

# Distribution of $\beta$ -lactamase Gene and Genetic Context of blaKPC-2 in Clinical Carbapenemase-producing *Klebsiella Pneumoniae* Isolates

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# Abstract

**Background:** This study is designed to characterize the dissemination mechanism and genetic context of *Klebsiella pneumoniae* carbapenemase (KPC) genes in carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates.

**Methods:** A retrospective analysis was performed on CRKP isolates from a teaching hospital of Wenzhou Medical University from 2015-2017. Polymerase chain reaction (PCR)-based amplification and whole-genome sequencing (WGS) were used to analyze the genetic context of the  $bla_{KPC-2}$  gene. Conjugation experiments were performed to evaluate the transferability of  $bla_{KPC-2}$ -bearing plasmids. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed to investigate the clonal relatedness of  $bla_{KPC-2}$ -producing strains.

**Results:** The  $bla_{KPC-2}$  gene was identified in 13.61% (40/294) of clinical *K. pneumoniae* isolates. Three different sequence types (ST11, ST15 and ST656) and 5 PFGE subtypes (A to E) were classified among them. ST11 was the dominant sequence type (92.50%, 37/40). Plasmid-oriented antibiotic resistance genes such as extended spectrum- $\beta$ -lactamases (ESBLs) and other antimicrobial resistance genes were also found in KPC-positive *K. pneumoniae* (KPC-*Kp*). Mapping PCR and genomic sequencing revealed that the  $bla_{KPC-2}$ -bearing sequence regions related to different mobile elements, including Tn 1721- and IS26-based transposons, were mainly located in but not restricted to IncFII-like plasmids and were structurally divergent.

**Conclusions:** The  $bla_{KPC-2}$  genes related to divergent mobile genetic elements encoded on transferable plasmids may transfer easily and widely, resulting in the spread of resistance among bacteria of different species or genera. Co-infection of KPC-*Kp* plasmids carrying additional non- $\beta$ -lactam antibiotic resistance genes simultaneously further limits the antibiotics available to treat infections with KPC-producing pathogens.

## Introduction

Carbapenems represent a critical class of  $\beta$ -lactam antimicrobials that are used against multidrug-resistant (MDR) gram-negative bacteria, especially strains expressing a high level of AmpC cephalosporinase or extended spectrum- $\beta$ -lactamases (ESBLs)<sup>[1]</sup>. Though *Klebsiella pneumoniae* is considered an opportunistic pathogen, the prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) associated with severe nosocomial and systemic infections has become an alarming threat to public health in recent years<sup>[2]</sup>. CRKP may serve as a reservoir for antimicrobial resistance because of its propensity to accumulate and transfer ESBLs and other antibiotic inactivating enzymes, mostly in the form of transferable plasmids and transposons, leading to MDR bacterial infections<sup>[3]</sup>.

By far, the CRKP pandemic is primarily driven either by the widespread dissemination of clonal complex (CC) 258, more specifically to multilocus sequence type (ST) 258, or the horizontal dissemination of

mobile genetic elements, especially insertion sequences and transposons encoded on the plasmids<sup>[4, 5]</sup>. ST258 is the international hyper-epidemic lineage clone contributing to the spread of *K. pneumoniae* carbapenemase (KPC)-positive *K. pneumoniae* (KPC-Kp), which has disseminated throughout the USA and European countries<sup>[6]</sup>. However, ST11, a single locus variant of ST258, is frequently reported as the dominant clone of CRKP in Asia due to its association with multidrug resistance determinants, hypervirulence and high transmission ability<sup>[7]</sup>.

The most frequent plasmid-carried Ambler class A carbapenemase is KPC, and the *bla*<sub>KPC-2</sub> gene is the main cause of a prolonged outbreak<sup>[3]</sup>. The *bla*<sub>KPC-2</sub> gene is carried frequently within the conserved Tn3 family transposon Tn4401 on transferable plasmids in Europe and the United States<sup>[8, 9]</sup>. However, non-Tn4401 mobile elements, especially Tn1721-like transposons among ST11 *K. pneumoniae*, are mainly responsible for the effective spread of the *bla*<sub>KPC-2</sub> gene in China<sup>[10]</sup>.

Despite the description of several novel structures and detailed data obtained in previous research, little is known about the genetic process of the conversion that accounts for the genetic differences in the *bla*<sub>KPC-2</sub> gene. The mechanism behind the transmission of this gene warrants further exploration. This research aimed to provide a molecular epidemiology investigation and the genetic features of retrospectively collected *bla*<sub>KPC-2</sub>-harboring *K. pneumoniae* in a Chinese tertiary hospital.

## Materials And Methods

### Bacterial strains and antibiotic susceptibility testing

Between January 2015 and December 2017, 294 clinical isolates of *K. pneumoniae* were obtained from a hospital in Wenzhou, China, and identified as *K. pneumoniae* by a VITEK 2 automated microbiology analyzer (BioMerieux Corporate, Craaponne, France). Antimicrobial susceptibility tests were determined by the agar dilution method for 14 antibiotics and by the broth microdilution method for tigecycline, colistin and ceftazidime/avibactam (CAZ/AVI). The results were interpreted by the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018)<sup>[11]</sup>, while for colistin, tigecycline, and CAZ/AVI, the breakpoints were defined according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org/>)<sup>[12]</sup>. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality controls. Isolates resistant to either or both carbapenems (imipenem and meropenem) tested were classified as CRKP<sup>[13]</sup>. The carbapenemase phenotype was further confirmed by the Carba NP test<sup>[14]</sup>. MDR, extensively drug-resistant (XDR) and pandrug-resistant (PDR) phenotypes were defined according to a previous report<sup>[15]</sup>.

### PCR amplification and sequencing

Bacterial DNA was extracted using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Scientific, Union City, CA, United States) from a single colony subcultured on MacConkey agar plates at 37 °C for 18 hours. Carbapenemase genes (*bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>), plasmid-mediated

AmpC  $\beta$ -lactamase genes (*bla*<sub>MOX</sub>, *bla*<sub>CIT</sub>, *bla*<sub>DHA</sub>, *bla*<sub>ACC</sub>, *bla*<sub>EBC</sub>, and *bla*<sub>FOX</sub>) and broad-spectrum  $\beta$ -lactamase genes (*bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub>) were analyzed by PCR as previously described<sup>[16]</sup>. PCR amplicons were sequenced and compared with sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST searches.

## Molecular typing

All CRKP isolates were classified by *Xba*I (Takara, Dalian, China)-digested pulsed-field gel electrophoresis (PFGE) using a CHEF Mapper System (Bio-Rad) as previously described<sup>[13]</sup>. A phylogenetic dendrogram of PFGE profiles was constructed using UPGMA clustering under appropriate tolerance (2%) settings. Cutoff lines at 85% and 95% were used to delineate PFGE clusters<sup>[17]</sup>. Multilocus sequence typing (MLST) of the *K. pneumoniae* isolates was performed with PCR and PCR products of seven housekeeping genes, namely, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*, were sequenced<sup>[10]</sup>. All the sequences were compared with information in the MLST database (<http://bigsdw.web.pasteur.fr/klebsiella>).

## Conjugation experiment

Plasmid conjugation experiments were carried out by filter mating using rifampicin-resistant *E. coli* EC600 as a recipient<sup>[18]</sup>. The transformants were selected on MacConkey agar supplemented with rifampicin (1,024 mg/L) and meropenem (2 mg/L). Putative transconjugant colonies were selected, further verification was performed based on 16S rRNA sequencing and PCR-amplified *bla*<sub>KPC-2</sub> detection of the transconjugant. PCR-based replicon typing was conducted and replicons were sequenced using previously reported primers<sup>[19]</sup>.

## Genetic environment analysis of the *bla*<sub>KPC-2</sub> gene

A series of primers based on *bla*<sub>KPC</sub>-surrounding sequences were designed. Mapping PCR was performed to compare the genetic context of the *bla*<sub>KPC</sub> gene according to previously reported conditions<sup>[10, 20]</sup>. When a standard primer pair failed to yield a product, alternative outer primers were used to span the region of variation and all amplification products obtained were sequenced.

## WGS and bioinformatic analyses

Genomic DNA was extracted using the aforementioned method. DNA was sequenced with an Illumina HiSeq 2500 and PacBio RS II instrument (Pacific Biosciences) at Personalbio Technology Co., Ltd. (Shanghai, China). The PacBio long reads were initially assembled by Canu v1.6<sup>[21]</sup> to obtain contigs of the genome sequences, and then two FASTQ sequence files generated using the Illumina HiSeq 2500 platform were mapped onto the primary assembly to control assembly quality. The potential open reading frames (ORFs) were predicted using Glimmer software (<http://ccb.jhu.edu/software/glimmer>) and annotated against UniProt/Swiss-Prot. BlastX (<https://blast.ncbi.nlm.nih.gov>) was used to annotate the predicted protein-coding genes against a non-redundant protein database with an e-value threshold of

1e-5. Annotation of resistance genes was performed using ISfinder, INTEGRALL and ResFinder with default parameters<sup>[22]</sup>.

## Comparative genomics analysis of the *bla*<sub>KPC-2</sub>-bearing plasmids and *bla*<sub>KPC-2</sub> gene-related regions

The plasmid and chromosome genome sequences used for comparative genomics analysis were downloaded from the NCBI database. The sequences with an identity and coverage of  $\geq 80\%$  with pKPC3020-124 were retained for later ortholog analysis using BlastP and InParanoid<sup>[23]</sup>. A map of the plasmid with GC content and GC skew was drawn using the online CGView Server and local GView 1.7 with a visual interface<sup>[22]</sup>. For the linear comparison of the *bla*<sub>KPC-2</sub> gene-related fragments, wild-type KPC-*Kp* isolates were mixed, and the plasmids were extracted using a Qiagen Plasmid Mega Kit (Qiagen, Valencia, CA, USA). Sequences containing the *bla*<sub>KPC-2</sub> gene were also obtained from the NCBI Nucleotide database using *bla*<sub>KPC-2</sub>, Tn 1721, IS*Kpn6*, and IS*Kpn8* as the key words. The results were filtered, and only sequences containing a complete *bla*<sub>KPC-2</sub> gene and more than 20 kb in length were retained. Multiple sequence alignments were performed by MAFFT using the 11 kb *bla*<sub>KPC-2</sub> gene-related fragment of pKPC3020-124 as a reference, and the sequences were clustered with an identity of 80%<sup>[24]</sup>. The sequence sharing the greatest similarity to the other sequences in each cluster was chosen as the candidate for ortholog analysis.

### Nucleotide Accession Number

The nucleotide sequences reported in this paper have been deposited in GenBank nucleotide database, and the accession numbers for the genome sequences of *K. pneumoniae* KP3020 are CP061354 (chromosome), CP061355 (pKPC3020-124), CP061356 (pKPC3020-54), CP061357 (pKPC3020-4) and CP061358 (pKPC3020-2), while CP061347, CP061346 and CP061348 were for the *bla*<sub>KPC-2</sub>-bearing plasmid pKPC1880 and sequences contig1846 and 41\_pilon, respectively.

## Results

### Prevalence of carbapenemase and distribution of the $\beta$ -lactamase genes

Ten  $\beta$ -lactamase genes were predicted through the sequencing of the pooled genomic DNA of 294 strains and were confirmed by the PCR screening method. Regardless of the intrinsic genes *bla*<sub>SHV</sub>, *bla*<sub>OKP</sub>, and *bla*<sub>LEN</sub> encoded in the chromosome, *bla*<sub>CTX-M-65</sub> and *bla*<sub>TEM-1</sub> were the most prevalent genes with positive rates of 27.21% and 18.71%, respectively, while *bla*<sub>OXA-10</sub> and *bla*<sub>OXA-1</sub> showed the lowest positive rates of 1.02% and 0.68%, respectively (Table S1). The *bla*<sub>KPC</sub> gene was identified in 13.61% (40/294) of the strains, all of which were verified to be carbapenemase producers. Sequencing results of

the PCR products revealed identical *bla*<sub>KPC-2</sub> alleles (NG\_049253) among these carbapenemase-producing *K. pneumoniae*. No other carbapenemase gene was identified. The KPC-*Kp* isolates also carried at least one other  $\beta$ -lactamase gene, such as the ESBL gene *bla*<sub>CTX-M</sub> (90%, 36/40) and the narrow-spectrum *bla*<sub>TEM</sub> (70%, 28/40) (Fig. 1). The *bla*<sub>SHV</sub> variants included *bla*<sub>SHV-2</sub> (n = 2), *bla*<sub>SHV-12</sub> (n = 10), *bla*<sub>SHV-28</sub> (n = 1) and *bla*<sub>SHV-11</sub> (n = 37), while *bla*<sub>CTX-M-65</sub> and *bla*<sub>TEM-1</sub> were the only *bla*<sub>TEM</sub> genotypes observed.

## Antimicrobial susceptibility and conjugation results of the CRKP isolates

The minimum inhibitory concentrations (MICs) of 17 antimicrobial agents against all forty CRKP isolates showed multidrug resistance profiles, particularly to  $\beta$ -lactams (including all carbapenems, ertapenem, meropenem and imipenem detected), quinolones and aminoglycosides (Table 1 and Table S1). Notably, all isolates were susceptible to CAZ/AVI, except for KP3034, which exhibited low level resistance to this combination with the MIC of 16/4 mg/L. Colistin was shown to be the most active agent (MIC<sub>90</sub> = 1 mg/L, 100% susceptible), followed by tigecycline (MIC<sub>90</sub> = 8 mg/L, 80% susceptible). The MIC values of aminoglycoside antibiotics were variable: 62.5% (25/40) of isolates showed resistance to gentamicin, and 57.5% (23/40) of isolates showed resistance to both tobramycin and amikacin. Chloramphenicol and fosfomycin exhibited low sensitivity (< 20%). In general, all isolates were not sensitive to at least one agent in three or more antimicrobial categories, while 8 isolates (20%, 8/40) were not sensitive to at least one agent in all categories except colistin, exhibiting MDR and XDR phenotypes.

Table 1

Antimicrobial susceptibility testing results of carbapenem-resistant *Klebsiella pneumoniae* isolates from 2015 to 2017 (mg/L)

Antibiotics	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC Interpretation		
				S (%)	I (%)	R (%)
Ceftazidime <sup>a</sup>	16–256	64	256	0	0	100
Ceftazidime/avibactam <sup>b</sup>	0.5–16	2	2	97.5	0	2.5
Cefotaxime <sup>a</sup>	8-512	256	512	0	0	100
Cefepime <sup>a</sup>	16–256	256	> 256	0	0	100
Aztreonam <sup>a</sup>	128-1,024	1,024	> 1,024	0	0	100
Imipenem <sup>a</sup>	8-512	32	64	0	0	100
Meropenem <sup>a</sup>	4-256	32	64	0	0	100
Ertapenem <sup>a</sup>	32 - 1,024	512	512	0	0	100
Gentamicin <sup>a</sup>	0.25-1,024	128	512	37.5	0	62.5
Tobramycin <sup>a</sup>	0.25-1,024	128	512	37.5	5	57.5
Amikacin <sup>a</sup>	0.125-1,024	1,024	> 1,024	42.5	0	57.5
Ciprofloxacin <sup>a</sup>	16–256	32	128	0	0	100
Levofloxacin <sup>a</sup>	8-128	32	64	0	0	100
Fosfomycin <sup>a</sup>	32 - 1,024	512	1,024	5	10	85
Chloramphenicol <sup>a</sup>	2-512	512	512	20	2.5	77.5
Tigecycline <sup>b</sup>	0.5–32	2	8	80	12.5	7.5
Colistin <sup>b</sup>	0.25-2	1	1	100	0	0
S, susceptible; I, intermediate; R, resistant.						
<sup>a</sup> The antimicrobial susceptibility test of these antibiotics was conducted by the agar diffusion method and interpreted by CLSI criteria.						
<sup>b</sup> For colistin, tigecycline and ceftazidime/avibactam, the antimicrobial susceptibility test was performed using broth microdilution method and interpreted by EUCAST criteria.						

To localize the *bla*<sub>KPC</sub> gene and determine the transferable ability of *bla*<sub>KPC</sub>-bearing plasmids in the *K. pneumoniae* strains, a conjugation experiment was performed. Despite repeated attempts, only 5 of 40

isolates consisting of different ST types successfully transferred carbapenem-resistant plasmids to *E. coli* EC600 by conjugation. The transconjugants exhibited a phenotype of resistance or reduced susceptibility to carbapenems compared with the recipient *E. coli* EC600 (Table 2).

Table 2  
Resistance genes and carbapenem MIC levels (mg/L) of wild strains and transconjugants

Strains	$\beta$ -lactam genes	ETA	MEM	IMP	Replicon types	ST types
KP1878	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>SHV-11</sub>	512	256	64	IncFII	11
pKPC1878/EC600	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub>	16	8	4	IncFII	-
KP1880	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>SHV-11</sub>	256	128	32	IncFII, IncFII <sub>K</sub>	11
pKPC1880/EC600	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub>	4	2	2	IncFII	-
KP2159	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-6</sub> , <i>bla</i> <sub>SHV-2</sub>	32	8	8	IncFIA, IncFII <sub>K</sub>	656
pKPC2159/EC600	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub>	8	2	2	IncFII <sub>K</sub>	-
KP3020	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>SHV-28</sub>	64	64	32	IncFII <sub>K</sub>	15
pKPC3020-124/EC600	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub>	8	2	2	IncFII <sub>K</sub>	-
KP4603	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>SHV-12</sub>	512	256	64	IncFII, IncFII <sub>K</sub>	11
pKPC4603/EC600	<i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>SHV-12</sub>	16	4	4	IncFII <sub>K</sub>	-
EC600	-	0.0075	0.03	0.015	-	-

ETA, ertapenem; MEM, meropenem; IMP, imipenem.

## Genetic relatedness of the CRKP isolates

The results of MLST and PFGE analyses divided 40 carbapenemase producers into three different ST types (ST11, ST15 and ST656) and five PFGE pulsotypes (A to E) (Fig. 1). ST11 (n = 37) was the most predominant sequence type, consisting of all strains of PFGE pulsotypes C (n = 11), D (n = 10) and E (n = 16), while ST15 (n = 1) and ST656 (n = 2) consisted of isolates of PFGE pulsotypes A and B, respectively. All the CRKP isolates harbored one or more other  $\beta$ -lactamase genes along with the *bla*<sub>KPC-2</sub> gene,

especially *bla*<sub>CTX-M-65</sub> or/and *bla*<sub>TEM-1</sub> in most ST11 *K. pneumoniae* isolates. The *bla*<sub>TEM-1</sub> gene was not identified in any strains of PFGE cluster C.

## Comparative genomics analysis of the plasmid pKPC3020-124 with homologous plasmids

Sequencing analysis of the conjugative *bla*<sub>KPC</sub>-bearing IncFII<sub>K</sub> plasmid pKPC3020-124 from KP3020 showed that the plasmid was 124,452 bp in length with an average GC content of 53.2% and was predicted to encode 152 ORFs. Using the plasmid sequence as a query, 15 sequences with the highest similarities (> 80% coverage and > 80% identity) with pKPC3020-124 were retrieved from the NCBI Nucleotide database. 14 of them were IncFII<sub>K</sub> plasmid sequences, including 2, 10 and 2 sequences from the *Enterobacteriaceae* species of *E. coli*, *K. pneumoniae* and *Serratia marcescens*, respectively, and one was from the chromosome sequence of *K. pneumoniae* 91a83dc8-b809-11e8-aae5-3c4a9275d6c8 (Table S2). These *bla*<sub>KPC</sub>-bearing plasmids could mainly be divided into two functional regions: a variable region and a conserved region (Fig. 2). The conserved region could be further divided into two fragments, with one fragment encoding genes related to the type IV secretion system (conjugative transfer region) and the other encoding backbone genes related to plasmid replication and stability (plasmid maintenance region). The variable region is full of insert sequences (such as IS*Kpn14*, IS*5075*, IS*Stma11*, and IS*Ec38*) and some resistance genes (MDR region), including *bla*<sub>KPC-2</sub>, *bla*<sub>TEM-1</sub> and four mercury resistance genes (*merACPT*). The difference between pKPC3020-124 and the other plasmids is that former has a unique region of approximately 15.5 kb in length encoding 5 insert sequences and four DNA metabolism enzyme genes (*resA*, *agp*, *nqrC* and *lold*) (Fig. 2, unknown function region).

## Comparison of genetic environments of *bla*<sub>KPC-2</sub>-encoding regions

Based on the results of the mapping PCR approach, four divergent forms of *bla*<sub>KPC-2</sub>-harboring structures were identified among these carbapenem-resistant plasmids (Table S3). The primers used and their positional counterparts are summarized in Table S4 and Fig. S1. The mapping PCR results were confirmed by subsequent sequencing and aligning with WGS and plasmid sequencing results. To analyze the structure of the *bla*<sub>KPC-2</sub> encoding region, all the sequences (including four obtained in this work) of approximately 5–20 kb in length (with the *bla*<sub>KPC-2</sub> gene at the center) were retrieved from the NCBI Nucleotide database. According to the similarities of the core sequences adjacent to the *bla*<sub>KPC-2</sub> gene, 10 sequences were chosen as representatives and are illustrated in Fig. 3, revealing that an approximately 4-kb fragment encoding *tpnR*-IS*Kpn8*-*bla*<sub>KPC-2</sub>- $\Delta$ IS*Kpn6* was conserved and present in the majority of the sequences. According to whether a Tn1721 (or its derivative) was adjacent to the *bla*<sub>KPC-2</sub>-encoding fragment, these sequences were roughly categorized into two groups. The first group consisted of the sequences without Tn1721 (or its derivative), while the second group contained Tn1721 (or its derivative) next to the *bla*<sub>KPC-2</sub>-encoding fragment. The sequences of the second group were mainly from *K.*

*pneumonia*, but those of the first group existed in various species such as *K. pneumonia*, *E. coli*, and *Citrobacter freundii*.

## Discussion

In this study, all carbapenem-resistant strains were confirmed to be carbapenemase producers and were highly resistant to the most common antimicrobial agents. All isolates showed MDR phenotypes, with 20% of them resistant to agents of all antimicrobial categories tested except for colistin, which could be evaluated as XDR pathogens according to a previous report<sup>[15]</sup>. The data showed that CAZ/AVI displayed potent activity against clinical KPC-*Kp* isolates with an MIC<sub>90</sub> of 2/4 mg/L. Intriguingly, in addition to the high level of carbapenem resistance with a meropenem MIC of 1,024 mg/L, the strain KP3034 exhibited a higher MIC level for CAZ/AVI (16/4 mg/L) than the other strains detected. As no history of previous antimicrobial exposure to CAZ/AVI treatment or specific mutations were observed in the *bla*<sub>KPC-2</sub> gene, hyper-expression of the *bla*<sub>KPC-2</sub> gene associated with porin deficiency in the outer membrane, or other unknown mechanisms might account for this low level of CAZ/AVI resistance<sup>[25]</sup>.

In addition to the *bla*<sub>KPC-2</sub> gene, one or more other  $\beta$ -lactamase genes (such as *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub>) were identified in these KPC-producing *K. pneumoniae* strains, with 90% (36/40) of the strains carrying the ESBL gene *bla*<sub>CTX-M</sub>. *bla*<sub>CTX-M-65</sub> identified in this work belongs to the group 9 CTX-M  $\beta$ -lactamase gene, which mediates cephalosporin resistance<sup>[26]</sup>, while *bla*<sub>SHV</sub>, a core chromosomal gene in *K. pneumonia*, mainly mediates ampicillin resistance<sup>[27]</sup>. CTX-M-65 is a variant of CTX-M-14 that differs by 2 amino acid substitutions (Ala78Val and Ser273Arg)<sup>[28]</sup> and was identified in the variable regions of F33:A:B- plasmids from food-producing animals in China<sup>[29]</sup>. In contrast to *bla*<sub>CTX-M-14</sub> reported by Yang et al<sup>[14]</sup>, *bla*<sub>SHV-11</sub>, *bla*<sub>CTX-M-65</sub>, and *bla*<sub>TEM-1</sub> were shown to be the predominant genotypes. The co-existence of these multiple broad-spectrum  $\beta$ -lactamase genes simultaneously in KPC-*Kp* confers resistance to 1st-, 2nd- and 3rd-generation cephalosporins, leading to difficulties in treating the corresponding bacterial infections. Other resistance genes, such as *rmtB* and *oqxA/B*, were also identified in these CRKP strains, corroborating the published literature that both the plasmid-mediated quinolone resistance genes (PMQRs) and 16S-RMTase-encoding genes were found in the KPC-*Kp* strain due to the selection pressure of antimicrobial agents, making it a potential reservoir for the spread of multidrug resistance<sup>[30]</sup>.

MLST results revealed that these 40 KPC-producing *K. pneumoniae* strains were composed of three ST types (ST11, ST15 and ST656), and most of these strains were ST11 (92.5%, 37/40). Consistent with previous reports, ST11 was the predominant ST attributed to the spread of carbapenem resistance in CRKP isolates<sup>[12]</sup>, while ST15 and ST656 appeared sporadically. Different *bla*<sub>SHV</sub> variants were found in CRKP strains of different ST types. *bla*<sub>SHV-11</sub> was the predominant subtype in ST11 *K. pneumoniae*, while

*bla*<sub>SHV-2</sub> and *bla*<sub>SHV-28</sub> were restricted to ST656 and ST15 *K. pneumoniae* in this work. *bla*<sub>SHV-28</sub> was previously found in ST15 *K. pneumoniae* isolated from dogs in a veterinary clinic, but these strains produced OXA-48 instead of KPC type carbapenemase<sup>[31]</sup>. Transmission of IncX plasmids co-producing *bla*<sub>KPC-2</sub> and *bla*<sub>SHV-12</sub> genes have been reported in many CRKP isolates<sup>[32]</sup>. In this study, *bla*<sub>SHV-12</sub> was located downstream of the Tn 1721 transposons in the KPC-bearing IncFII plasmids among 10 strains in PFGE plusotype C (Fig. 3, variant 2). We also found a substantial proportion of ST11 *K. pneumoniae* from various samples sharing identical PFGE profiles and similar MIC values, indicating a nosocomial transmission and outbreak of ST11-type KPC-*Kp* in the hospital. ST11 was proven to be a successful clone that contributed to the outbreak of KPC-2-producing *K. pneumoniae*, leading to severe nosocomial infections<sup>[7, 33]</sup>.

Our results are consistent with those from previous studies in which the *bla*<sub>KPC-2</sub> gene is associated with the core *bla*<sub>KPC</sub> platform ( $\Delta$ ISKpn6-*bla*<sub>KPC-2</sub>-ISKpn8) and can be broadly classified as the NTE<sub>KPC-II</sub> (*bla*<sub>KPC</sub>-bearing non-*Tn4401* elements type II) group due to the absence of *bla*<sub>TEM</sub> between ISKpn8 and *bla*<sub>KPC-2</sub><sup>[27, 34]</sup>. Sequencing data revealed that the *bla*<sub>KPC-2</sub> gene was embedded in divergent IncFII-like plasmids, which had a superiority to capture *bla*<sub>KPC-2</sub> by the mobile gene elements, resulting in gradual acquisition or accumulation of carbapenem resistance in ST11 *K. pneumoniae*<sup>[35]</sup>. Tn 1721 transposon is located adjacent to *bla*<sub>KPC-2</sub> in the plasmid pKPC3020-124. Tn 1721 was a result of an integration of the Tn3-based transposon and the partial Tn4401 segment reported in the USA and European countries and was confirmed to transport the carbapenem resistance gene *bla*<sub>KPC-2</sub> in China<sup>[36]</sup>. Interestingly, the Tn 1721-based transposons are more flexible than expected when the IS26 was inserted into Tn 1721-*tnpA* in p44-2<sup>[10]</sup>, and the insertion of the *fosA3* gene sandwiched by two IS26 was found in a transferable *bla*<sub>KPC-2</sub>-carrying plasmid pHS102707 at the same position<sup>[37]</sup>, resulting in additional fosfomycin resistance (Fig. 3). Moreover, a 5-bp target site duplication (TSD), a characterized signature of the transposition event of the Tn 1721-like transposon, was also confirmed in this study<sup>[36]</sup>. An identical *bla*<sub>KPC-2</sub>-bearing region was found in 15 sequences investigated in comparative genomics analysis, suggesting that spread of the *bla*<sub>KPC-2</sub> gene could occur between species such as *K. pneumoniae*, *E. coli*, and even *S. marcescens* due to horizontal gene transfer mediated by the conjugative plasmids.

However, the *bla*<sub>KPC-2</sub>-carrying plasmids were transferable and did not appear to be restricted to a certain ST or PFGE. We observed the identical Tn 1721-ISKpn6-*bla*<sub>KPC-2</sub>-ISKpn8- $\Delta$ Tn3-IS26 configuration located in conjugative plasmids within ST656 *K. pneumoniae* strain KP2159 and ST15 strain KP3020. Congruent with previous reports<sup>[36]</sup>, the *bla*<sub>KPC-2</sub> gene can be transferred from one strain to another, even if not embedded in Tn 1721 transposons. The *bla*<sub>KPC-2</sub> gene was detected in the ST656 *K. pneumoniae* isolate FK2181 in China, as determined previously in the same hospital<sup>[38]</sup>. Moreover, while KP2171 exhibited the identical ST type, PFGE profile and similar susceptibility patterns with KP2159, we speculate that the sporadic spread of the *bla*<sub>KPC</sub> gene among these *bla*<sub>KPC</sub>-producing ST656 and ST15 *K. pneumoniae* is

presumably caused by the transmission of conjugative plasmids or mobilization of genetic elements, such as Tn 1721 transposons and the insertion sequence IS26.

## Conclusion

In summary, carbapenemase-producing *K. pneumoniae* isolates in this study differed in clonal backgrounds, mainly consisting of ST11 or PFGE pulsotypes C, D and E. The mobile genetic elements related to the *bla*<sub>KPC-2</sub> gene that are accompanied by multiple resistance genes, such as ESBLs, PMQRs and 16S-RMTase encoded on the transferable plasmid, may indicate the widespread resistance among bacteria of different species. Moreover, vigilance should be heightened with regard to the occurrence of CAZ/AVI resistance without previous exposure. Effective prevention and control measures should be adopted to control nosocomial infection and reduce the spread of CRKP strains in hospitals.

## Abbreviations

CRKP: Carbapenem-resistant *Klebsiella pneumoniae*; PCR: Polymerase chain reaction; WGS: Whole-genome sequencing; MLST: Multi-locus sequence typing; PFGE: Pulsed-field gel electrophoresis; KPC-*Kp*: KPC-producing *K. pneumoniae*; ESBLs: extended spectrum- $\beta$ -lactamases; CLSI: Clinical and Laboratory Standards Institute; MIC: minimum inhibitory concentration; CAZ/AVI: ceftazidime/avibactam; MDR: Multidrug-resistant; XDR extensively drug-resistant; PDR: pandrug-resistant.

## Declarations

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## Authors' contributions

Conceived and designed the experiments: YH, TX QB and HZ. Performed the experiments: HLiu, HLin, XZhu, XZhang, QL, JL and XL. Analyzed the data: ZS, LL, KL, MZ and TX. Wrote the paper: HLiu, QB and HZ.

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# Availability of data and materials

The datasets used and analyzed during the current study are included in this published article.

# Ethics approval and consent to participate

Not required for this type of work without individual patient data.

# Consent for publication

All authors read and approved the final manuscript and gave consent for publication.

# Competing interests

The authors have no competing interests.

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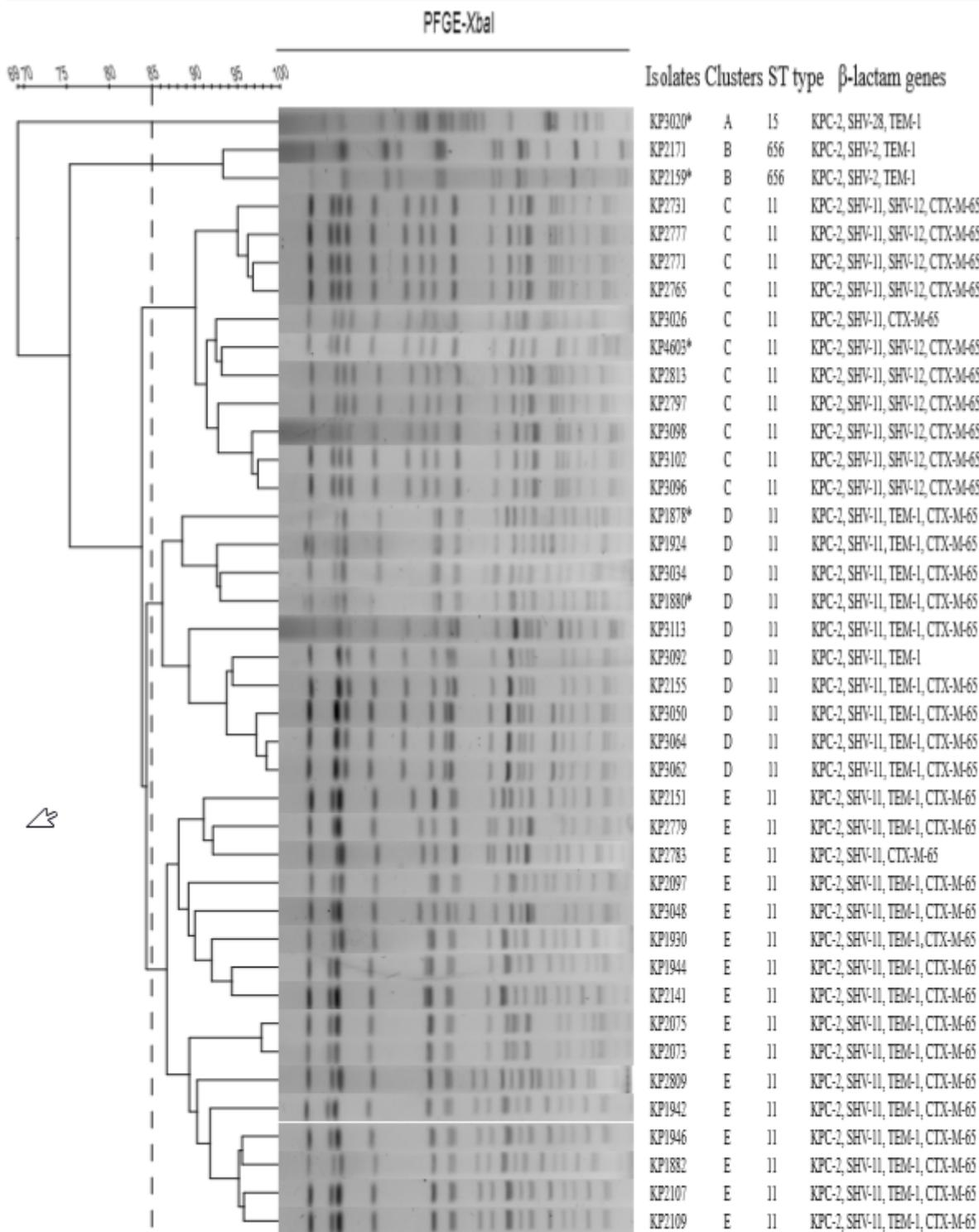
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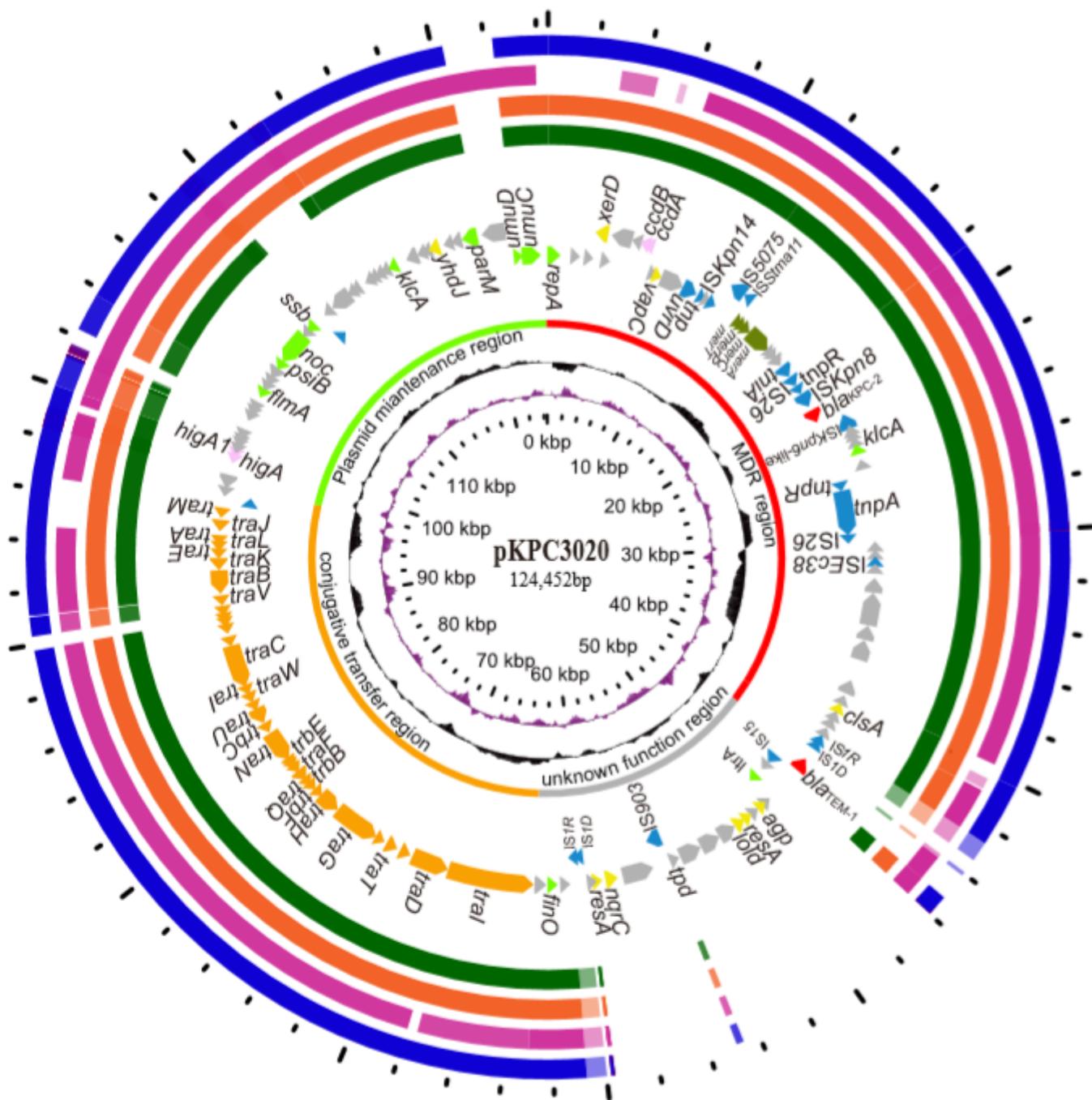
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## Figures



**Figure 1**

Dendrogram of XbaI-digested genomic DNA of 40 CRKP isolates. Five different clusters were identified based on 85% similarity of PFGE profiles. An asterisk (\*) indicates that the blaKPC-2 plasmid in the strain was transferable by conjugation.



**Figure 2**

Circular map of the plasmid pKPC3020-124 and comparative genomics analysis with its humongous plasmids. Counting from the center toward outside: (1) the scale in bp. (2) GC skew (G-C/G+C), with a positive GC skew toward the outside and a negative GC skew toward the inside. (3) GC content, with an average of 50%, whereby a G+C content of more than 50% is shown toward the outside, otherwise, inward. (4) Functional regions of multidrug resistance, conjugation, maintenance and unknown function. (5) Genes encoded in the leading strand (outwards) or the lagging strand (inwards). The plasmid pKPC3020-124 (CP061355) was used as the reference sequence and was compared to the sequences of (6) pBK34397 (KU295132.1), (7) p628-KPC (KP987218.1), (8) pKPHS2 (CP003224.1) and (9) pS1-KPC2 (MN615880.1). Genes with different functions are shown in different colors: red, antibiotic resistance;



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