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ORIGINAL RESEARCH

Distribution of β-Lactamase Genes and Genetic Context of bla_{KPC-2} in Clinical Carbapenemase-Producing *Klebsiella pneumoniae* Isolates

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Background: This study was 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 strains isolated from a teaching hospital of Wenzhou Medical University during 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 from 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-β-lactamases (ESBLs) and other antimicrobial resistance genes, were also found in KPC-positive *K. pneumoniae* (KPC-*Kp*) isolates. Mapping PCR and genomic sequencing revealed that the bla_{KPC-2}-bearing sequence regions, which are related to different mobile elements, including Tn1721- and IS26-based transposons, were mainly located in but not restricted to IncFII-like plasmids and were structurally divergent.

Conclusion: The bla_{KPC-2} genes related to divergent mobile genetic elements encoded on transferable plasmids may transfer widely, facilitating the spread of carbapenem resistance among bacteria with different genetic backgrounds. The dissemination of bla_{KPC}-bearing plasmids that collectively carry additional multidrug resistance genes has caused widespread public concern, further limiting the antibiotics available to treat infections caused by KPC-producing pathogens.

Keywords: carbapenemase, CRKP, bla_{KPC-2}, KPC-*Kp*, ST11, Tn*1721*

Introduction

Carbapenems represent a critical class of β-lactam antimicrobials that are used against multidrug-resistant (MDR) gram-negative bacteria, especially those expressing high levels of AmpC cephalosporinases or extended spectrum-β-lactamases (ESBLs).[1] Although *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.[2] 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.[3]

To date, 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.[4,5] ST258 is an international hyperepidemic lineage clone that contributes to the spread of *K. pneumoniae* carbapenemase (KPC)-positive *K. pneumoniae* (KPC-*Kp*) and has disseminated throughout the USA and European countries.[6] 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.[7]

The most common plasmid-carried Ambler class A carbapenemase is KPC, and the bla_{KPC-2} gene is the main cause of the prolonged outbreak.[3] The bla_{KPC-2} gene is carried frequently within the conserved Tn*3* family transposon Tn*4401* on transferable plasmids in Europe and the United States.[8,9] However, non-Tn*4401* mobile elements, especially Tn*1721*-like transposons among ST11 *K. pneumoniae*, are mainly responsible for the effective spread of the bla_{KPC-2} gene in China.[10]

Despite the description of several novel structures and detailed data obtained in previous research, the genetic process of the conversion that accounts for the genetic differences in the bla_{KPC-2} gene has not yet been fully elucidated. The mechanism behind the transmission of this gene deserves further exploration. This research aimed to provide a molecular epidemiological investigation and the genetic features of retrospectively collected bla_{KPC-2}-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, Craponne, 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).[11] 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/).[12] *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality controls. Strains resistant to either or both carbapenems (imipenem and meropenem) tested were classified as CRKP.[13] The carbapenemase phenotype was further confirmed by the Carba NP test.[14] MDR, extensively drug-resistant (XDR) and pandrug-resistant (PDR) phenotypes were defined according to a previous report.[15]

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_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48}), plasmid-mediated AmpC β-lactamase genes (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{FOX}) and broad-spectrum β-lactamase genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) were analyzed by PCR as previously described.[16] PCR amplicons were sequenced and compared with sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST searches. The coding sequences of the outer membrane protein (Omp) genes OmpK35 and OmpK36 were amplified by PCR as previously described.[17] The PCR products were sequenced and compared with the reference sequences of *K. pneumoniae* strain ATCC13883 (NZ_JOOW00000000.1).

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.[13] 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.[18] 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.[10] All the sequences were compared with information in the MLST database (http://bigsdb.web.pasteur.fr/klebsiella).

Conjugation Experiment

Plasmid conjugation experiments were carried out by filter mating using rifampicin-resistant *E. coli* EC600 as a -recipient.[19] The transformants were selected on MacConkey agar supplemented with rifampicin (1,024 mg/L) and meropenem (2 mg/L). Putative transconjugant colonies were selected, and further verifications were performed based on 16S rRNA sequencing and PCR-amplified bla_{KPC-2} detection of the transconjugants. PCR-based replicon typing was conducted, and replicons were sequenced using previously reported primers.[20]

Genetic Environment Analysis of the bla_{KPC-2} Gene

A series of primers based on bla_{KPC}-surrounding sequences were designed. Mapping PCR was performed to compare the genetic context of the bla_{KPC} gene according to previously reported conditions.[10,21] 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 by 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[22] 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 nonredundant protein database with an e-value threshold of 1e-5. Annotation of resistance genes was performed using ISfinder, INTEGRALL and ResFinder with default parameters.[23]

Comparative Genomics Analysis of the bla_{KPC-2}-Bearing Plasmids and bla_{KPC-2} 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 ≥80% with pKPC3020-124 were retained for later ortholog analysis using BlastP and InParanoid.[24] 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.[23] For the linear comparison of the bla_{KPC-2} 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_{KPC-2} gene were also obtained from the NCBI nucleotide database using bla_{KPC-2}, Tn*1721*, IS*Kpn6*, and IS*Kpn*8 as the key words. The results were filtered, and only sequences that contained a complete bla_{KPC-2} gene and were more than 20 kb in length were retained. Multiple sequence alignments were performed by MAFFT using the 11 kb bla_{KPC-2} gene-related fragment of pKPC3020-124 as a reference, and the sequences were clustered with an identity of 80%.[25] 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 the 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_{KPC-2}-bearing plasmid pKPC1880 and sequences contig1846 and 41_pilon, respectively.

Results

Prevalence of Carbapenemase and Distribution of the β-Lactamase Genes

Ten β-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_{SHV}, bla_{OKP}, and bla_{LEN} encoded in the chromosome, $bla_{CTX-M-65}$ and bla_{TEM-1} were the most prevalent genes, with positive rates of 27.21% and 18.71%, respectively, while bla_{OXA-10} and bla_{OXA-1} showed the lowest positive rates of 1.02% and 0.68%, respectively (Table S1). The bla_{KPC} gene was identified in 13.61% (40/294) of the strains, and they all exhibited a carbapenem-resistant phenotype. The sequencing results of the PCR products revealed identical bla_{KPC-2} alleles (NG_049253) among these carbapenemase-producing *K. pneumoniae* strains. No other

carbapenemase genes were identified in the other two carbapenem nonsusceptible isolates (Table S1). The KPC-*Kp* isolates also carried at least one other β-lactamase gene, such as the ESBL gene bla_{CTX-M} (90%, 36/40) and narrow-spectrum bla_{TEM} (70%, 28/40) (Figure 1). The bla_{SHV} variants included bla_{SHV-2} (n=2), bla_{SHV-12} (n=10), bla_{SHV-28} (n=1) and bla_{SHV-11} (n=37), while $bla_{CTX-M-65}$ and bla_{TEM-1} were the only variants observed.

Antimicrobial Susceptibility and Conjugation Results of the KPC-*Kp* Isolates

The minimum inhibitory concentrations (MICs) of 17 antimicrobial agents against all forty KPC-*Kp* isolates showed multidrug resistance profiles, particularly to β-lactams (including all carbapenems, ertapenem, meropenem and imipenem detected), quinolones and aminoglycosides (Tables 1 and S1). Colistin was shown to be the most active agent (MIC_{90} = 1 mg/L, 100% susceptible), followed by tigecycline (MIC_{90} = 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%). On the whole, all isolates were not susceptible to at least one type of agent in three or more antimicrobial categories, while 8 isolates (20%, 8/40) were not susceptible to at least one agent in all categories except colistin, exhibiting MDR and XDR phenotypes. Notably, all isolates were susceptible to CAZ/AVI, except for KP3034, which exhibited low-level resistance to this combination with an MIC of 16/4 mg/L. Sequencing results of the Omp genes amplified from KP3034 revealed that two amino acid duplications (Gly136-Asp137) in the coding sequences of the OmpK36 gene were identified, and no amino acid variation was found in the OmpK35 protein sequence.

According to the conjugation experiment, 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).

Genetic Relatedness of the KPC-*Kp* 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) (Figure 1). ST11 (n=37) was the most predominant sequence type, which consisted 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 KPC-*Kp* isolates harbored one or more other β-lactamase genes along with the bla_{KPC-2} gene, especially $bla_{CTX-M-65}$ and/or bla_{TEM-1} in most ST11 *K. pneumoniae* isolates. The bla_{TEM-1} 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_{KPC}-bearing $IncFII_K$ 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 reference query, 15 sequences with the highest similarities (>80% coverage and >80% identity) with pKPC3020-124 were retrieved from the NCBI nucleotide database. Fourteen of them were $IncFII_K$ 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_{KPC}-bearing plasmids could mainly be divided into two functional regions: a variable region and a conserved region (Figure 2). The conserved region could be further divided into two fragments, 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_{KPC-2}, bla_{TEM-1} and four mercury resistance genes (*merACPT*). The difference between pKPC3020-124 and the other plasmids is that the 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*) (Figure 2, unknown functional region).

Comparison of Genetic Environments of bla_{KPC-2}-Encoding Regions

Based on the results of the mapping PCR approach, four divergent forms of bla_{KPC-2}-harboring structures were identified among these carbapenem-resistant plasmids

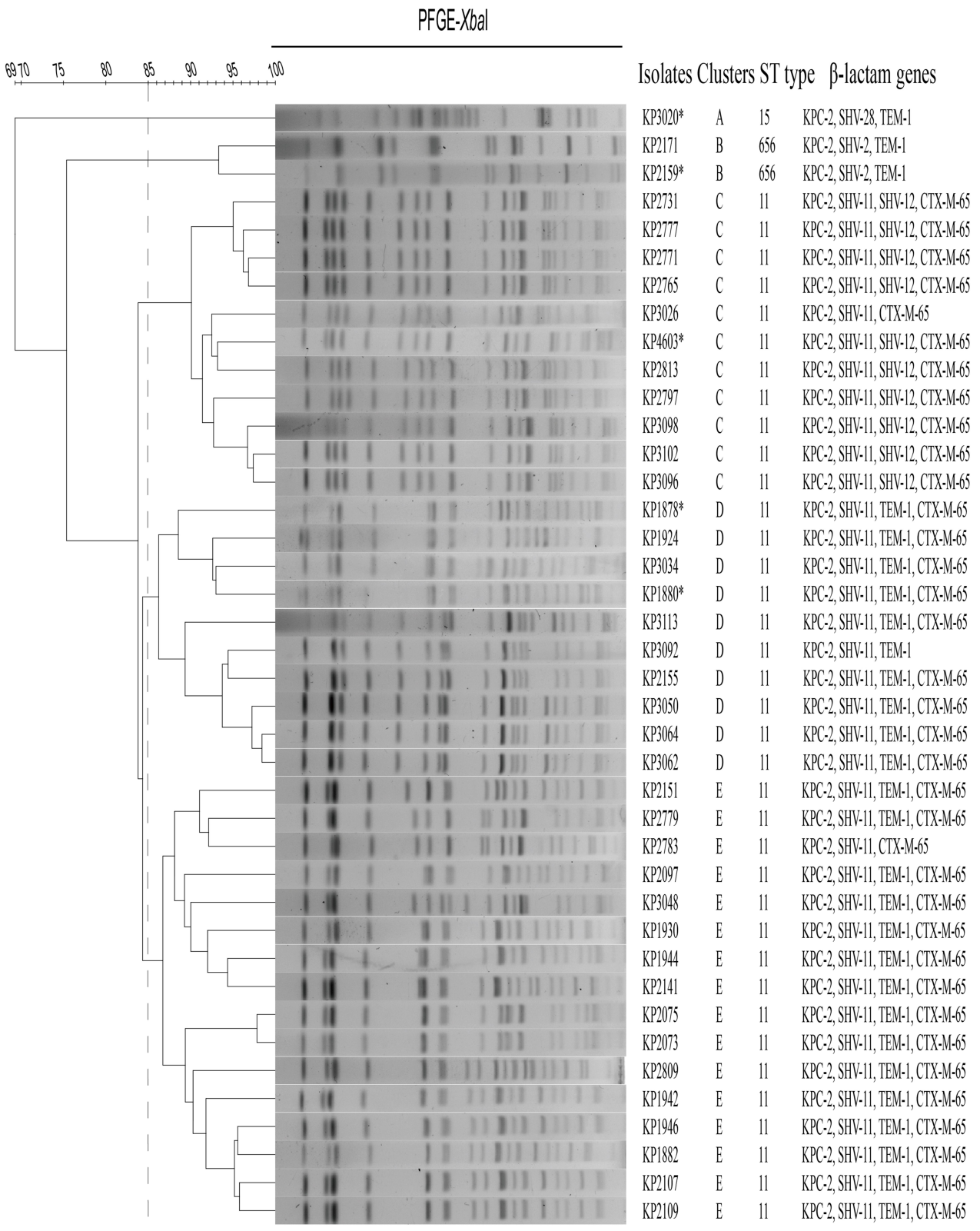

Figure 1 Dendrogram of *Xba*I-digested genomic DNA of 40 KPC-*Kp* isolates. Five different clusters were identified based on 85% similarity of PFGE profiles. An asterisk (*) indicates that the bla_{KPC-2} plasmid in the strain was transferable by conjugation.

Table 1 Antimicrobial Susceptibility Testing Results of Carbapenemase-Producing *Klebsiella pneumoniae* Isolates from 2015 to 2017 (mg/L)

Antibiotics	Range	MIC_{50}	MIC_{90}	MIC Interpretation		
				S (%)	I (%)	R (%)
Ceftazidime[a]	16–256	64	256	0	0	100
Ceftazidime/avibactam[b]	0.5–16	2	2	97.5	0	2.5
Cefotaxime[a]	8–512	256	512	0	0	100
Cefepime[a]	16–256	256	>256	0	0	100
Aztreonam[a]	128–1,024	1,024	>1,024	0	0	100
Imipenem[a]	8–512	32	64	0	0	100
Meropenem[a]	4–1,024	32	64	0	0	100
Ertapenem[a]	32–1,024	512	512	0	0	100
Gentamicin[a]	0.25–1,024	128	512	37.5	0	62.5
Tobramycin[a]	0.25–1,024	128	512	37.5	5	57.5
Amikacin[a]	0.125–1,024	1,024	>1,024	42.5	0	57.5
Ciprofloxacin[a]	16–256	32	128	0	0	100
Levofloxacin[a]	8–128	32	64	0	0	100
Fosfomycin[a]	32–1,024	512	1,024	5	10	85
Chloramphenicol[a]	2–512	512	512	20	2.5	77.5
Tigecycline[b]	0.5–32	2	8	80	12.5	7.5
Colistin[b]	0.25–2	1	1	100	0	0

Notes: [a]The antimicrobial susceptibility test of these antibiotics was conducted by the agar diffusion method and interpreted by CLSI criteria; [b]For colistin, tigecycline and ceftazidime/avibactam, the antimicrobial susceptibility test was performed using broth microdilution method and interpreted by EUCAST criteria.
Abbreviations: S, susceptible; I, intermediate; R, resistant.

Table 2 Resistance Genes and Carbapenem MIC Levels (mg/L) of Wild Strains and Transconjugants

Strains	β-Lactam Genes	ETA	MEM	IPM	Replicon Types	ST Types
KP1878	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$, bla_{SHV-11}	512	256	64	IncFII	11
pKPC1878/EC600	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$	16	8	4	IncFII	-
KP1880	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$, bla_{SHV-11}	256	128	32	IncFII, $IncFII_K$	11
pKPC1880/EC600	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$	4	2	2	IncFII	-
KP2159	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-6}$, bla_{SHV-2}	32	8	8	IncFIA, $IncFII_K$	656
pKPC2159/EC600	bla_{KPC-2}, bla_{TEM-1}	8	2	2	$IncFII_K$	-
KP3020	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$, bla_{SHV-28}	64	64	32	$IncFII_K$	15
pKPC3020-124/EC600	bla_{KPC-2}, bla_{TEM-1}	8	2	2	$IncFII_K$	-
KP4603	bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-65}$, bla_{SHV-11}, bla_{SHV-12}	512	256	64	IncFII, $IncFII_K$	11
pKPC4603/EC600	bla_{KPC-2}, $bla_{CTX-M-65}$, bla_{SHV-12}	16	4	4	$IncFII_K$	-
EC600	-	0.0075	0.03	0.015	-	-

Abbreviations: ETA, ertapenem; MEM, meropenem; IPM, imipenem.

(Table S3). The primers used and their positional counterparts are summarized in Table S4 and Figure 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_{KPC-2}-encoding region, all the sequences (including four obtained in this work) of approximately 5–20 kb in length (with the bla_{KPC-2} gene at the center) were retrieved from the NCBI nucleotide database. According to the similarities of the core sequences adjacent to the bla_{KPC-2} gene, 10 sequences were chosen as representatives and are illustrated in Figure 3, revealing that an approximately 4-kb fragment encoding *tnpR*-IS*Kpn8*-bla_{KPC-2}-ΔIS*Kpn6* was conserved and presented in the majority of the sequences. According to whether Tn*1721* (or its derivative) was adjacent to the bla_{KPC-2}-encoding fragment, these sequences were roughly categorized into two groups. The first group consisted of the sequences without Tn*1721* (or its derivative), while the second group contained Tn*1721* (or its derivative) next to the bla_{KPC-2}-encoding fragment. The sequences of the first group existed in various species, such as *K. pneumoniae, E. coli, and*

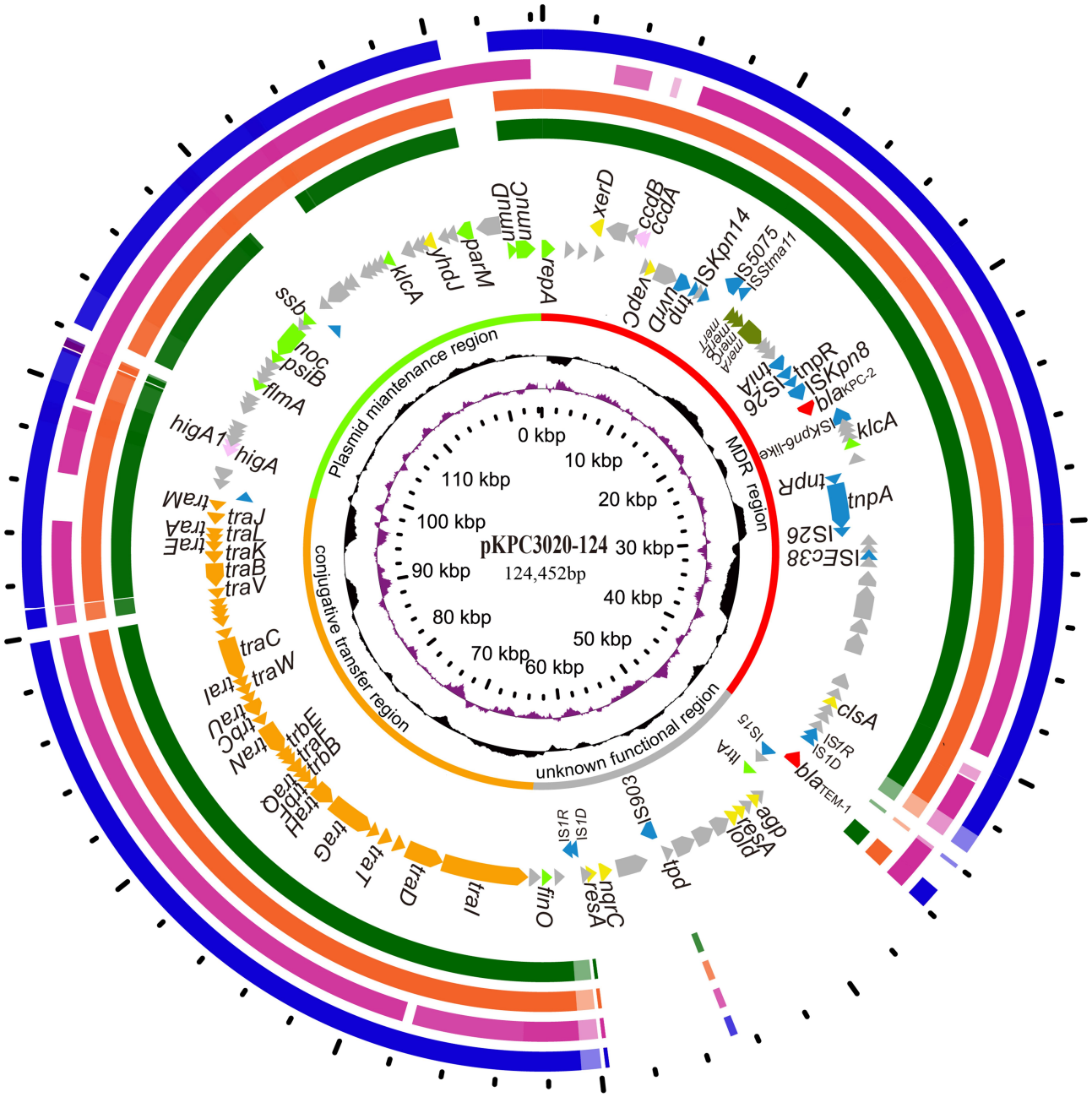

Figure 2 Circular map of the plasmid pKPC3020-124 and comparative genomics analysis with its humongous plasmids. Counting from the center toward outside: (1) the scale in bp. (2) GC skew (G-C/G+C), with a positive GC skew toward the outside and a negative GC skew toward the inside. (3) GC content, with 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; blue, mobile genetic elements; orange, transfer conjugation; light green, plasmid stability and replication; brown, heavy metal resistance; pink, toxin-antitoxin system; yellow, DNA metabolism; and gray, hypothetical proteins/genes with unknown functions.

Citrobacter freundii, while those in the second group were mainly from *K. pneumoniae*.

Discussion

In this study, all KPC-*Kp* 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.[15] The data showed that CAZ/AVI displayed potent activity against clinical KPC-*Kp* isolates with

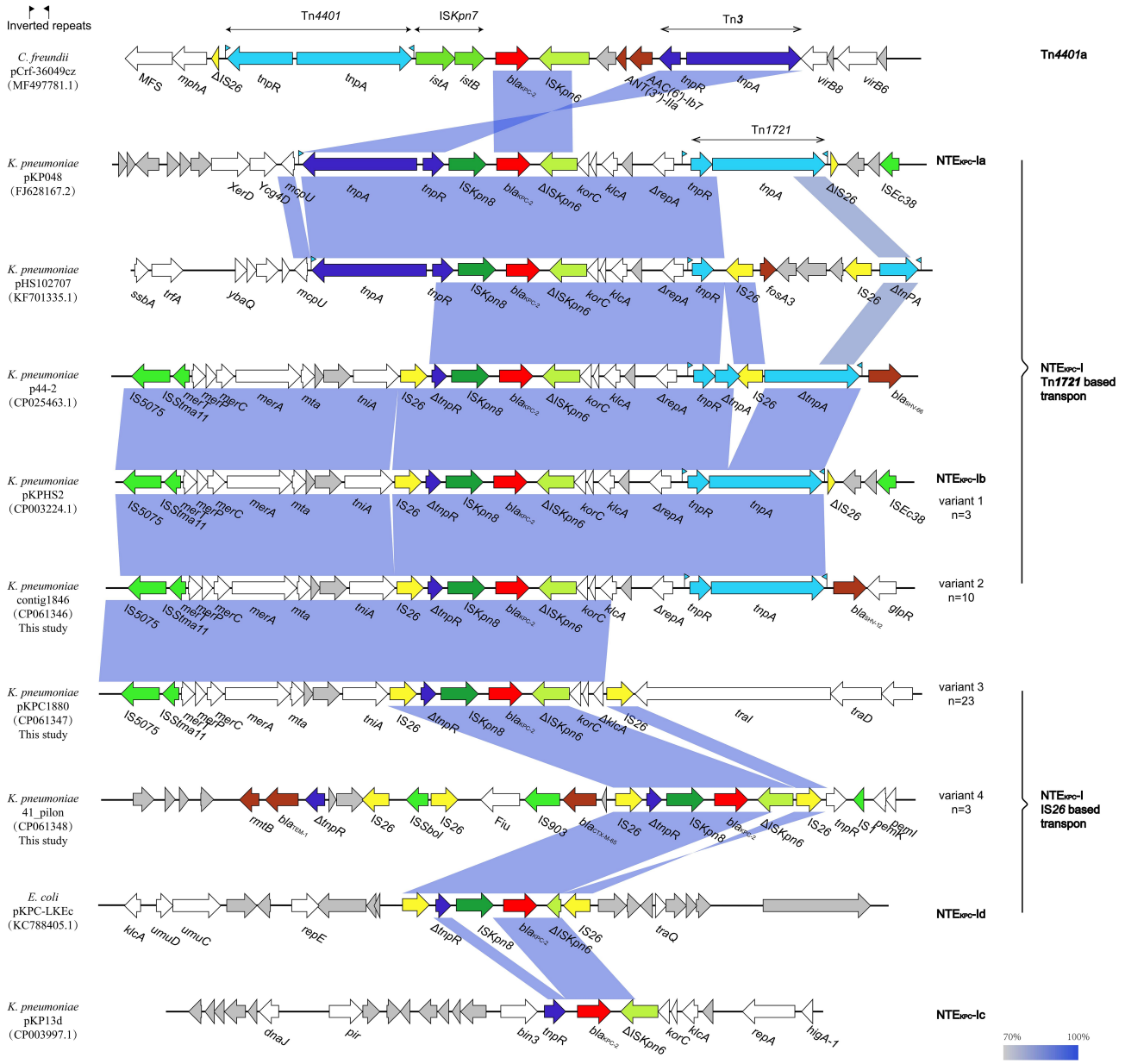

Figure 3 Comparison of the bla_{KPC-2}-encoding regions between 10 representative plasmid sequences. The arrows represent the direction of transcription. ORFs are depicted by arrows and are colored based on predicted gene function. Light blue shading denotes homology of regions in each adjacent plasmid. The bla_{KPC-2} genes are shown in red.

an MIC_{90} of 2/4 mg/L. Intriguingly, in addition to the high level of carbapenem resistance with a meropenem MIC of 1,024 mg/L, strain KP3034 exhibited a higher MIC level for CAZ/AVI (16/4 mg/L) than the other strains detected. In contrast to the alteration recently reported by Coppi et al[26] two-amino-acid duplications (Gly136-Asp137) within transmembrane β-strand loop 3 (L3) of OmpK36 were observed in KP3034, which was previously associated with reduced susceptibility to carbapenems.[27] Since there is no history of previous antimicrobial exposure to CAZ/AVI treatment or specific mutations were observed in the bla_{KPC-2} gene, hyperexpression of the bla_{KPC-2} gene associated with alteration in the outer membrane or other unknown mechanisms might account for this low level of CAZ/AVI resistance.[28] The transconjugants exhibited reduced resistance levels to carbapenems compared with clinical isolates. The copy number of genes and selective expression of promoters in strains of different species or genera,[29] restoration of deficient Omp proteins, and antibiotic selection pressure could influence the MIC level of the transconjugants.[30]

In addition to the bla_{KPC-2} gene, one or more other kinds of β-lactamase genes (such as bla_{CTX-M}, bla_{SHV} and

bla_{TEM}) were identified in these KPC-producing *K. pneumoniae* strains, with 90% (36/40) of the strains carrying the ESBL gene bla_{CTX-M}. $bla_{CTX-M-65}$ identified in this work belongs to the group 9 CTX-M β-lactamase gene, which mediates cephalosporin resistance,[31] while bla_{SHV}, a core chromosomal gene in *K. pneumoniae*, mainly mediates inherent ampicillin resistance.[32] CTX-M-65 is a variant of CTX-M-14 that differs by 2 amino acid substitutions (Ala78Val and Ser273Arg),[33] and it was identified in the variable regions of F33:A-:B- plasmids from food-producing animals in China.[34] In contrast to $bla_{CTX-M-14}$ reported by Yang et al[14] bla_{SHV-11}, $bla_{CTX-M-65}$, and bla_{TEM-1} were shown to be the predominant genotypes. The coexistence of these multiple broad-spectrum β-lactamase genes simultaneously in KPC-*Kp* confers resistance to 1st-, 2nd- and 3rd-generation cephalosporins, leading to difficulties in treating the corresponding bacterial infections.

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 major ST type attributed to the spread of carbapenem resistance in CRKP isolates,[12] while ST15 and ST656 appeared sporadically in this study. Different bla_{SHV} variants were found in CRKP strains of different ST types. bla_{SHV-11} was the predominant subtype in ST11 *K. pneumoniae*, while bla_{SHV-2} and bla_{SHV-28} were restricted to ST656 and ST15 *K. pneumoniae* in this work. bla_{SHV-28} was previously found in ST15 *K. pneumoniae* strains isolated from dogs in a veterinary clinic but produced OXA-48 instead of KPC-type carbapenemase.[35] We also found a substantial proportion of ST11 *K. pneumoniae* from various samples sharing identical PFGE profiles and similar MIC values, indicating nosocomial transmission and outbreaks of ST11-type KPC-*Kp* in the hospital. ST11 was proven to be a successful clone that contributed to the outbreak of KPC-*Kp*, leading to severe nosocomial infections.[7,36]

Our results are consistent with those from previous studies in which the bla_{KPC-2} gene is associated with the core bla_{KPC} platform (ΔIS*Kpn6*-bla_{KPC-2}-IS*Kpn8*) and can be broadly classified as the NTE$_{KPC-II}$ (*bla*$_{KPC}$-bearing non-Tn*4401* elements type II) group due to the absence of bla_{TEM} between IS*Kpn8* and bla_{KPC-2}.[32,37] Sequencing data revealed that the bla_{KPC-2} gene was embedded in divergent IncFII-like plasmids, which had a superiority to capture bla_{KPC-2} by mobile gene elements, resulting in gradual acquisition or accumulation of carbapenem resistance in ST11 *K. pneumoniae*.[38] The Tn*1721* transposon was located adjacent to bla_{KPC-2} in the plasmid pKPC3020-124. Tn*1721* was a result of an integration of the Tn*3*-based transposon and the partial Tn*4401* segment reported in the USA and European countries and was confirmed to transport the carbapenem resistance gene bla_{KPC-2} in China.[39] Interestingly, the Tn*1721*-based transposons were more flexible than expected when IS*26* was inserted into Tn*1721-tnpA* in p44-2,[10] and the insertion of the *fosA3* gene sandwiched by two IS*26* sequences was found in the transferable bla_{KPC-2}-carrying plasmid pHS102707 at the same position,[40] resulting in additional fosfomycin resistance (Figure 3). In this study, Tn*1721*-related bla_{KPC-2} and bla_{SHV-12} cocarriers were found in ten KPC-bearing plasmids in strains of PFGE plusotype C (Figure 3, variant 2), and these strains exhibited elevated ceftazidime resistance compared with those with a single bla_{KPC-2} gene (Table S1). An identical bla_{KPC-2}-bearing region with a Tn*1721*-like transposon was found in 15 sequences investigated in comparative genomics analysis, suggesting that the bla_{KPC-2} gene could transfer across different *Enterobacteriaceae* species, such as *E. coli* and *S. marcescens*, through horizontal transfer of mobile genetic elements or conjugative plasmids. Moreover, an identical Tn*1721*-IS*Kpn6*-bla_{KPC-2}-IS*Kpn8*-ΔTn*3*-IS*26* configuration was observed in conjugative plasmids within ST656 *K. pneumoniae* strain KP2159 and ST15 strain KP3020. Congruent with previous reports,[39] the bla_{KPC-2}-carrying plasmid was transferable, even if not embedded in Tn*1721* transposons and was not restricted to a certain ST or PFGE subtype.

Conclusion

In summary, although carbapenemase-producing *K. pneumoniae* isolates in this study differed in clonal backgrounds, similarities were found in the phenotypic and genotypic profiles of antibiotic resistance, as well as the identical core configuration of the ΔIS*Kpn6*-bla_{KPC-2}-IS*Kpn8* region. Transmission of the bla_{KPC-2} gene with the assistance of Tn1721- and IS26-based transposons increases the difficulty of clinical use of carbapenems. Effective prevention and control measures should be adopted to control nosocomial infection caused by the spread of CRKP strains in hospitals.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

Individual patient data was not involved, and only anonymous clinical residual samples during routine hospital laboratory procedures were used in this study. This study followed the principles stated in the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/) and was approved by the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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