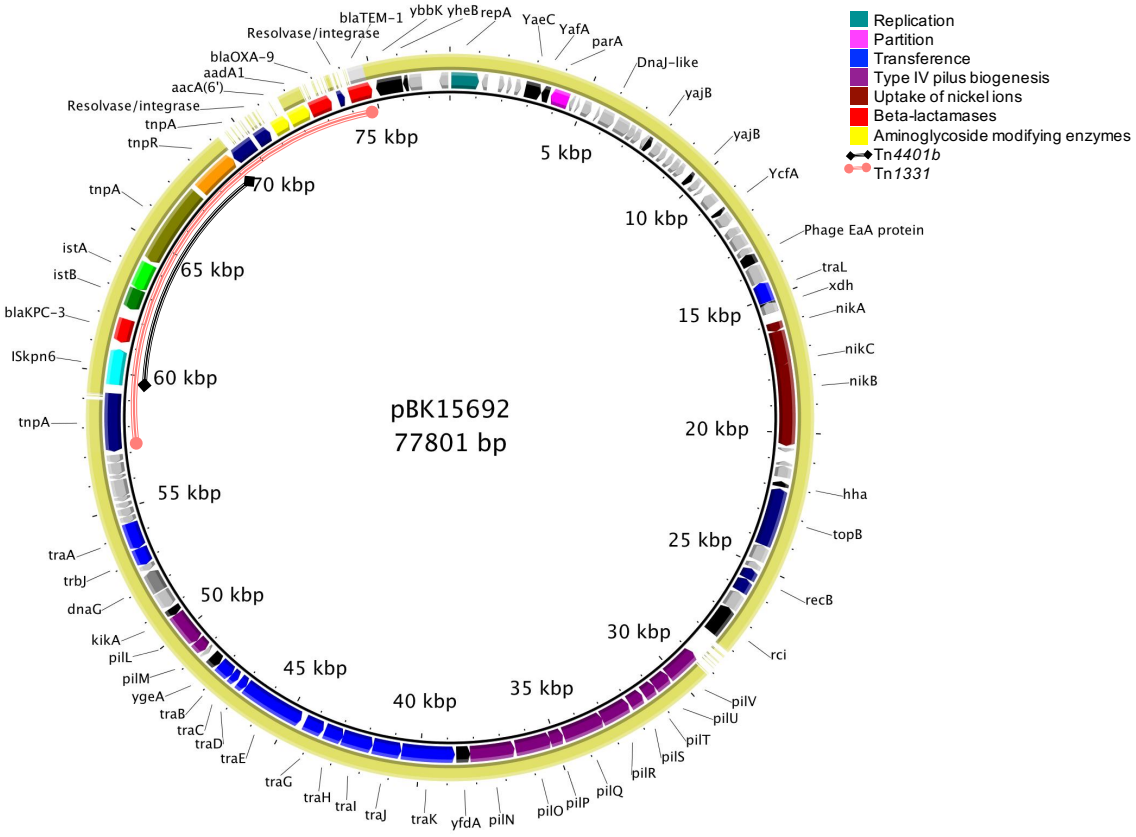


Figure 4-7. Comparison of the IncI2 plasmid pBK15692 (NC_022520.1) harboring *bla*$_{KPC-3}$ with KPC_48 isolate.

Outer ring (yellow) corresponds to the BLASTn result of KPC_48 contigs relative to the annotated (inside ring) pBK15692 plasmid reference.

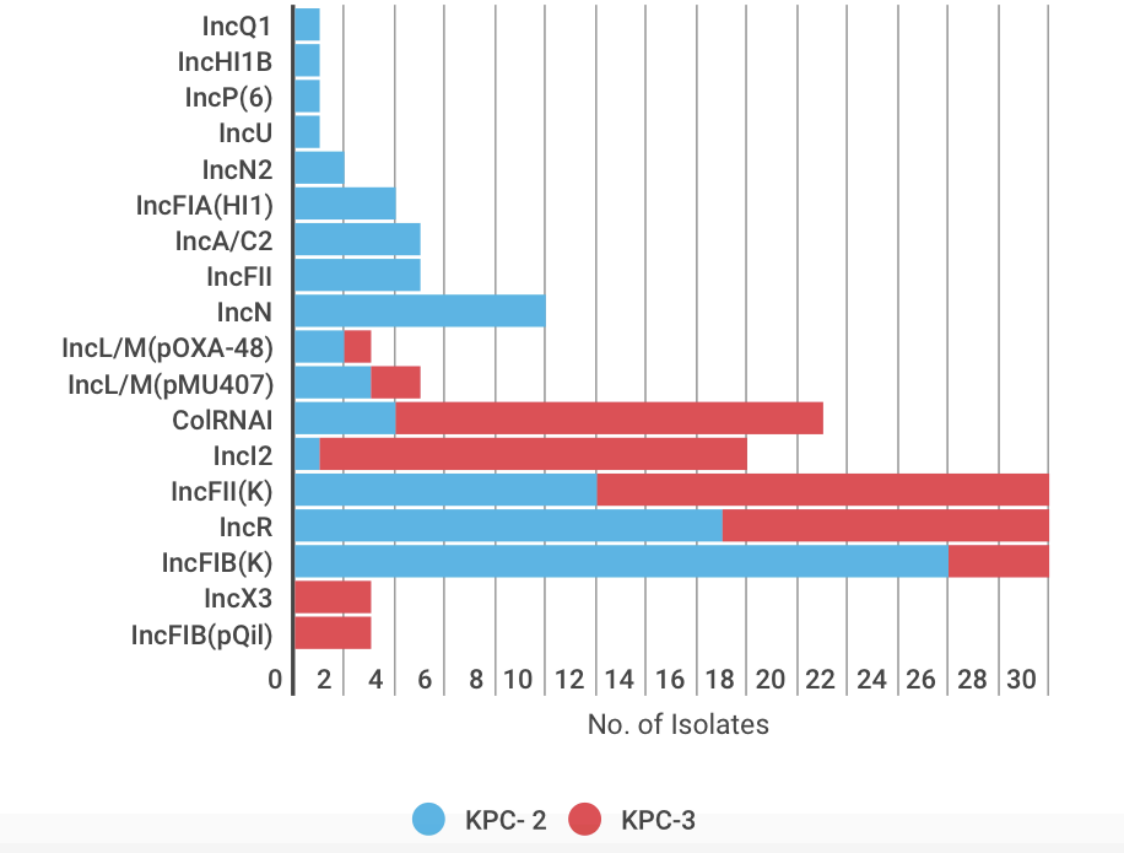


Figure 4-8. Plasmid replicons identified in the Colombian *bla*$_{KPC}$ harboring *K. pneumoniae* isolates using PlasmidFinder.

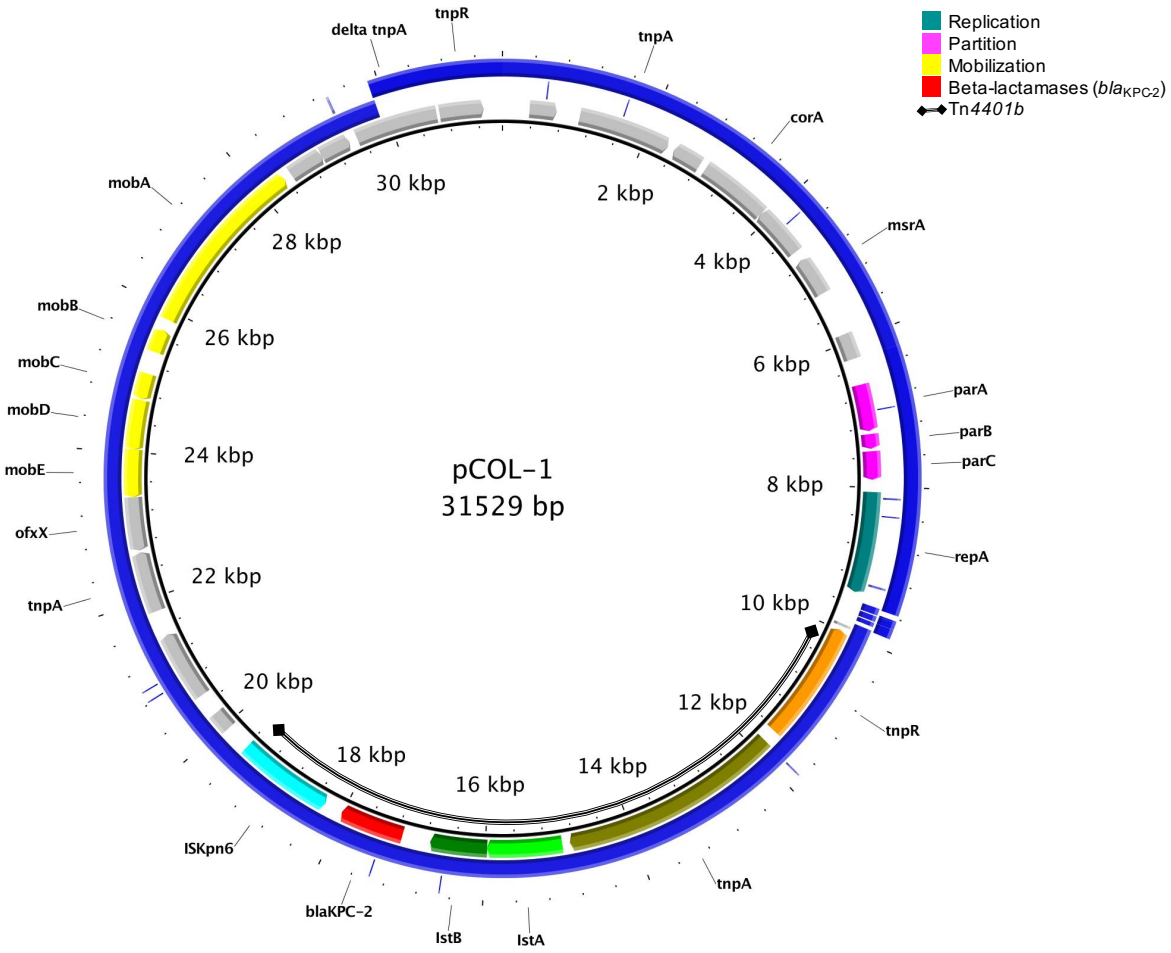


Figure 4-9. Comparison of the IncP6 plasmid pCOL-1 (NC_022346.1) harboring *bla*$_{KPC-2}$ with COLKpn_66 isolate.

Outer ring (blue) corresponds to the BLASTn result of COLKpn_66 contigs relative to the annotated (inside ring) pCOL-1 plasmid reference.

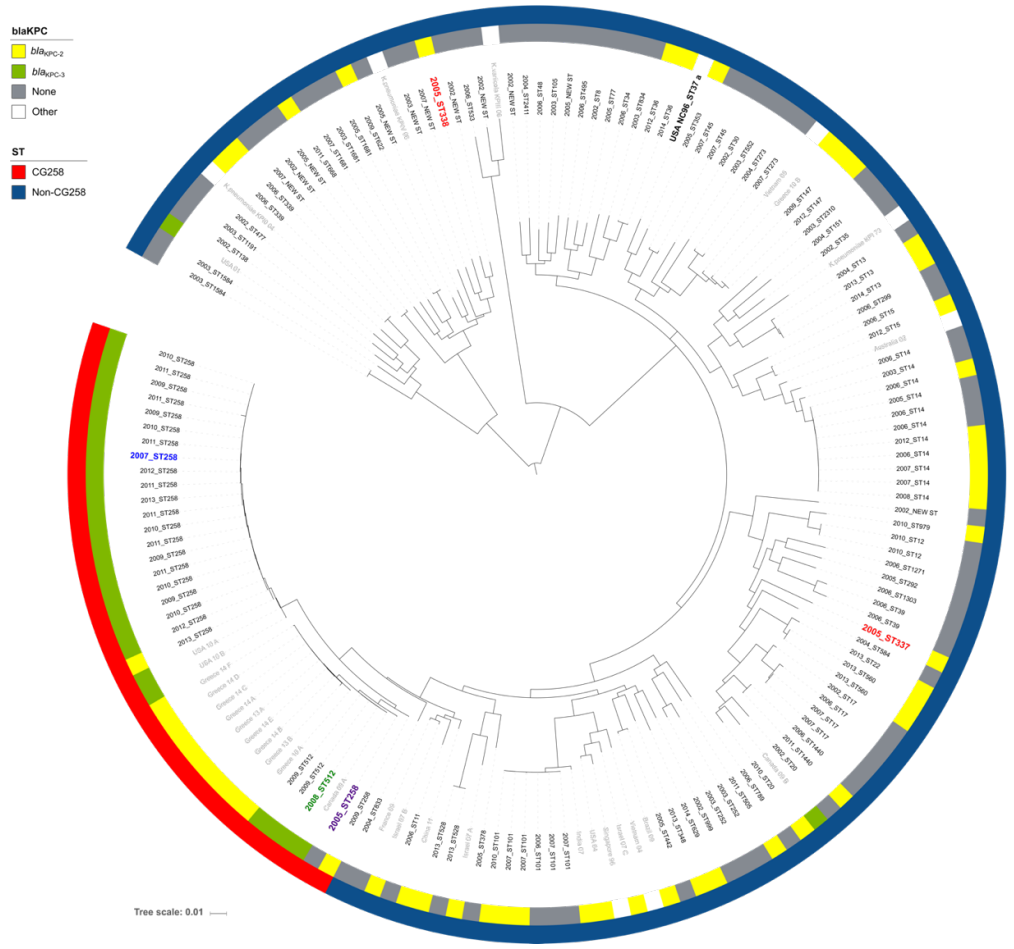


Figure 4-10. Circular representation of the phylogenetic (retaining annotation based exclusions) showing the genetic relationships among 133 Colombian *K. pneumoniae* isolates.

Figure 4-10. (continued) Phylogenetic tree was generated by RAxML and drawn using the online iTOL program. Isolates in gray correspond to *K. pneumoniae* isolates from different parts of the world added as reference. Isolates in red correspond to the first two Colombian *K. pneumoniae* isolates described, carrying bla_{KPC-2}. Isolate in purple, corresponds to the oldest ST258 isolate in our collection. Isolate in blue, corresponds to the oldest ST258 KPC-Kpn isolate in our collection. Isolate in green corresponds to the index isolate from the "Israeli outbreak" in Medellin. [a] First KPC-Kpn reported in the world (North Carolina, USA

CHAPTER 5 - COLISTIN RESISTANCE IN CARBAPENEM-RESISTANT *Klebsiella pneumoniae*: LABORATORY DETECTION AND IMPACT ON MORTALITY

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKp) infections remain a significant challenge associated with morbidity and mortality worldwide (96, 163). Few antimicrobials retain activity against CRKp (289). These include aminoglycosides, tigecycline, and the recently approved ceftazidime/avibactam. In addition, polymyxins including colistin are an important treatment option (289). After being mostly abandoned in the 1970's, these cationic antimicrobial peptides have re-emerged as a "last-line" therapeutic option for several multidrug resistant organisms (MDRO) (290, 291). In severe CRKp infections, colistin is often used in combination with other antibiotics including tigecycline, meropenem, gentamicin, or fosfomycin (291).

With the rise in consumption of colistin, cases of colistin-resistant *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains are reported globally (113). This emergence of colistin resistance (ColR) in CRKp creates a therapeutic challenge that threatens to return clinicians

and patients to a "pre-antibiotic era". Here, data from the multicenter Consortium on Resistance against Carbapenems in *Klebsiella pneumoniae* (CRACKLE) were analyzed to determine risk factors for ColR and to compare outcomes of patients with ColR CRKp vs colistin-susceptible (ColS) CRKp.

Methods

Patients

The Consortium on Resistance against Carbapenems in *Klebsiella pneumoniae* (CRACKLE) is a prospective, multi-center, observational study of hospitalized patients with CRKp in the Great Lakes Region of the United States (96, 292-294). In this nested cohort within CRACKLE, patients were included once at the time of their first culture positive for a CRKp isolate for which *in vitro* colistin susceptibility testing was performed as a part of clinical care at each study site during December, 2011 to October, 2014. All the health systems involved in this study had approval from their respective Institutional Review Boards.

Microbiology

CRKp were defined as *K. pneumoniae* isolates with non-susceptibility to any of the following carbapenems: meropenem, imipenem, or ertapenem, as outlined by the Clinical and Laboratory Standards Institute (CLSI) (197). Bacterial identification and routine antimicrobial susceptibility

testing was performed with MicroScan (Siemens Healthcare Diagnostics), or Vitek2 (bioMérieux), supplemented by GN4F Sensititre tray (Thermo Fisher), or Etest (bioMérieux), as indicated. Colistin minimum inhibitory concentration (MIC) determination in clinical microbiology laboratories was performed using Etest and was reported as "no interpretation" due to a lack of CLSI and Food and Drug Administration (FDA) breakpoints.

Colistin (sulfate salt, Sigma-Aldrich) and polymyxin B (Sigma-Aldrich) MIC determination in the central research laboratory was performed in duplicate using a broth macrodilution method in glass sterile tubes using *Escherichia coli* ATCC25922 as quality control, according to CLSI guidelines (181). If the two MIC values were >1 dilution apart, further repeats were performed to determine the final MIC. The final colistin MIC thus determined was used to designate ColR vs. ColS isolates. *In vitro* polymyxin resistance was defined per European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines as MIC of >2 mg/L (295). Very major and major error rates respectively were defined as described (296). Briefly, very major errors were those in which the clinical laboratory result was susceptible and the reference method result was resistant, and major errors were defined as those in which the clinical laboratory result was resistant and the reference method result was susceptible. Very major and major error rates were calculated using the 31 ColR CRKp isolates and the 215 ColS CRKp isolates as the denominator, respectively.

Detection of carbapenemase genes and repetitive extragenic palindromic polymerase chain reaction (rep)-PCR strain typing was performed as previously described (96). Briefly, PCR amplification of *bla*$_{KPC}$, *bla*$_{NDM}$ *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{OXA-48}$ genes was conducted using validated primers and methods; amplicons were sequenced at a commercial sequencing facility (MCLAB, San Francisco, CA), and analyzed (207, 297). rep-PCR was performed using the DiversiLab Strain typing system (Bacterial BarCodes, bioMerieux, Athens, GA). Isolates with ≥ 95% similarity were considered of the same rep-PCR type (298). In addition, all ColR CRKp were tested for the presence of *mcr-1* and *mcr-2* through PCR using the following primers: *mcr-1*_F *5'*- ATGATGCAGCATACTTCTGTGTGG -3', *mcr-1*_R *5'*- GTGCGGTCTTTGACTTTGTCC-*3' (*amplifying the complete mcr-1 ORF according to GenBank sequence KU341381.1), *mcr-2*_IF 5' – TGTTGCTTGTGCCGATTGGA -3', *mcr-2*_IR 5' - AGATGGTATTGTTGGTTGCTG -3' (299).

Clinical data

Clinical data was entered into a centralized database using the electronic medical record (EMR). The index hospitalization was recorded as the first hospital stay within the study period during which the CRKp was obtained and tested for colistin susceptibility. CRKp infections were defined by the standardized criteria described previously (96). Hospital acquisition was defined as timing of the first positive culture more than 2 days into the admission. Critical illness was determined as a Pitt bacteremia score ≥ 4

points on the day of the index culture (300). The Charlson comorbidity index was calculated, as described elsewhere (301).

Statistics

Differences between groups were analyzed using Wilcoxon Rank Sum for continuous variables. Fisher Exact and Pearson testing were used for categorical variables where appropriate. Kaplan-Meier curves were constructed comparing time to 30-day in-hospital mortality between groups. Adjusted hazard ratios (aHR) were calculated using Cox proportional hazards modeling on time to 30-day in-hospital mortality. In the Cox model, all variables were considered which were associated at the $p<0.1$ level with the outcome, followed by backwards selection. P values of ≤ 0.05 were considered statistically significant. JMP 10.0.1 software (SAS, Inc, Cary, North Carolina) was used for all analyses.

Results

Colistin susceptibility testing

A total of 522 unique patients were included in the CRACKLE study during the study period. Of these 522 patients, 246 (47%) patients had at least one isolate that was shipped to the central research laboratory that had been tested for colistin susceptibility as part of routine clinical care. These 246 patients were included in the study. Colistin resistance per research laboratory determination ("ColR") was detected in the CRKp isolates for 31 (13%) patients as compared to 21 (9%) per clinical laboratory testing. Of

note, additional colistin MIC testing beyond duplicates was required in 25 (10%) isolates; these isolates all had been reported by the clinical laboratory as susceptible. In addition, 25 (10%) of 240 tested isolates were *in vitro* resistant to polymyxin B as determined by the central research laboratory. In the remaining 6 (2%) isolates, a definitive polymyxin B MIC could not be determined in spite of repetitive testing, due to the observance of "skipped wells" and trailing end points.

The distributions of colistin MIC as determined by the central research laboratory as compared to the clinical microbiology laboratories and as compared to polymyxin B MIC are shown in Figure 5-1. Testing in the research laboratory resulted in a different interpretation in 12/246 (5%) isolates (Figure 5-1). The very major error rate was 35% (11/31), and the major error rate was 0.4% (1/215).

Clinical characteristics

Characteristics of patients with ColR vs. ColS CRKp were similar (Table 5-1). Overall, 111 (45%) patients met criteria for CRKp infection. The anatomic sources of CRKp were urine (59%), blood (18%), respiratory system (11%), wound (5%), and others (7%). Remarkably, time from admission to first positive culture was shorter in patients in ColR vs. ColS group; at a median of 0 days (IQR 0-3 days) in the ColR group vs. 1 day (IQR, 0-9 days) in the ColS group (p=0.01). The total length of stay (LOS) was also shorter for patients with ColR isolates; a median of 8 days (IQR 5-12 days) as compared to 13 days (IQR 7-26 days) in the ColS group

(p=0.01). When only patients who survived their hospital stay were analyzed, this difference in LOS was similar, albeit no longer significant (median 8 vs. 12 days, p=0.08). Finally, a marginal association was observed with pre-hospitalization origin; patients with ColR CRKp were more likely to be admitted from home, or a long term acute care facility (LTAC), whereas patients with ColS CRKp were more likely to be admitted from skilled nursing facility (SNF), or to be transferred from another hospital (p=0.05). No specific SNF or LTAC appeared to be associated with ColR; 12 patients with ColR isolates were admitted from 4 LTACs and 5 SNFs.

Previous Antibiotic Exposure

Antibiotic exposures in the 14 days preceding the first positive culture were analyzed (Table 5-2). In 82 (33%) patients antibiotic use was not documented. In the remaining 164 (67%), vancomycin was the single most common drug exposure, and was given to 80 (33%) patients. Twenty-seven (11%) patients received more than 3 classes of antibiotics in the 14-day period before the positive culture. Exposure to fluoroquinolones was less common in the ColR group as compared to the ColS group. Otherwise, antibiotic exposures in this time period were not different between patients with, or without ColR CRKp.

Documented previous colistin use in 14 days leading up to the day of first positive culture was uncommon in the cohort as a whole, and patients in the ColR group did not receive colistin during this limited time period.

Colistin exposure data beyond the 14-day period was available for 27 patients with ColR isolates, based on EMR of the healthcare system of the index hospitalization. In total, 6/27 (22%) patients were found to have previous colistin exposure at any time prior to first positive culture with a ColR CRKp.

Microbiology

The susceptibilities for other antibiotics other than colistin were determined as clinically indicated by clinical microbiology laboratories (Figure 5-2). ColR CRKp were significantly less likely to be amikacin susceptible; 8/15 (53%) of tested ColR CRKp were amikacin susceptible as compared to 104/126 (83%) of tested ColS CRKp (p=0.02). In contrast, ColR CRKp were more likely to be gentamicin susceptible; 21/31 (68%) of tested ColR CRKp were gentamicin susceptible as compared to 97/212 (46%) of tested ColS CRKp (p=0.03). Furthermore, ColR isolates were also more likely to be tigecycline non-susceptible; 13/21 (62%) of tested ColR CRKp were tigecycline susceptible as compared to 138/168 (82%) of tested ColS CRKp (p=0.04).

Overall, a non-clonal distribution was observed. rep-PCR suggested that most strains belonged to clades ST258A (n=76, 31%) and ST258B (n=102, 41%). The remaining 68 (28%) isolates belonged to 28 other rep-PCR types. Likewise, in the ColR group a non-clonal pattern was observed (Figure 5-3). rep-PCR types corresponding to clades ST258A and ST258B together accounted for 77% of ColR isolates.

The presence of carbapenemase genes was confirmed in 238 (97%) isolates. The carbapenemase genes *bla*$_{KPC-2}$, and *bla*$_{KPC-3}$ were the most common and were found in 113 (46%) and 124 (50%) of CRKp isolates, respectively. The distribution of carbapenemase genes did not differ between ColR and ColS CRKp. Of note, *bla*$_{NDM-1}$ was found in only one isolate. This ColS isolate was extensively described previously using whole genome sequencing (96). In the remaining 8 (3%) isolates, carbapenemase genes were not detected. The plasmid-mediated colistin resistance genes, *mcr-1* and *mcr-2* were not detected in any of the ColR CRKp.

Treatment and mortality

In the first 7 days after first positive culture, 143 (58%) patients received directed treatment. Treatment regimens used in these 143 patients are summarized in Table 5-3. Treatment with colistin was uncommon; colistin, either alone, or in combination with other antibiotics was given to 31 (22%) of treated patients. Colistin treatment was given to 30 of 127 (24%) of treated patients in the ColS group, as compared to 1/16 (6%) treated patients in the ColR group. Other treatment regimens were similar between the two groups. The most commonly used antibiotics were aminoglycosides and tigecycline. Aminoglycosides were given to 72 (50%) of treated patients; 36 (25%) patients received only aminoglycosides in the first 7 days. Similarly, 63 (44%) of treated patients received tigecycline, of whom 21 (15%) received tigecycline monotherapy in the first 7 days.

We evaluated time to all-cause in-hospital mortality, censored at 30 days (Figure 5-4). In univariable Kaplan-Meier survival curves, patients with ColR CRKp were at increased hazard of 30-day mortality ($p<0.0001$). After adjustment, the aHR of ColR vs. ColS was 3.48 (95% confidence interval [CI] 1.73-6.57, $p<0.001$). Other variables that impacted survival included age, pre-hospitalization origin, and anatomical source of the positive CRKp culture (Table 5-4). Treatment variables and infection vs. colonization status were not associated with mortality and inclusion of these into the model did not significantly alter the association between colistin and mortality. Of note, when the colistin resistance as determined by the clinical microbiology laboratory was entered into this model instead of ColR as determined by the research laboratory, this was still associated with increased mortality but to a lesser extent (aHR 2.78, 95% CI 1.22-5.71, $p=0.02$, data not shown).

Discussion

In this nested cohort of patients from CRACKLE – a prospective, ongoing, multi-center evaluation of hospitalized patients with carbapenem-resistant *Enterobacteriaceae* (CRE) – a ColR rate of 13% was observed in CRKp isolates. This rate was underestimated by testing as performed by clinical microbiology laboratories. In addition, even testing in optimal research conditions resulted in the need for repeat colistin MIC testing in 10% of isolates. Furthermore, polymyxin B MIC testing was also fraught with difficulties; a definitive polymyxin B MIC could not be determined in

6 isolates, even with repetitive testing. The difficulty in determining colistin MIC has been previously reported by Hindler et al (302). Broth macrodilution as used here, was found to be most reliable (302). The American Society for Microbiology recently released a white paper on the issue of colistin testing, recommending that "laboratories that choose to test for colistin susceptibility should validate a broth microdilution method and report MIC values only." (303). Clearly, the absence of FDA breakpoints and the resulting impossibility of a FDA-approved test for colistin susceptibility remains a major issue.

The association between ColR and mortality was also seen in an Italian case-control study on CRKp bloodstream infections (304). In that study 30-day all-cause mortality rates were 51% in the ColR group (n=142) vs. 39% in the ColS group (n=284), p=0.02. The increased mortality may be directly related to decreased colistin susceptibility of the organism. Alternatively, the observed mortality differences may be secondary to either variance in baseline characteristics of patients with ColR vs. ColS CRKp, or to an alteration in the virulence of the pathogen (305). Regardless of causality, it is clear that patients with ColR CRKp are at increased risk of hospital mortality.

We observed that – similar to patients with tigecycline-resistant CRKp – isolation of ColR CRKp tended to occur earlier during hospitalization as compared to ColS CRKp (292). Our findings suggest that the introduction of ColR isolates may be present on admission to acute care hospitals. In

the majority of patients, colistin exposure prior to the positive culture was not documented. This may be explained in a number of ways. First, some patients may have had unrecorded colistin exposure at non-study hospitals. The intuitive link between colistin exposure and ColR has been previously documented for CRKp and carbapenem-resistant *Acinetobacter baumannii* (CRAB) (290, 304). Second, patients with ColR CRKp without colistin exposure, may have received ColR CRKp from either another person exposed to colistin, or potentially from a food source. The latter option would appear less likely as no such food-related transmission has been reported in the United States for *K. pneumoniae*. In contrast, person-to-person transmission of ColR CRKp without further colistin exposure of subsequently infected patients was shown to be an important mechanism of spread in an outbreak-related report (306). The lack of clonality in ColR CRKp described in that report, argues against a single clonal outbreak but obviously does not rule out multiple person-to-person transmission events.

Previously we described various chromosomally mediated mechanisms of ColR for a subset of isolates in this cohort (110). Of concern, two recently described plasmid-mediated, readily transmissible ColR mechanisms – *mcr-1* and *mcr-2* – are being recognized in *Enterobacteriaceae* from animals and humans around the world (114, 299). Even though *mcr-1* has already been reported in the United States, this gene was not detected in any of the isolates described here (307, 308). Reproducibility issues in

polymyxin/colistin MIC testing, as well as variability between testing methods illustrated here and in other reports may complicate tracking of *mcr-1* and *mcr-2* carrying isolates (302). If screening of *mcr* carriage is only performed on isolates based on colistin MIC by Etest, the most common method used by clinical labs, the spread of *mcr* is likely to be underestimated.

There are a number of limitations to this work. We purposefully selected isolates in which colistin susceptibility testing was performed by the clinical microbiology laboratories as clinically indicated, and patients with CRKp isolates which were not tested are not included in this study. Therefore, our estimate of 13% ColR at first test may not necessarily be representative of the whole patient population with CRKp. Furthermore, follow-up for patients in this observational study is limited to the duration of hospitalization. Consequently, we cannot comment on long-term outcomes. Another potential limitation of our work is that we included both patients who met standardized criteria for infection as well as those who did not. Nevertheless, standardized criteria have limitations in distinguishing patients with infection from those who are colonized. In addition, patients who do not meet criteria for infection often receive antibiotic treatment and are an important source of transmission. Finally, our study population was predominantly derived from the Great Lakes region of the United States. Therefore, these data may not accurately reflect incidence of ColR in other CRKp in the United States. In a study

performed by the Centers for Disease Control and Prevention (CDC) in 2012-2013 in 7 US communities, 12 CRE isolates were tested for colistin susceptibility. Nine of these were reported to be non-susceptible, based on testing performed in clinical laboratories (309). Additional data from other US regions are expected from ongoing surveillance programs and from the recently started national expansion of CRACKLE. Additionally, there are important differences in ColR rates in CRE isolates outside of the US as well (114, 299).

In summary, we observed a 13% ColR rate in this multicenter, observational cohort of hospitalized patients with CRKp in U.S. hospitals. Colistin *in vitro* susceptibility testing is characterized by reproducibility issues. Patients with ColR CRKp were at higher risk for mortality. They also tended to present earlier during admission. This, in combination with the polyclonality of the observed ColR CRKp isolates, suggests that direct patient-to-patient transmission of CRKp isolates within short-term acute care hospitals is unlikely to be the primary mechanism driving resistance rates. Alternative, not mutually exclusive explanations include within-patient development of ColR and transmission in healthcare settings outside of short-term acute care hospitals, such as LTACs or SNFs.

Table 5-1. Clinical Characteristics

Characteristic	All	Colistin resistant	Colistin susceptible	P value[a]
N	246	31	215	
Age in years, median (IQR)	67 (54-77)	62 (53-73)	67 (54-78)	0.43
Female	139 (57)	20 (65)	119 (55)	0.44
Race/Ethnicity				0.22
White	135 (55)	13 (42)	122 (57)	
Black	95 (39)	16 (52)	79 (37)	
Hispanic	7 (3)	0	7 (3)	
Other	9 (4)	2 (6)	7 (3)	
Charlson comorbidity index, median (IQR)	3 (2-5)	3 (2-4)	3 (2-5)	0.78
Diabetes mellitus	118 (48)	15 (48)	103 (48)	1.0
Renal failure[b]	75 (30)	7 (23)	68 (32)	0.40
Heart disease	121 (49)	14 (45)	107 (50)	0.70
COPD	62 (25)	8 (26)	54 (25)	1.0
Malignancy	41 (17)	8 (26)	33 (15)	0.19
Infection	111 (45)	13 (42)	98 (46)	0.85
Source				0.22
Urine	144 (59)	16 (52)	128 (60)	
Blood	45 (18)	5 (16)	40 (19)	
Respiratory	28 (11)	4 (13)	24 (11)	
Wound	13 (5)	1 (3)	12 (6)	
Other	16 (7)	5 (16)	11 (5)	
Days to first positive culture, median (IQR)	1 (0-7)	0 (0-3)	1 (0-9)	0.01
Hospital acquisition	115 (47)	10 (32)	105 (49)	0.12
Origin				0.05
Home	90 (37)	14 (45)	76 (35)	
Skilled nursing facility	76 (31)	6 (19)	70 (33)	
Hospital transfer	59 (24)	5 (16)	54 (25)	
Long term acute care	21 (9)	6 (19)	15 (7)	
Critical illness[c]	86 (35)	7 (23)	79 (37)	0.16
Length of stay, days, median (IQR)	12 (6-24)	8 (5-12)	13 (7-26)	0.01

All data expressed as n (%), unless otherwise indicated. IQR, interquartile range. LOS, length of stay.
[a]univariable relationship between variable of interest and colistin resistance
[b]renal failure defined as creatinine >2 mg/dL upon admission
[c]critical illness defined as Pitt bacteremia score ≥4 at the time of index culture

Table 5-2. Antibiotic exposures

Variable	All (n=246)	colistin resistant (n=31)	colistin susceptible (n=215)	P value
Classes of antibiotics				0.68
None	82 (33)	10 (32)	72 (33)	
1	51 (21)	9 (29)	42 (20)	
2	51 (21)	5 (16)	46 (21)	
3	35 (14)	5 (16)	30 (14)	
>3	27 (11)	2 (6)	25 (12)	
Tigecycline	7 (3)	2 (6)	5 (2)	0.22
Carbapenem	36 (15)	4 (13)	32 (15)	1.0
Fluoroquinolone	44 (18)	1 (3)	43 (20)	0.02
Colistin	2 (1)	0	2 (1)	1.0
Vancomycin	80 (33)	8 (26)	72 (33)	0.54
β-lactam/ β-lactamase inhibitor	52 (21)	7 (23)	45 (21)	0.82
Cephalosporin	25 (10)	5 (16)	20 (9)	0.22
Aminoglycoside	14 (6)	2 (6)	12 (6)	0.69
Daptomycin	10 (4)	2 (6)	8 (4)	0.37
Metronidazole	19 (8)	4 (13)	15 (7)	0.27
Other	90 (37)	11 (35)	79 (37)	1.0

Shown is the documented exposure to antibiotics in 14 days prior to first positive culture. All data expressed as n (%)

Table 5-3.Treatment characteristics

Characteristic	All	Colistin resistant	Colistin susceptible
n	143	16	127
Any colistin in first 7 days	31 (22)	1 (6)	30 (24)
One antibiotic in first 7 days[a]	90 (63)	10 (63)	80 (63)
aminoglycoside	36 (25)	5 (31)	31 (24)
colistin	10 (7)	1 (6)	9 (7)
tigecycline	21 (15)	3 (19)	18 (14)
trimethoprim/sulfamethoxazole	13 (9)	0	13 (10)
fosfomycin	10 (7)	1 (6)	9 (7)
>1 antibiotic in first 7 days	53 (37)	6 (38)	47 (37)
2 antibiotics	38 (27)	5 (31)	33 (26)
3 antibiotics	13 (9)	1 (6)	12 (9)
4 antibiotics	2 (1)	0	2 (2)

Treatment is shown in the subset of patients who received at least one antibiotic with potential *in vitro* anti-CRKp activity in the first 7 days after first positive CRKp culture. [a]Only antibiotics with potential *in vitro* anti-CRKp activity were included.

Table 5-4. Cox proportional hazards model on time to 30-day in-hospital mortality

Variable	Adjusted Hazard ratio	95% Confidence Interval	P value
Colistin resistance	3.48	1.73-6.57	<0.001
Age (by decade)	1.24	1.03-1.53	0.02
Origin			<0.01
Home (ref.)	-	-	
Skilled nursing facility	2.52	1.12-6.03	
Hospital transfer	2.64	1.18-6.35	
Long term acute care	5.18	2.17-12.82	
Source			<0.01
Blood (ref.)	-	-	
Urine	0.35	0.19-0.66	
Other	0.29	0.13-0.60	

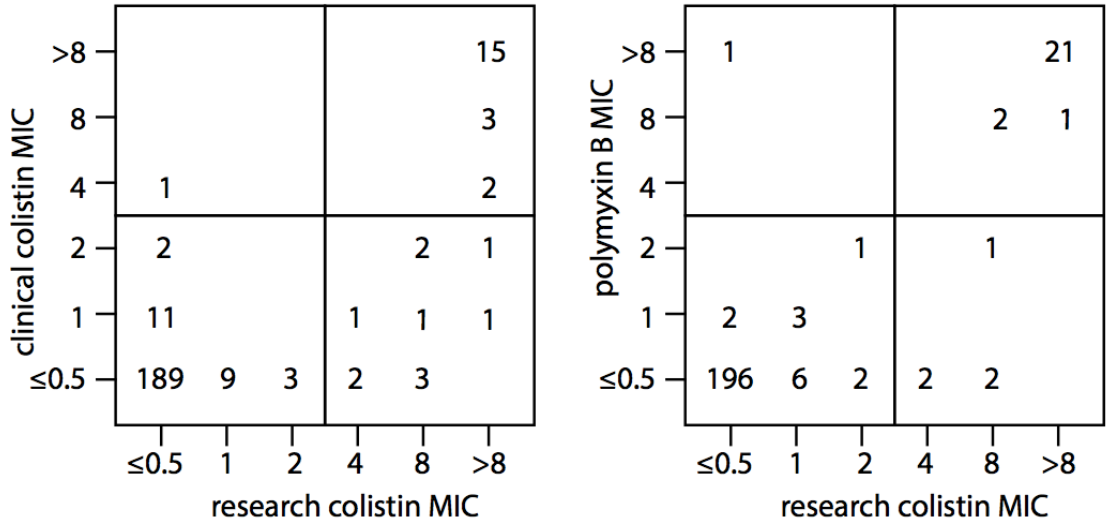


Figure 5-1. Distributions of colistin MIC as determined by the central research laboratory compared to the MIC determined by the clinical microbiology laboratories, and compared to polymyxin B MIC determined by the central research laboratory.

The numbers of isolates that correspond to each minimum inhibitory concentration (MIC) value for the MIC as reported by the clinical microbiology laboratories ("clinical colistin MIC") and the centralized research laboratory ("research colistin MIC"), as well as the polymyxin B MIC are shown.

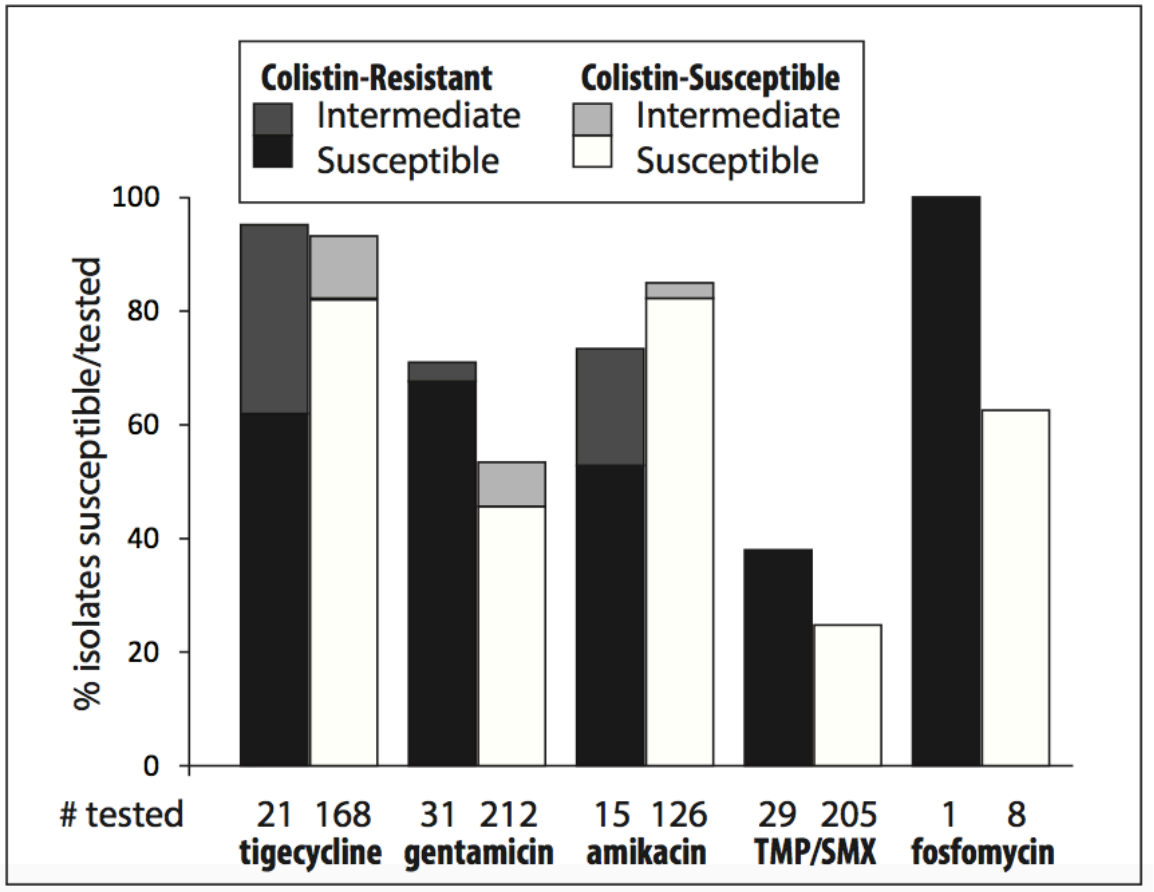


Figure 5-2. Percentages of susceptible and intermediate isolates for each drug for colistin-resistant (ColR) and colistin-susceptible (ColS) carbapenem-resistant *Klebsiella pneumoniae* (CRKp)

Underneath each bar is the number of CRKp tested for each drug in the ColR and ColS groups. The black and white bars represent the percentage of susceptible/tested CRKp isolates in the ColR and ColS groups, respectively. The dark-gray and light-gray bars on top of the black and white bars represent the percentage of intermediate/tested CRKp isolates in the ColR and ColS groups, respectively. Abbreviation: TMP/SMX, trimethoprim/sulfamethoxazole.

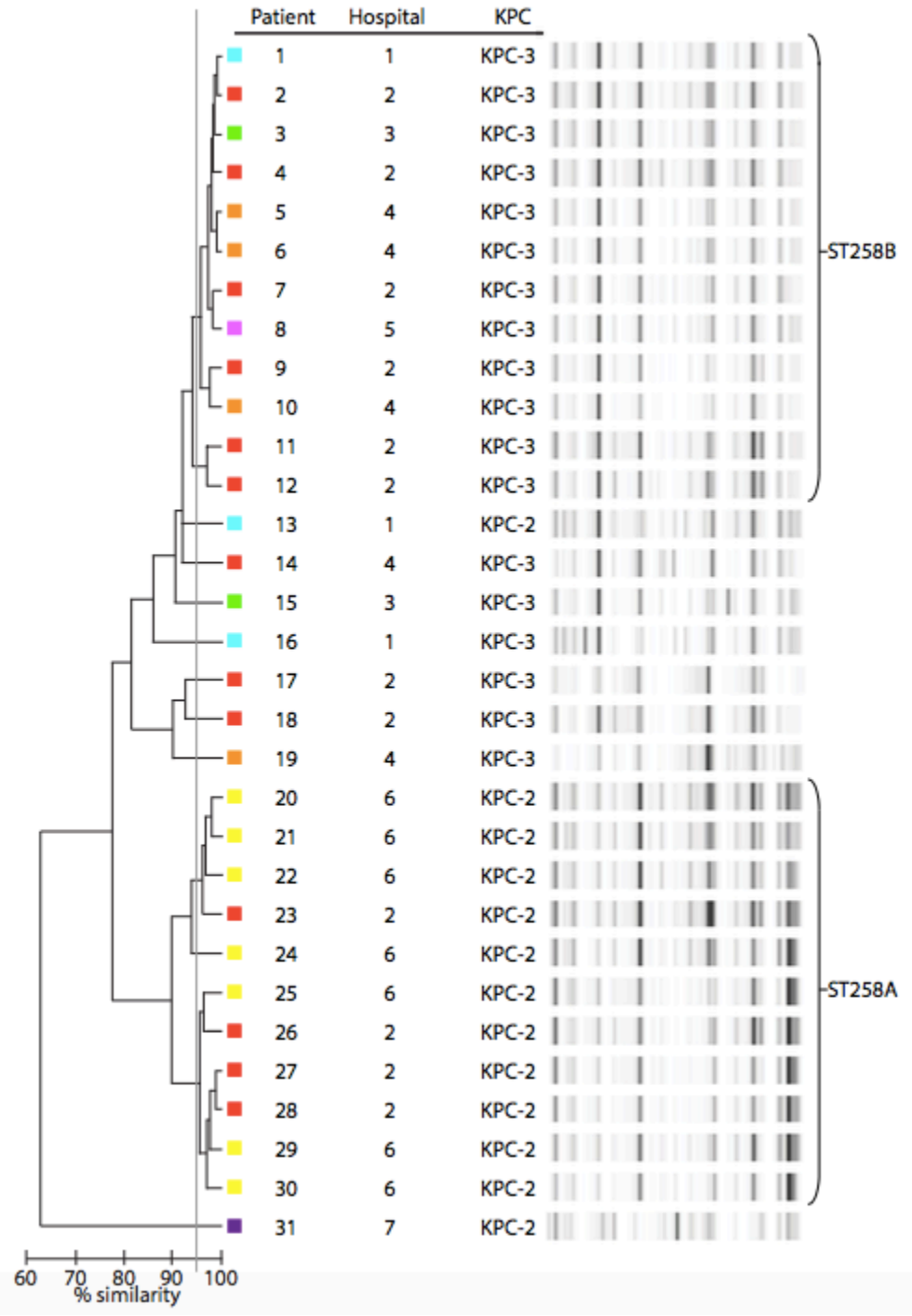


Figure 5-3. Repetitive extragenic palindromic–polymerase chain reaction (rep-PCR) dendrogram of colistin-resistant carbapenem-resistant *Klebsiella pneumoniae* isolates.

A ≥95% similarity cutoff (gray line) was used to consider isolates of the same rep-PCR type. Colored boxes next to each strain represent the various hospitals from which isolates were recovered.

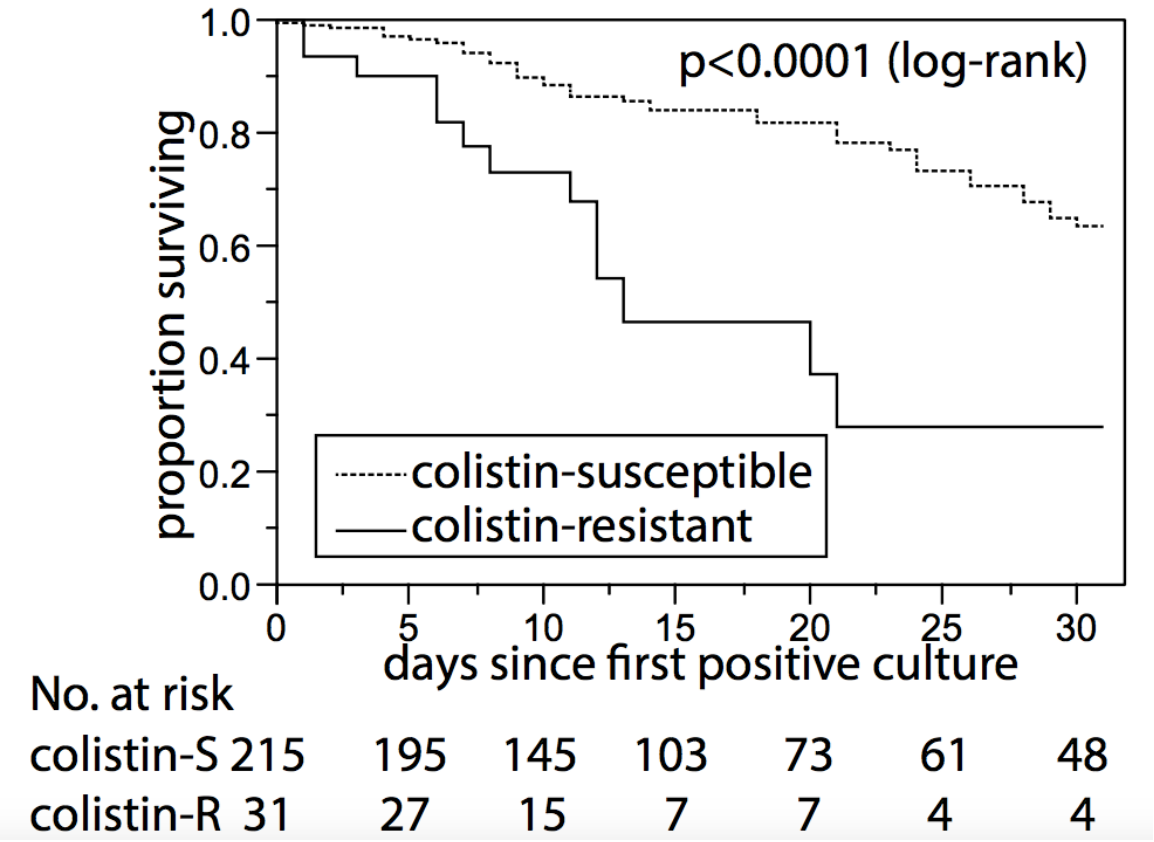


Figure 5-4. Kaplan-Meier curve showing the 30-day in-hospital survival for patients with colistin-resistant carbapenem-resistant *Klebsiella pneumoniae* (CRKp) as compared to colistin-susceptible CRKp.

Patients were censored at the time of hospital discharge.

CHAPTER 6 - SUMMARY AND FUTURE DIRECTIONS

In this chapter, we provide a synopsis of the research described in this dissertation emphasizing the main findings and contributions; followed by limitations of the work presented and finally, potential ways to address new research questions derived from each project.

The global spread of Gram-negative bacteria (GNB) resistant to nearly all available antibiotics is currently the most pressing issue in bacterial resistance. In the last decade, the crisis in drug resistance has spread worldwide since the speed of dissemination and the lack of development of new antimicrobial drugs, are rapidly outpacing available treatment options. In GNB, the biggest concern is the increasing resistance to β-lactams, especially carbapenems, the drugs of "last resort" to treat severe community and hospital acquired infections. Carbapenem resistance is primarily mediated by β-lactamases that hydrolyze carbapenems - carbapenemases. Among them, the worldwide distribution of *Klebsiella pneumoniae* carbapenemase (KPC) and the rapidly emerging metallo-β-lactamase (MBL) NDM, are currently the most concerning. Since the pipeline for the development of new antibacterial drugs is prohibitively slow, preserving the efficacy of existing drugs is essential. The results presented here aimed at contributing to this end by i) studying what factors drive evolution of novel genotypes and phenotypes in clinically

important Gram negative bacteria in order to find ways of minimizing the development and spread of resistance; and ii) exploring new treatment options for CRE-infections by studying a "recycled" antibiotic, as well as new potential ß-lactamase inhibitors.

Chapter 2 - Boronic acid transition state inhibitors as an alternative for class A ß-lactamases

In this chapter, a series of BATSIs with selectively modified regions (R1, R2, and amide group) were strategically designed and tested against representative class A β-lactamases of *Klebsiella pneumoniae*, KPC-2 and SHV-1. Among the compounds that inhibited KPC-2 and SHV-1 β -lactamases, nine possessed 50% inhibitory concentrations (IC50s) of <600 nM. The most active compounds contained the thiopheneacetyl group at R1 and for the chiral BATSIs, a carboxy- or hydroxy-substituted aryl group at R2. Compound 2b (S02030) was the most active, and demonstrated antimicrobial activity against *Escherichia coli* DH10β carrying bla_{SHV} variants and bla_{KPC-2} or bla_{KPC-3} and against clinical strains of *Klebsiella pneumoniae* and *E. coli* producing different class A β -lactamase genes showing MICs decreases up to 5-fold (from 16 to 0.5 mg/liter).

Although we demonstrated that the compounds are active against these two class A β-lactamases, studies against other important β-lactamases are warranted; nevertheless, this is a promising lead. As compounds become more focused and pathogen directed, the overarching goal is to

design specific chemotypes to treat particular resistant bacteria (i.e., *K. pneumoniae*) in that regard, the approach taken here is an important step towards "precision medicine" (13).

Since compound 2b (S02030) demonstrated inhibitory activity against the tested enzymes, as well as antimicrobial activity when paired with Cefepime, these promising results compelled us to perform experiments *in vitro* to assess its bactericidal effect, and *in vivo* (animal model) to test efficacy and toxicity. To evaluate the *in vitro* antibacterial efficacy of S0203 in combination with cefepime, we performed time-kill curves with a KPC-producing *K.pneumoniae* isolate. Minimum inhibitory concentrations (MICs) with cefepime alone and in combination with S02030 (4 μg/mL) for *K.pneumoniae* KP1: a clinical, KPC-harboring, pan-drug-resistant bloodstream isolate, were determined by agar dilution according to CLSI guidelines (310). *In vitro* time-kill assays were performed for the KP1 strain as previously described (311). Briefly, cultures were grown in Muller-Hinton broth at 37°C with shaking until they reached approximately 10^8 CFU/mL. The bacterial culture was then diluted with fresh media to 10^6 CFU/mL, at which point cefepime was added so that the final concentration was 1×, 2×, 4×, or 8× the agar MIC for the cefepime-S02030 combination (0.5 μg/mL cefepime + 4 μg/mL S02030), while S02030 was held constant at 4 μg/mL. Cefepime-S02030 resulted in an astounding 3-log reduction in bacterial density over the first four hours for all conditions tested, however regrowth was seen at 24 h (Figure 6-1). To test if this

regrowth could be potentially attributed to inhibitor degradation, additional S02030 was added at 4 h. Addition of 4 µg/mL S02030 at 4 h caused an additional, albeit modest decrease in bacterial density 2 h later for all conditions tested. Although regrowth was still observed at 24 h for most conditions, the 8× MIC condition demonstrated a remarkable 5-log cumulative decrease in bacterial density when replenished with S02030 at 4 h (Figure 6-1). Regrowth has been previously observed in other BL-BLI combinations, including the recently developed ceftazidime/avibactam (11). In general, evaluating BL/BLI combinations in vitro by performing time-kill studies is challenging since usually the β-lactam is tested at multiples of the combination MIC, while inhibitor is always tested at a fixed concentration. Under such circumstances it is possible that the threshold concentration of inhibitor may not be sufficient to protect the activity of the β-lactam over the 24 hours, which may contribute to regrowth. Nonetheless, as it has been observed for other commercially available BL/BLI combinations such as piperacillin-tazobactam, regrowth in time-kill studies is likely an in vitro phenomenon that does not necessarily translate to an effect on clinical efficacy, possibly in part due to the repeat dosing that is administered clinically in vivo in addition to the host's immune defenses.

To determine if *in vitro* activity predicted *in vivo* efficacy, our collaborator from the University of Southern California Dr. Brad Spellberg and his team, tested the efficacy of cefepime with or without S02030 in mice

infected intravenously (IV) with the same *K. pneumoniae* strain and treated intraperitoneally (IP) at the time of infection with placebo control, cefepime, or cefepime + S02030 (at 1:1 or 1:4 ratio). Doses were repeated the following morning and afternoon. All mice treated with placebo or cefepime alone died, whereas all mice treated with either ratio of S02030 plus cefepime survived (Figure 6-2A). In a repeat experiment evaluating bacterial burden in the mice, cefepime alone did not significantly reduce blood bacterial density compared to placebo control, but combination therapy at a 1:4 molar ratio caused a reduction of >2-log in bacterial burden at 1 h post-infection, which persisted to 19 h post-infection (Figure 6-2B). These results demonstrate that S02030 restores cefepime activity in a pan-drug-resistant KPC-producing clinical isolate of *K. pneumoniae*.

With this promising set of results, a new BATSI derived from S0203 was synthesized. MB_076, has improved solubility and demonstrated comparable antimicrobial activity when paired with Cefepime. Additionally, the *in vivo* efficacy of this new compound in combination with cefepime, was assessed using the same *K.pneumoniae* strain and bacteremia murine model as previously done for S02030. Results showed that mice treated with placebo or cefepime alone died, whereas all mice treated with MB_076 plus cefepime 1:4 survived (Figure 6-3). Given the increased solubility of MB_076, the following experiments will be aimed at assessing its efficacy in a pneumonia/aspiration model.

To summarize, we have demonstrated bactericidal activity and in vivo efficacy of cefepime in combination with two BATSIs -S02030 and the novel MB_076, which support the continued development of this combinations as a new treatment option for infections caused by Class A carbapenemase-producing *Enterobacteriaceae*, particularly KPC-*K. pneumoniae*.

Chapter 3 - NDM & OXA-48, emerging carbapenemases

In this chapter we describe the sentinel detection and genomic characterization of several Gram negative strains harboring one or two of the emerging carbapenemases NDM and OXA, isolated in Colombia and the USA. First, we report the complete genome sequences of four *bla*$_{\text{NDM-1}}$-harboring isolates recovered between June and October 2012 (soon after the first outbreak in Colombia). *Acinetobacter baumannii* (Aba), *Acinetobacter nosocomialis* (Ans), *Escherichia coli* (Eco) *and Kpn* isolates were multidrug resistant (MDR), and contained multiple plasmids. *bla*$_{\text{NDM-1}}$ was located in plasmids of 193Kb-Inc FIA (*E. coli*), 178Kb-Inc A/C2 (*K. pneumoniae*) and a 47Kb plasmid of unknown Inc type (*A. baumannii* and *A. nosocomialis*). Interestingly, we identified that the Inc A/C2 plasmid in *E. coli* contained a novel complex transposon (Tn125 and Tn5393 with three copies of *bla*$_{\text{NDM-1}}$) and a recombination "hot spot" for the acquisition of new resistance determinants. Noteworthy, unlike other genomic studies in *Acinetobacter* spp. resistance islands (RIs) were not found in these two

strains; however, *bla*$_{\text{ADC-25}}$ (both isolates), *bla*$_{\text{OXA-94}}$ (a *bla*$_{\text{OXA-51}}$ derivative) and plasmidic *aphA6* were discovered (in Aba). In contrast, Kpn and Eco carried several other resistance determinants including *bla*$_{\text{CTX-M 15}}$, *bla*$_{\text{CARB-2}}$, *aac, cat* and *sul*. MLST analysis revealed that isolates belong to ST10 (Eco), ST392 (Kpn), ST322 (Aba) and ST464 (Ans). All strains were recovered from elderly patients with severe systemic hospital-acquired infections; three of four patients presented several co-morbidities and died within 30 days of recovery of the isolate. In this case, the emergence of *bla*$_{\text{NDM}}$ in different genetic contexts among a variety of species and different geographic locations without any epidemiological link raises the public health concern that there is widespread dissemination of this MBL in Colombia.

In the second part, we describe a carbapenem-resistant *Klebsiella pneumoniae* ST147 isolate harboring *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-181}}$ from a young man who underwent abdominal surgery in India and was subsequently transferred to the US and admitted at one of the hospitals participating in the CRACKLE network. In this strain *bla*$_{\text{NDM-5}}$ was located on an IncFII plasmid of 90 kb, whereas *bla*$_{\text{OXA-181}}$ was chromosomally encoded. Resistome and genome analysis demonstrated multiple copies of the transposable element IS26 and a “hot-spot region” in the IncFII plasmid.

Widespread dissemination of NDM-producing bacteria has occurred primarily in endemic areas in Southeast Asia, the Middle East, and the Balkans. Similarly, OXA-48, has achieved broad distribution among *K.*

pneumoniae and other *Enterobacteriaceae*, especially in Turkey and neighboring regions of Southern Europe, North Africa, and the Middle East. By contrast, in the United States, OXA-48 and NDM had remained rare and carbapenem resistance is more frequently mediated by *K. pneumoniae* carbapenemase (KPC). However, these sentinel detections are of special epidemiological and clinical importance not only because carbapenem-resistant infections pose a serious and life-threatening challenge, but also because the appearance of these cases in the Americas raises a concern and highlights the vital importance of continuous surveillance.

In that regard, we have been able to identify additional cases through the Consortium on Resistance Against Carbapenems in *Klebsiella pneumoniae* and Other *Enterobacteriaceae* (CRACKLE), an ongoing, prospective multicenter observational study that includes 60 hospitals in all five census regions of the continental US. As part as this surveillance effort, more than 1,000 patients with CRE infection or bacterial colonization have been enrolled so far and as a result, isolates harboring *bla*$_{\text{IMP}}$ (n=1), *bla*$_{\text{VIM}}$ (n=2), *bla*$_{\text{NDM}}$ (n=30), *bla*$_{\text{OXA-48-}like}$ (n=24), and a combination of *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48-}like}$ (n=6) have been found.

In particular, we were able to characterize the genetic background of two of these isolates harboring multiple carbapenemases. In the first case, we were described an extensively drug resistance (XDR) *K. pneumoniae* producing CTX-M-15, OXA-48 and NDM-1 in a young woman

from Turkey who underwent a multivisceral transplantation in one of the hospitals part of the network. Multilocus sequence typing determined that the isolate belonged to sequence type (ST) 14 and plasmids of 3 types, HIB-M/FIB-M, L, and R, were also present. The most valuable contribution of this molecular characterization is how the medical team was able to design a tailored perioperative antibiotic prophylaxis regime of ceftazidime/avibactam and aztreonam. Postsurgical infections did not occur during this regimen or after its discontinuation despite evidence of rectal colonization with XDR *K. pneumoniae* in surveillance cultures. At the time of the publication (312), the patient was doing well at 9 months post-transplant. In another case, a 57-year-old man with a history of diabetes mellitus type 1 underwent kidney and pancreas transplantation 15 years before presentation with dysuria and acute kidney failure. Noteworthy, he did not have any relevant traveling history. A urine culture grew a carbapenem resistant *Escherichia coli* and we were able to identify $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-14-like}}$, and $bla_{\text{CTX-M-15-like}}$, by PCR amplification and sequencing. As in the previous case, the genotypic characterization combined with the antimicrobial susceptibility data helped the medical team to choose an empiric regimen to avoid nephrotoxicity in this kidney transplant recipient (double carbapenem – meropenem, ertapenem- and fosfomycin). The patient tolerated treatment well and clinical signs of infection resolved; subsequent urine culture obtained 1 week into antibiotic therapy did not show the presence of *E. coli* (313).

Further studies will include a better estimation of the nationwide prevalence of these carbapenemases, thanks to an extended number of CRACKLE surveillance sites, including not only the US but also South America and China. Additionally, since it is just a matter of time until we start seeing local dissemination beyond imported cases future studies should aim at evaluating the effectiveness of combination therapies as an alternative for treating these resistant *Enterobacteriaceae*. One such example is the combination of CAZ/AVI and aztreonam, that has proven useful for *Enterobacteriaceae* strains harboring metallo-β-lactamases (314). One of the evaluated strains (*Enterobacter cloacae*) even contained the combination described in this chapter: NDM-1 and OXA-48, and the MIC for each antibiotic alone went down from >512 to 0.5 μg/mL. Since avibactam is a very potent β-lactamase inhibitor of class A and C enzymes, and most strains of *Enterobacter* spp. possess class C enzymes and in this case a class D carbapenemase as well, avibactam prevented the hydrolysis of ceftazidime and aztreonam. Even though other MBL-*K.pneumoniae* were evaluated, showing similar results, none of them had both an MBL and a class D carbapenemase, therefore other strains with this background should be tested, including the ones described in this chapter. Although avibactam cannot restore susceptibility in MBL-producing strains, aztreonam is not susceptible to hydrolysis by MBLs. Therefore, by inhibiting class A and C and D β-lactamases with avibactam and using aztreonam to "bypass" the class B metallo-β-lactamase, susceptibility can

be restored, leading to a successful microbiological and possible clinical outcome. Knowing the genetic background of a strain, and based upon an understanding of the biochemical mechanisms of action of antibiotics and inhibitors, tailored combinations can be explored.

Chapter 4 - An analysis of the epidemic of KPC-producing Klebsiella pneumoniae: convergence of two evolutionary mechanisms creates the "perfect storm"

In this chapter we investigated the evolution and dynamics of spread of KPC-*K.pneumoniae* in Colombia. Carbapenem resistance is a critical health care challenge worldwide, however the widespread dissemination of *Klebsiella pneumoniae* carbapenemase (KPC) is particularly concerning. *K. pneumoniae* harboring bla_{KPC} (KPC-Kpn) is endemic in many areas including United States, where the epidemic was primarily mediated by the clonal dissemination of Kpn ST258, but we postulated that the spread of bla_{KPC} in other regions occurs by different and more complex mechanisms. To test this, we investigated how KPC disseminated within the *K.pneumoniae* population in Colombia, where KPC became rapidly endemic after emerging in 2005. We sequenced the genomes of 133 clinical isolates recovered from 24 tertiary-care hospitals located in ten cities throughout Colombia, between 2002 (before the emergence of KPC-Kpn) and 2014. Phylogenetic reconstructions and evolutionary mapping were performed to determine temporal and genetic associations between the

isolates. Our findings indicate that the start of the epidemic was driven by horizontal dissemination of mobile genetic elements carrying *bla*$_{KPC-2}$, followed by the introduction and subsequent spread of clonal group 258 (CG258) isolates containing *bla*$_{KPC-3}$. The combination of two evolutionary mechanisms of KPC-Kpn within a challenged health system of a developing country created the "perfect storm" for sustained endemicity of these multidrug-resistant organisms in Colombia.

Some of the limitations of this work include that as a non-governmental sentinel surveillance system, CIDEIM's antimicrobial resistance network relies in the voluntary collaboration of hospitals around the country. This in turn, limited our pool of samples and naturally introduced a bias in the strains received per city and per year, included in the study. Also, given its retrospective nature, this work does not delineate the true incidence of Kpn-KPC infections in the population nor it discriminates between community-acquired or nosocomial infection. However, this constitutes a varied enough sample that could be considered representative of the situation in the country. Moreover, although horizontal transmission was identified as a contributing mechanism for dissemination, individual *bla*$_{KPC}$-harboring plasmids were not elucidated. Identifying specific plasmids and their compositions, host range specificity and incompatibility groups, will help to understand the compartmentalization of the two variants described (KPC-2 in variety of species, while KPC-3 remains exclusive of *K.pneumoniae*). Additionally, although *K.pneumoniae*

is the most common carrier of KPC, other bacterial species populations should be explored, not only because of their individual clinical importance, but also to understand the genetic elements that have contributed to widespread local dissemination. This will ultimately contribute to having a broader epidemiological picture of this carbapenemase in Colombia, an endemic country where dissemination continues.

To start addressing this question, in collaboration with Dr. Villegas from the International Center for Medical Training and research (CIDEIM) in Colombia, we looked at the *bla*$_{KPC}$-harboring species different from *K. pneumoniae* from 2006-2016 (Figure 6-4). Preliminary findings indicate that in this collection, KPC is commonly carried by *P. aeruginosa*, *Enterobacter cloacae* and *Serratia marcesens*, so these 3 species could serve as starting populations to investigate KPC dissemination dynamics. Alternatively, representative isolates from a variety of species could be selected in order to establish possible commonality between the plasmids aiding to the *bla*$_{KPC}$ dissemination. In any case, since this surveillance network often times receives sets of isolates to investigate if they are part of an outbreak, the first step should be to determine if they are clonally related, for example via Pulsed Field Gel Electrophoresis, or member of epidemiologically important clones via Multi Locus Sequence typing. Subsequent analyses should include determination of their resistomes

and presence of mobile genetic elements, including the KPC-associated Tn*4401* and plasmids of different incompatibility groups.

To start, we have selected *Enterobacter* spp. to establish the diversity of molecular vehicles used by *bla*$_{KPC}$ to spread within this population in Colombia. *Enterobacter spp.* is a member of the normal human gut flora and is not usually a primary pathogen, however over recent decades, *Enterobacter* spp. has gained clinical significance emerging as nosocomial pathogen with greater incidence in intensive care patients. Members of the *E. cloacae complex* have emerged as a troublesome pathogen for healthcare institutions globally accounting for up to 5% of hospital-acquired sepsis, 5% of nosocomial pneumonias, 4% of nosocomial urinary tract infections and 10% of postsurgical peritonitis cases (315, 316). The increasing presence of *bla*$_{KPC}$ in *E. cloacae* has dramatically changed outcome of infections caused by this microorganism, given the difficulty in treating these already complicated infections. In the US an increasing trend of carbapenem-nonsusceptibility rate among *Enterobacter cloacae* complex (*E. cloacae, E. asburiae, E. kobei, E. hormaechei, E. xiafangensis*) has been reported (317). In Colombia, *E. cloacae* is the third most frequent pathogen carrying *bla*$_{KPC}$, however, very little is known about the evolution and spread of *bla*$_{KPC}$ in *Enterobacter* spp. In order to have a snapshot on the molecular epidemiology of this GNB in Colombia, we selected 20 isolates collected between 2009 and 2011 that screened positive for KPC by PCR. Whole genome sequences were obtained and MLST, plasmid types, and

resistance genes content was evaluated in order to understand the genetic background, *bla*$_{KPC}$ type, and nature of the plasmids and transposons. Our preliminary results provide a glance of the population structure of KPC-harboring Enterobacter spp. in Colombia (Figure 6-6). An overall variability was found, since multiple subspecies were found, belonging to variety of STs. The majority of isolates were found to be *Enterobacter hormacheii sp. steigelwartii*, and ST510 was the most widespread. As has been found in previous studies describing carbapenem resistant Gram negative bacteria in Colombia, only KPC-2 was found in these isolates. Interestingly, Tn*4401* was found only in a few isolates, suggesting that a different transposable element might be responsible for the dissemination. However, plasmids of incompatibility group IncP6, were the most commonly predominant carriers of *bla*$_{KPC.}$ Of note, this group of plasmids haven been found in our previously described *K. pneumoniae* isolates as well as in the first KPC-*P.aeruginosa* isolate ever described (318). This could indicate that these broad spectrum plasmids could be playing an important role in the dissemination of KPC among Colombian Gram negative isolates. Further studies will focus in determining the plasmids responsible for harboring *bla*$_{KPC}$, and an in-depth analysis of the rearrangements and changes in backbones and transposable elements in order to attempt a reconstruction of the dissemination of this determinant in such a variety of Gram negatives in Colombia. In addition, the plasmid analyses (incompatibility types, conjugability) will shed light on why only

bla$_{KPC-2}$ has been able to expand to variety of species while *bla*$_{KPC-3}$ keeps confined to the *K.pneumoniae* population.

Chapter 5 - Colistin resistance in carbapenem-resistant Klebsiella pneumoniae: laboratory detection and impact on mortality

In this chapter we studied a cohort of patients with infection, or colonization with carbapenem resistant *Klebsiella pneumoniae* (CRKp) in the US, nested within the Consortium on Resistance against Carbapenems in *Klebsiella pneumoniae* (CRACKLE). Each patient was included once, at the time of their first colistin-tested CRKp positive culture during the study period of December 2011 to October 2014. Isolates were tested for colistin susceptibility at each participating hospital, and subsequently reference colistin resistance determination was performed by broth macrodilution and compared to results from clinical microbiology laboratories (Etest) and to polymyxin resistance testing. Of 246 patients with CRKp, 13% possessed ColR CRKp however, ColR was underestimated by Etest: very major error rate = 35% (corresponding to the isolates determined to be resistant by the clinical lab when the reference method indicates they are susceptible), major error rate = 0.4% (corresponding to the isolates determined to be susceptible by the clinical lab when the reference method indicates they are resistant). Carbapenem resistance was mediated primarily by *bla*$_{KPC-2}$ (46%) and *bla*$_{KPC-3}$ (50%), however a variety of rep-PCR strain types were encountered in both the ColS and the ColR groups.

Time to 30-day in-hospital all-cause mortality was evaluated by Kaplan-Meier curves and Cox proportional hazard modeling and we found that ColR was associated with increased hazard for in-hospital mortality (aHR 3.48; 95% confidence interval, 1.73-6.57; P < .001). Since the plasmid-associated ColR genes, *mcr-1* and *mcr-2* were not detected in any of the ColR CRKp, and there is an apparent polyclonal nature of the isolates, we hypothesize that de novo emergence of ColR is the primary factor driving ColR.

As evidenced by our results, determining an accurate MIC for polymyxins (colistin and polymyxin B) is quite difficult due to the physical characteristics of these molecules. More worrisome, unacceptably high error rates occur when polymyxins are tested using disk or gradient (Etest) diffusion methods, commonly preferred by clinical labs to determine MICs. Due to the in-vitro susceptibility testing reproducibility issues, evidenced in our study and taking into account that polymyxins are often prescribed as the last resort for patients with complicated infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE), it is of vital importance to have unified testing and interpretation methodologies. Unfortunately, there are no FDA breakpoints which prevents manufacturers from providing an FDA approved method to clinical laboratories in the United States. Since the Clinical and Laboratory Standards Institute (CLSI) has clinical breakpoints for polymyxins only for *Acinetobacter* spp. and *P. aeruginosa,* but not *Enterobacteriaceae* (319), European Committee on

Antimicrobial Susceptibility Testing (EUCAST) colistin breakpoints for *Enterobacteriaceae* (susceptible, ≤ 2 μg/mL and resistant, >2 μg/mL) are often used (295). In 2017, CLSI published an epidemiologic cutoff value (ECV) of ≤ 2 μg/mL for colistin that applies to five species of *Enterobacteriaceae (Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, Enterobacter aerogenes, Raoultella ornitholytica)* with explicit instructions to not use the ECV as a clinical breakpoint (i.e., do not assign interpretations of "susceptible" to the wild type or "resistant" to the non-wild type population) (319). According to our experience in the analyzed population we propose a new way of looking at colistin/polymyxin resistance where isolates with an MIC below 0.5 μg/mL are considered "truly sensitive" but all others whose MIC is not above the breakpoint of >2 μg/mL still present a risk of treatment failure therefore can be placed in a "quasi resistant vulnerable zone" (Figure 6-5).

Further studies should be aimed at determining the molecular mechanisms of the colistin resistance in this *K. pneumoniae* population. Colistin resistance is caused by weakening of the overall negative charge of the outer membrane by changing the phosphate groups of lipid A to 4-amino- 4-deoxy-l-arabinose and/or phosphoethanolamine (L-Ara4N). In *K. pneumoniae*, the most common modification is the addition of L-Ara4N, which is achieved by plasmid mediated phosphoetanolamine transferases (encoded by *mcr* genes) or activation of the *pmrHFIJKLM* operon caused by alterations in any of the components regulating this operon: two-

component regulatory systems (TCRS) PhoPQ and PmrAB or MgrB, a negative regulator that influences PhoQ-PhoP phosphorylation.

To determine the genetic basis of the colR phenotype among these KPC-harboring *K.pneumoniae*, the plasmid-mediated *mcr-1* and *mcr-2* genes were queried using PCR amplification, and *mgrB phoP, phoQ, pmrAB* mutations were investigated by PCR and sequencing. Our results indicate that the isolates possess heterogeneity of resistance mechanisms leading to the colR phenotype (Figure 6-7): 23/41 isolates possess alterations on the *mgrB* locus including point mutations leading to single amino acid changes, insertional inactivation of the gene (ISKpn 26 & ISKpn18 and other insertions and deletions leading to frameshifts resulting in truncated versions of the protein. Five isolates had a complete deletion of *mgrB* locus (Figure 6-7). Only nine isolates were found to have mutation in the two component systems investigated: 8/41 isolates had mutations in PhoQ and 1/41 isolate had mutations in PmrB. None of the isolates carried plasmid mediated phosphoetanolamine transferases encoded by *mcr-1* or *mcr-2.* Surprisingly, 9/41 isolates possess WT *mgrB*, *phoPQ, pmrAB* suggesting that other mechanism heretofore uncharacterized may be present.

Due to the variety of mechanisms contributing to the increase of colistin resistance, combined with the *in-vitro* susceptibility testing reproducibility issues, the significant difficulty in dosing and variation in individual pharmacokinetic and pharmacodynamic handling of this drug, it is

worrisome that clinicians use colistin as primary monotherapy. Additional two-component regulatory systems could be involved in the generation of colistin resistance via activation of *pmrHFIJKLM* operon, e.g. *ccrAB*, therefore the next step would be to look for substitutions that result in an alteration of this TCRS, or novel undescribed TCRS. It has been shown that mutations that derive in CrrB inactivation activate PmrAB via the newly named crrC (originally reported as ORF H239_3062). The now phosphorylated PmrAB then upregulates the transcription of arnBCADTEF and *pmr*C (111).

Also, since insertional inactivation of *mgrB* seems to be one of the most common colR mechanisms, further studies should aim to determine if transposable elements present in bla_{KPC}-encoding plasmids are linked to the emergence of colistin resistance in Kpn. Since a variety of IS-like elements that are identical or very similar to elements that have been found to be involved in insertional inactivation of *mgrB* are found on several KPC-encoding plasmids, the objective would be to investigate if possessing these plasmids could facilitate the emergence of colistin resistance by providing a source of insertion sequences capable of targeting the *mgrB* gene.

One strategy to answer this question could be by selecting a set of fully sequenced strains: one KPC negative strain without plasmids, and one representative of each one of the KPC plasmid harboring strains. All strains could be grown under colistin selective pressure until colistin

resistance phenotype is induced (as measured by MICs). Subsequently, *mgrB* locus will be sequenced to investigate the mutations responsible for the colistin resistance. Since colistin resistance could also be caused by alterations in PhoP/Q and PmrB, mutations in those genes will be also detected by sequencing. The insertion sequences found to be responsible *mgrB*-insertional-inactivation colistin resistant phenotype will be compared to sequences carried by KPC plasmids to try to establish if they originated from those plasmids.

Alternatively, other less frequent routes of colistin resistance in *K. pneumoniae* could be explored, including the intrinsic regulator RamA known to play a significant role in the overall response to antimicrobials by regulating genes that are linked to permeability barriers and therefore may be involved in reduced susceptibility. It was recently shown that increased levels of this regulator caused LPS alterations and consequently reduced susceptibility to polymyxins (320), therefore experiments aimed at measuring its expression levels, via qPCR, could be explored.

Another potential mechanism to explore is the hyperproduction of capsule polysaccharide (CPS). CPS is well recognized as one of the primary determinants of antigenicity associated with *K. pneumoniae* and capsule switching is a species-specific mechanism used to escape the host immune response. Additionally, DNA exchange in-and-around the *cps* region has been suggested as an important mechanism used by *K. pneumoniae* to rapidly diversify and evolve (267). A recent study showed that CPS acts as

a protective barrier against polymyxins in *K. pneumoniae* (321). *K.pneumoniae* has the ability of releasing anionic capsular polysaccharides from its surface leading to the trapping of cationic antimicrobial peptides, such as polymyxins, thus decreasing the amount of antibiotic reaching the bacterial surface (322). The CPS is connected to the bacterial surface through an ionic interaction with the LPS, and this interaction is stabilized by divalent cations. As a consequence, the release of CPS in the presence of polymyxins is likely due to perturbation of the cation-dependent bridges between the molecules of LPS (323). In line with this, the upregulation of capsular biosynthesis genes could be explored, via qPCR, since an increased CPS production will translate into reduced interactions of polymyxins with the bacterial surface, leading to polymyxin resistance. In addition to exploring if capsular genes are upregulated in strains with high colistin MICs and no other mechanism previously found, a set of *K.pneumoniae* strains with different capsule types (from the Colombian collection) could also be evaluated with and without exposing to sub-inhibitory concentrations of colistin aiming to establish if certain capsular types are more prone to displaying capsule-mediated colistin resistance. Finally, since both the capsule and LPS are key molecules in the immune response mechanisms elicited in the host, the implications in immune recognition and response generated by alterations in any of these two important immunogenic components constitute another very important area to be explored.

General conclusions

The rapid spread of carbapenem resistant *Enterobacteriaceae* has made necessary for the scientific and medical community to adopt innovative approaches combining knowledge about the complexity of resistance mechanisms and genetic heterogeneity in host and bacteria, PK/PD considerations, and systematic clinical experience in order to advance in the treatment of infections caused by these bacteria. The more we understand about the mechanisms of resistance and the genetic elements linked to their successful dissemination, better prevention and control strategies can be designed, and most importantly better guides for empiric therapy can be established.

In that regard, this dissertation makes three important contributions i) our surveillance studies provide insights into the molecular epidemiology of CRE in two endemic regions (The US and Colombia) allowing us to prove our hypothesis that both horizontal transmission and clonal spread play a key role in dissemination; ii) we describe useful alternative therapeutic options including a promising new boronic acid transition state inhibitor that could be a good candidate to become one of the new β-lactam/carbapenemase inhibitor to have clinical impact in the future, and while the studies on the new molecules advance, we have described new ways to treat MDR Gram negative bacilli by using combination therapy. Finally, iii) we discuss the use of polymyxins (colistin and polymyxin B), the difficulty of antimicrobial susceptibility testing, and show how as

hypothesized, alterations in the LPS composition linked to colistin resistance have an impact in mortality.

As recognized by the United Nations and the World Health Organization, Antimicrobial resistance threatens public global health, development, and security, and requires a collaborative multidisciplinary response from the international community. We are confident that the lessons learned in this work and the pursuit of the future directions discussed constitute a step forward in understanding the full scale of the problem in order to prevent further expansion, and find innovative approaches using alternatives to antimicrobials and new technologies for diagnosis and vaccines. We are eager to continue striving to help accomplish this goals.

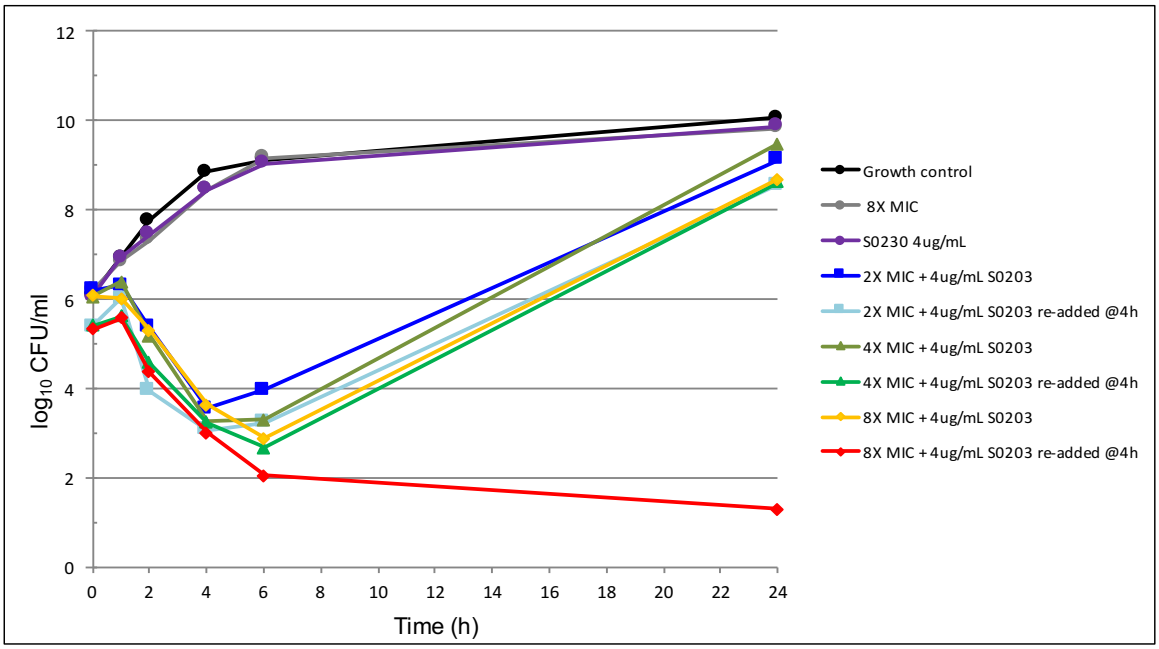


Figure 6-1. Time kill curve for isolate *K. pneumoniae* Kp1

Time-kill curves with and without additional inhibitor added at 4 h. S0203 was present at 4 µg/ml from time zero for each treatment (concentrations of cefepime corresponding to 2x, 1 µg/ml; 4x, 2 µg/ml; and 8x, 4 µg/ml) the MIC of the combination cefepime-S0203 by agar dilution (0.5 ug/ml). A growth control (no antibiotics added), and cefepime alone at 8X the combination MIC were included. Additional S0203 at 4 ug/ml was added at 4 h (indicated by re-added at 4 h). Three replicates were conducted for each of the conditions reported in the time kill assay.

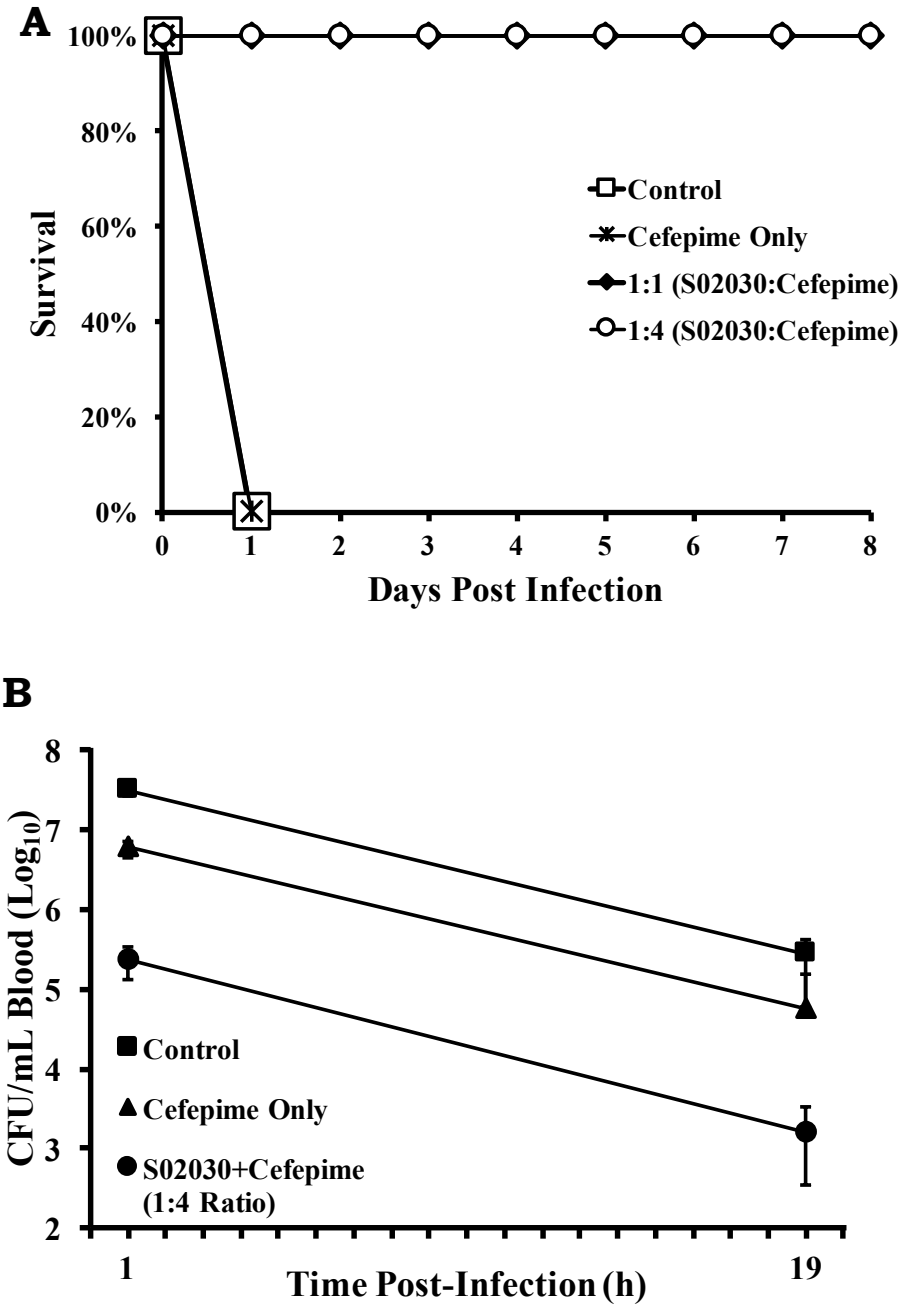


Figure 6-2. Survival curve of mice infected IV with *K.pneumoniae* Kp1 and treated with Cefepime alone or in combination with S02030 (A); and bacterial burden (CFU/mL) after 1 and 19 h (B).

Five male C57BL/6 mice per treatment were infected IV via the tail-vein with 2.1×10^8 CFU/mouse and treated with placebo control (phosphate-buffered saline), cefepime (100 mg/kg/dose), or cefepime and S02030. Animal experiments were performed by Brad Spellberg at the University of Southern California.

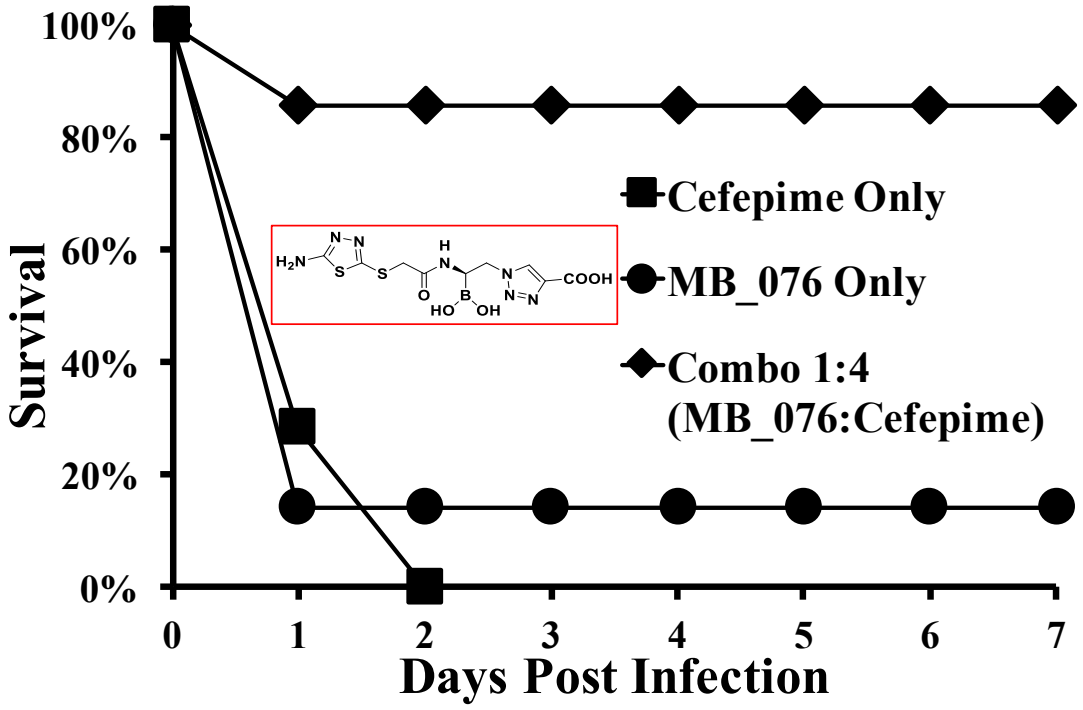


Figure 6-3. Survival curve of mice infected IV with *K.pneumoniae* Kp1 and treated with Cefepime alone or in combination with MB_076.

Five male C57BL/6 mice per treatment were infected IV via the tail-vein with 2.1×10^8 CFU/mouse and treated with placebo control (phosphate-buffered saline), cefepime (100 mg/kg/dose), or cefepime and MB_076. Animal experiments were performed by Brad Spellberg at the University of Southern California.

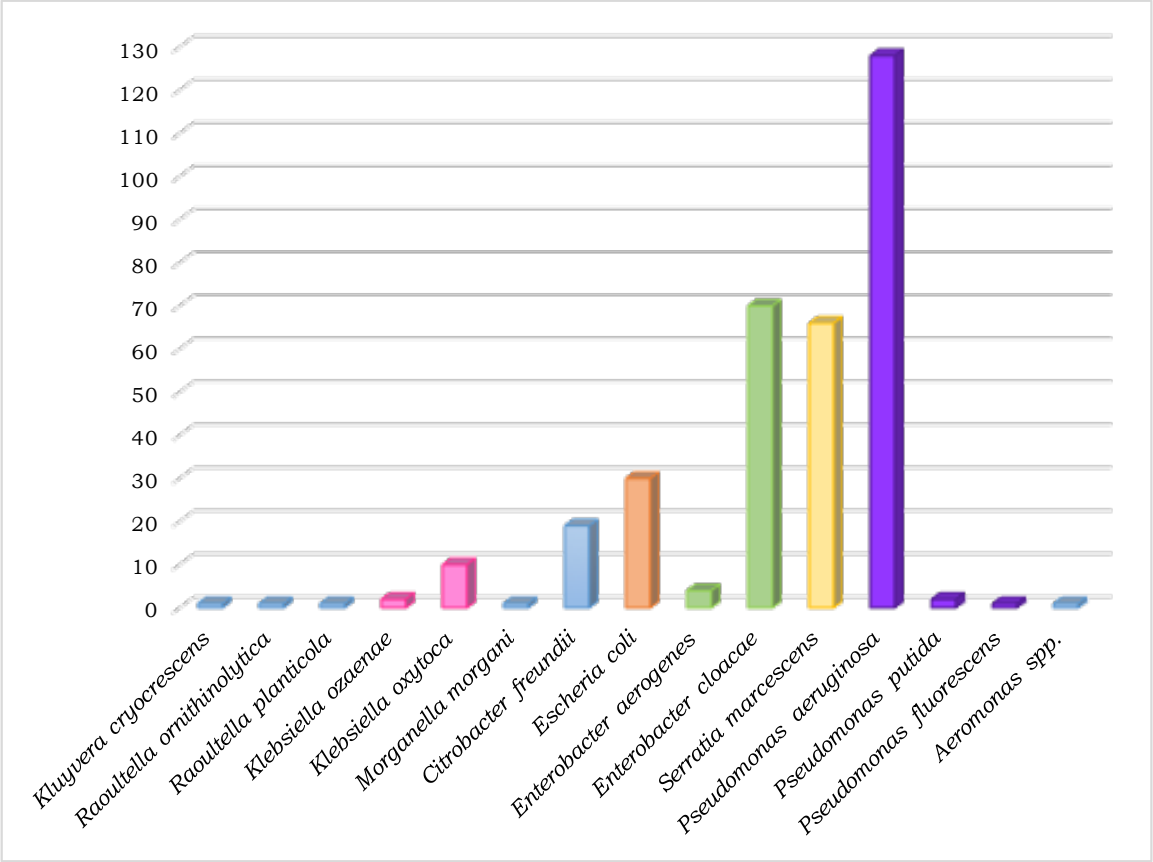


Figure 6-4. Distribution of bacterial species other than *K.pneumoniae* harboring bla_{KPC} from 2006-2016 in Colombia.

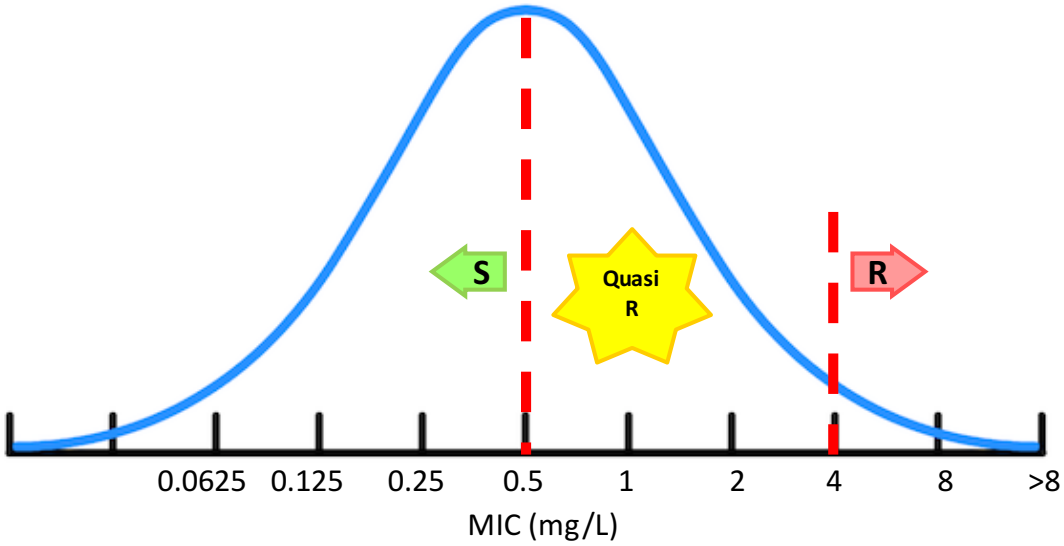
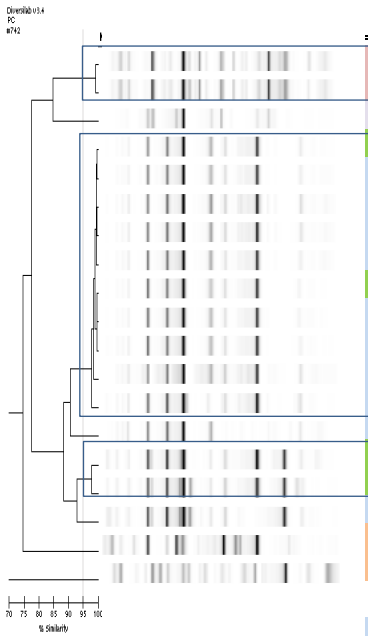


Figure 6-5. The quasiresistant vulnerable zone



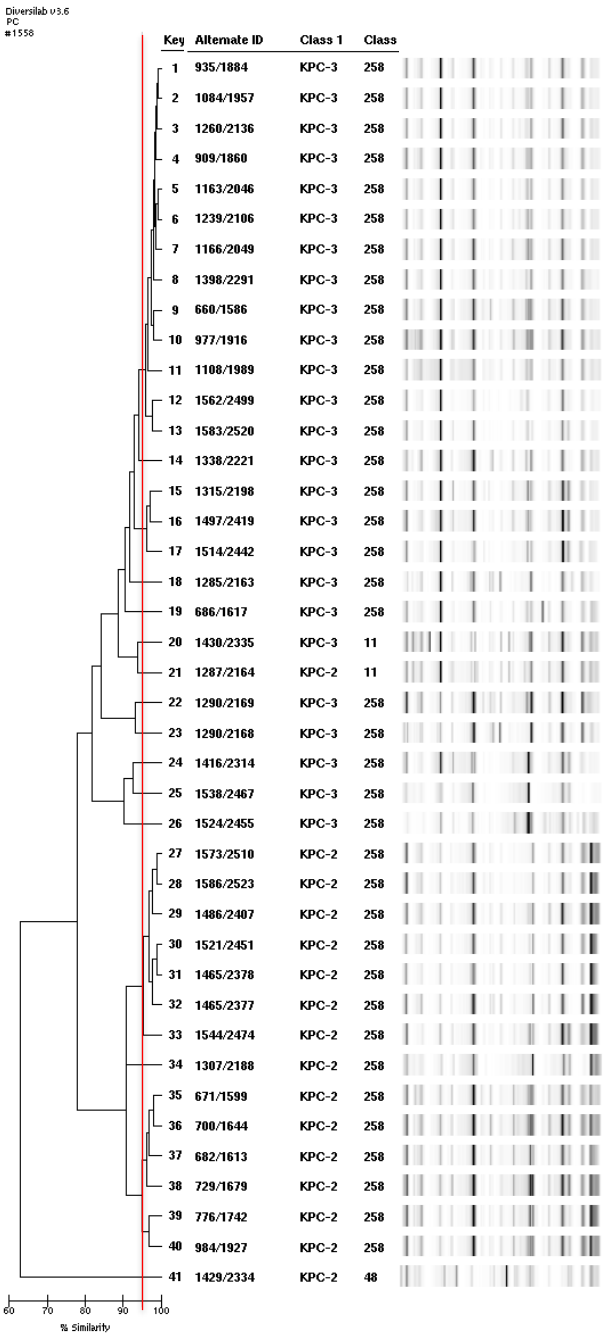
ST	City	Year	Origin	Ward	KPC	Tn 4401 Isoform	Plasmid Type	Plasmid size	Resistance genes	ETP	IMI	MEM	DOR	CIP	AMK	POL	TGC
27	Cali	2009	Blood	ICU	2	b, ΔISKpn6	ND	ND	*ere(A), aadA10, catA1, strB, strA, bla* $_{TEM-1B}$*, aac(3)-IIa, tet(B), bla* $_{SHV-12}$*, oqxB, oqxA, sul1, bla* $_{OXA-2}$*, dfrA29, bla* $_{ACT-10}$*, fosA, qnrB19*	= 8	= 16	= 16	= 16	<= 0,5	= 64	= 1	= 1
27	Cali	2009	Rectal swab	ICU	2	b, ΔISKpn6	ND	ND	*qnrB19, oqxB, oqxA, bla* $_{ACT-10}$*, fosA*	> 32	> 64	> 64	> 64	<= 0,5	<= 8	= 1	= 2
712	Cali	2011	Blood	HOSP	2	Other	IncP6	39511	*bla* $_{CMH-3}$*, bla* $_{TEM-1A}$*, fosA, oqxB, oqxA*	= 2	= 2	= 1	= 2	<= 0,5	<= 8	= 32	= 0,5
264	Cali	2013	Blood	HOSP	2	Other	IncP6	34498	*bla* $_{TEM-1A}$*, bl* $_{aCTX-M-12}$*, bla* $_{ACT-16}$*, qnrB19, rmtG, dfrA14, fosA, sul2, strA, strB, oqxA, oqxB*	= 4	= 2	= 2	= 2	<= 0,5	>= 64	= 2	<= 0,5
510	Cali	2011	Soft Tissue	Surgery	2	Other	IncP6	28052	*bla* $_{TEM-1A}$*, bla* $_{ACT-7}$*, tet(D), QnrB19, aac(3)-IId, dfrA12, aadA2, sul1, sul2, mph(A), fosA, oqxB, oqxA*	= 8	= 4	= 4	= 2	> 8	<= 8	= 2	= 1
510	Pereira	2010	Urine	HOSP	2	Other	IncP6	29343	*aadA1, mph(A), bla* $_{ACT-7}$*, tet(D), aac(6')Ib-cr, aac(6')-Ib, aac(3)-IId, blaOXA-9, qnrB19, sul2, sul1, aadA2, oqxB, oqxA, dfrA12, bla* $_{TEM-1A}$*, fosA*	= 16	= 4	= 4	= 4	> 8	<= 8	<= 0,5	= 2
510	Pereira	2010	Urine	HOSP	2	Other	IncP6	28052	*bla* $_{TEM-1A}$*, bla* $_{ACT-7}$*, bla* $_{OXA-9}$*, qnrB19, aadA1, aac(6')Ib-cr, aac(6')-Ib, , dfrA1, tet(D), sul2, aac(3)-IId, mph(A), sul1, aadA2, dfrA12, fosA, oqxB, oqxA*	= 16	= 4	= 4	= 4	> 8	<= 8	<= 0,5	= 1
510	Cali	2010	Urine	Emergency	2	Other	IncP6	29326	*bla* $_{TEM-1A}$*, bla* $_{OXA-9}$*, bla* $_{ACT-7}$*, qnrB19, aac(6')Ib-cr, aac(6')-Ib, aadA1 ,dfrA1, sul2, fosA, oqxB, oqxA*	= 16	= 4	= 4	= 4	> 8	= 16	= 1	= 2
182	Cali	2012	ND	HOSP	2	Other	ND	ND	*bla* $_{TEM-1A}$*, bla* $_{ACT-16}$*, qnrB6, qnrB19, aadA16, aac(6')Ib-cr, ARR-3, sul1, fosA, oqxA, oqxB,dfrA27*	>= 8	>= 16	= 8	= 64	>= 4	= 16	= 2	> 8
510	Cali	2012	Urine	HOSP	2	Other	IncP6	29343	*bla* $_{TEM-1A}$*, bla* $_{OXA-9}$*, bla* $_{ACT-7}$*, qnrB19, tet(D), aadA1, aac(6')Ib-cr, aac(6')-Ib, aac(3)-IId, aadA2, sul1, sul2, mph(A), dfrA12, fosA, oqxB, oqxA*	= 16	= 4	= 4	= 4	> 8	<= 8	= 1	= 1
510	Cali	2012	Respiratory tract	ICU	2	Other	IncP6	28052	*bla* $_{TEM-1A}$*, bla* $_{ACT-7}$*, qnrB19, tet(D), sul2, aac(3)-IId, dfrA12, aadA2, sul1, mph(A), oqxB, oqxA, fosA*	> 4	> 8	> 8		> 2	<= 8	ND	ND
510	Cali	2011	Blood	ICU	2	Other	IncP6	29343	*bla* $_{TEM-1A}$*,bla* $_{OXA-9}$*,bla* $_{ACT-7}$*,qnrB19,dfrA1, aadA1, aac(6')Ib-cr, aac(6')-Ib,, aac(3)-IId, , aadA2, tet(D), mph(A), sul1, sul2, dfrA12, oqxB, oqxA, fosA*	= 16	= 4	= 4	= 4	> 8	= 16	= 1	<= 0,5
88	B/manga	2011	ND	Emergency	2	b	ND	ND	*bla* $_{TEM-1A}$*, bla* $_{OXA-1}$*, bla* $_{QES-2}$*, bla* $_{ACT-7}$*, bla* $_{CTX-M-15}$*, qnrB19, aadA2, aadB, aacA4, aac(6')Ib-cr, , aph(3')-Via, catB3, mph(A), sul1, dfrA12, oqxA, oqxB*	>= 8	<= 1	= 1		>= 4	<= 64	ND	ND
711	Cali	2009	Rectal swab	ICU	2	b, missing ISKpn6	IncP6	89089	*oqxA, oqxB, bla* $_{ACT-7}$*, fosA, qnrB19*	= 8	= 32	= 8	= 4	<= 0,5	<= 8	= 1	= 0,5
710	Pereira	2009	Blood	Surgery	2	Other	ND	ND	*strA, strB, ere(A), aadA16, sul1, bla* $_{TEM-1A}$*, aadA2, dfrA12, tet(A), bla* $_{ACT-16}$*, qnrB19, fosA, oqxA, oqxB*	= 4	= 4	= 4		<= 1	<= 8	<= 0,5	= 1
171	Pasto	2011	Urine	HOSP	2	Other	IncP6	29591	*bla* $_{ACT-7}$*, qnrB19, aac(3)-IId, strB, strA, tet(D), dfrA12, aadA2, sul1, aph(3')-Ic, mph(A), , fosA, oqxA, oqxB*	= 16	= 4	= 8	= 8	> 8	= 32	<= 0,5	<= 0,5
510	Cali	2012	Blood	HOSP	2	Other	IncP6	28052	*bla* $_{TEM-1A}$*, bla* $_{OXA-9}$*, bla* $_{ACT-7}$*, qnrB19, tet(D), aadA1, aac(6')Ib-cr, aac(6')-Ib, aac(3)-IId, aadA2, sul1, sul2, mph(A), dfrA12, fosA, oqxB, oqxA*	>= 8	>= 16	= 8		>= 4	= 16		
747	Pereira	2009	Blood	ICU	2	b	ND	ND	*oqxA, oqxB, aadA16, ere(A), sul1, strB, strA, bla* $_{CMG}$*, qnrB19, fosA*	= 4	= 8	= 4		<= 1	<= 8	= 32	= 0,5
414	B/quilla	2010	Blood	ICU	2	b	ND	ND	*bla* $_{TEM-1A}$*, bla* $_{SHV-12}$*, bla* $_{ACT-6}$*, sul1, qnrB19, aac(3)-IIa, fosA, oqxA, oqxB*	= 4	= 2	= 1	= 0,5	> 8	= 64	= 1	= 8

E. hormachei sp. steigerwartii
E. hormachei sp. oharae
E. absuriae
E. cloacae sp. cloacae
E. cloacae complex

Figure 6-6. Summary of features of KPC-harboring *Enterobacter* spp. complex.

(Legend in next page)

Figure 6-4 (legend). MLST was performed using the *Enterobacter* cloacae complex scheme. Species determination was performed based on whole genome sequence. Resistance determinants detected in the isolates are: narrow spectrum β-lactamases (TEM-1, SHV-1 SHV-11, OKP-1, LEN-1/12/16,); extended spectrum β-lactamases (SHV-5/11/12/25/27/31/33/101/108/129, CTX-M 2/12/15/96, OXA-1/2/9/47), carbapenemases (KPC-2/3, VIM-24); aminoglycoside modifying enzymes [aminoglycoside N-acetyltransferases (AAC), aminoglycoside O-nucleotidyltranferases (ANT), aminoglycoside O-phosphotransferases (APH)]; 16S rRNA modifying enzymes [methyltransferases (RMT)]; quinolone conferring resistance enzymes [plasmid-mediated quinolone-resistance (QNR), OqxA-B efflux pump, and N-acetyltransferase Aac(6′)-Ib-cr]; fosfomycin resistance proteins (FosA, FosB); macrolides, lincosamides and streptogramins (MLS) resistance determinants [ErmB rRNA methylase (ERM) and macrolide phosphorylases (MphA/B)]; chloramphenicol resistance determinants [chloramphenicol acetyltransferase (CatA1) and chloramphenicol resistance efflux protein, CmlA]; rifampin ADP-ribosyltransferase (Arr); trimethoprim dihydrofolate reductase (DfrA); tetracycline efflux pump (TetA - C); sul1, sul2 and sul3 genes encoding dihydropteroate synthetase (DHPS). Minimum inhibitory concentrations against the following antibiotics ETP, ertapenem; IMI, imipenem; MEM, meropenem; DOR, doripenem; CIP, ciprofloxacin; AMK, amikacin; POL, polymyxin; TGC, tygecycline are expressed in mg/L.



PolB MIC	Colistin MIC	*mcr-1*	*mcr-2*	*mgrB*	*phoQ*	*phoP*	*pmrB*	*pmrA*
>8	>8	NEG	NEG	WT	Asn255Tyr	WT	WT	WT
>8	>8	NEG	NEG	WT	Asn255Tyr	WT	WT	WT
2	8	NEG	NEG	WT	Gly385Asp	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
>8	>8	NEG	NEG	WT	Gly385Asp	WT	WT	WT
SW	4	NEG	NEG	WT	Gly385Asp	WT	WT	WT
≤0.5	8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	Truncated protein (nt128: A-->T)	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	NS	NS	NS	WT
8	8	NEG	NEG	WT	Gly385Asp	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
2	4	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
>8	>8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	Δ114T (frameshift)	NS	NS	NS	NS
>8	>8	NEG	NEG	WT	WT	WT	Thr134Pro	WT
≤0.5	4	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	Truncated protein (nt59: G-->A)	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn18	NS	NS	NS	NS
>8	>8	NEG	NEG	WT	NS	NS	NS	NS
>8	>8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	WT	Phe398Leu	WT	WT	WT
>8	>8	NEG	NEG	Deletion	WT	WT	WT	WT
8	>8	NEG	NEG	Deletion	WT	WT	WT	WT
>8	>8	NEG	NEG	Cys39Ser	WT	WT	WT	WT
8	8	NEG	NEG	Asp31Asn	WT	WT	WT	WT
>8	>8	NEG	NEG	Deletion	NS	NS	NS	NS
>8	>8	NEG	NEG	Deletion	NS	NS	NS	NS
>8	>8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	NS	NS	NS	NS
≤0.5	4	NEG	NEG	Asp31Asn	WT	WT	WT	WT
≤0.5	8	NEG	NEG	WT	Ser188Ile	WT	WT	WT
8	8	NEG	NEG	WT	WT	WT	WT	WT
>8	>8	NEG	NEG	ISKpn26	WT	WT	WT	WT
>8	>8	NEG	NEG	Cys39Ser	WT	WT	WT	WT
>8	>8	NEG	NEG	Deletion	NS	NS	NS	NS
>8	>8	NEG	NEG	Frameshift nt3: insA, truncated protein	WT	WT	Gly233Arg Val257Leu	WT

Figure 6-7. rep-PCR based dendogram of colistin resistant KPC- *K.pneumoniae* isolates and their associated colistin resistance mechanism.

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