

Comparative analysis of *bla*_{KPC-2}- and *rmtB*-carrying IncFII-family pKPC-LK30/pHN7A8 hybrid plasmids from *Klebsiella pneumoniae* CG258 strains disseminated among multiple Chinese hospitals

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Background: We recently reported the complete sequence of a *bla*_{KPC-2}- and *rmtB*-carrying IncFII-family plasmid p675920-1 with the pKPC-LK30/pHN7A8 hybrid structure. Comparative genomics of additional sequenced plasmids with similar hybrid structures and their prevalence in *bla*_{KPC}-carrying *Klebsiella pneumoniae* strains from China were investigated in this follow-up study.

Methods: A total of 51 *bla*_{KPC}-carrying *K. pneumoniae* strains were isolated from 2012 to 2016 from five Chinese hospitals and genotyped by multilocus sequence typing. The *bla*_{KPC}-carrying plasmids from four representative strains were sequenced and compared with p675920-1 and pCT-KPC. Plasmid transfer, carbapenemase activity determination, and bacterial antimicrobial susceptibility test were performed to characterize resistance phenotypes mediated by these plasmids. The prevalence of pCT-KPC-like plasmids in these *bla*_{KPC}-carrying *K. pneumoniae* strains was screened by PCR.

Result: The six KPC-encoding plasmids p1068-KPC, p20049-KPC, p12139-KPC and p64917-KPC (sequenced in this study) and p675920-1 and pCT-KPC slightly differed from one another due to deletion and acquisition of various backbone and accessory regions. Two major accessory resistance regions, which included the *bla*_{KPC-2} region harboring *bla*_{KPC-2} (carbapenem resistance) and *bla*_{SHV-12} (β-lactam resistance), and the MDR region carrying *rmtB* (aminoglycoside resistance), *fosA3* (fosfomycin resistance), *bla*_{TEM-1B} (β-lactam resistance) and *bla*_{CTX-M-65} (β-lactam resistance), were found in each of these six plasmids and exhibited several parallel evolution routes. The pCT-KPC-like plasmids were present in all the 51 *K. pneumoniae* isolates, all of which belonged to CG258.

Conclusion: There was clonal dissemination of *K. pneumoniae* CG258 strains, harboring *bla*_{KPC-2}- and *rmtB*-carrying IncFII-family pKPC-LK30/pHN7A8 hybrid plasmids, among multiple Chinese hospitals.

Keywords: *Klebsiella pneumoniae*, multidrug resistance, carbapenemase, plasmids

Introduction

Klebsiella pneumoniae belongs to the family Enterobacteriaceae and is usually found in human gastrointestinal tract and the environment especially in the hospital settings.¹ *K. pneumoniae* has emerged as one of the most important opportunistic bacterial pathogens associated with over 70% of human infections from bloodstream, respiratory tract, and urinary tract.^{2,3} Carbapenems are used as the first-line therapeutic agents to treat life-threatening infections caused by multidrug-resistant (MDR) *K. pneumoniae*,

but carbapenem-resistant *K. pneumoniae* strains have spread worldwide and posed a severe threat to public health with the mortality up to 40%–50%.⁴ The production of carbapenemases, such as *K. pneumoniae* carbapenemase (KPC), is an important mechanism of carbapenem resistance in *K. pneumoniae*.⁵

KPC enzymes belong to Ambler class A and are able to hydrolyze all β -lactam antimicrobial agents including carbapenems.^{4,6} KPC-2 is the first variant of KPC enzymes found in 1996,⁵ and, to date, at least 31 different KPC variants have been identified (<https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/>). The *bla*_{KPC} genes are mainly carried on plasmids of different incompatibility (Inc) groups such as IncFII, FIA, I2, A/C, N, X, R, P, U, W, and L/M.⁵ IncFII plasmids commonly harbor one or more replicons in addition to FII, and they have been frequently isolated from Enterobacteriaceae⁵ and found to carry various antibiotic resistance genes, including those responsible for resistance to carbapenems (such as *bla*_{KPC}⁷ and *bla*_{NDM}⁸), extended-spectrum cephalothins (such as *bla*_{CTX-M-65}⁹ and *bla*_{CTX-M-14}³), aminoglycosides (such as *armA*¹⁰ and *rmtB*¹¹), and fosfomycins (such as *fosA39*).

The most predominant isolates of KPC-producing *K. pneumoniae* belong to the clonal group 258 (CG258), which mainly includes the sequence type 258 (ST258; allelic profile 3–3–1–1–1–1–79 corresponding to the collection of seven housekeeping genes) and its single-locus variation ST11 (allelic profile 3–3–1–1–1–1–4) as defined by multi-locus sequence typing (MLST).⁵ The genome of ST258 is a hybrid of ST11 (80%) and ST442 (20%), and the ST258 strains can be segregated into clades I and II based on the distinct capsule polysaccharide gene (*cps*) regions; clade I (*cpsI*) has been evolved from clade II (*cps2*) as a result of horizontal transfer of *cps* (*cps2*) region from ST42 to clade II, which is mediated by the mobile element ICEKp258.2 of clade II.^{5,12} The ST258 and ST11 strains have been identified worldwide – ST258 is mostly prevalent in America and Europe, while ST11 is the highly dominant clone in Asia (especially in China) and Latin America.⁵

We recently reported the complete nucleotide sequence of a *bla*_{KPC-2}-carrying IncFII plasmid p675920-1 (accession number, MF133495) from an MDR *K. pneumoniae* isolate 675920.¹³ p675920-1 was essentially a hybrid of the *rmtB*-carrying IncFII plasmid pHN7A8 (accession number, JN232517)⁹ and the *bla*_{KPC-2}-carrying IncR plasmid pKPC-LK30 (accession number, KC405622),¹⁴ and almost all of the backbone and accessory regions of pKPC-LK30 and pHN7A8 were presented in p675920-1.

This follow-up study presented four fully sequenced *bla*_{KPC}-carrying plasmids, p1068-KPC, p20049-KPC, p12139-KPC, and p64917-KPC, which were genetically closely related to

p675920-1. A total of nine available sequenced plasmids (including the abovementioned five plasmids) constituted a novel group of IncFII plasmids with pHN7A8/pKPC-LK30 hybrid structures. This group of plasmids was disseminated among *K. pneumoniae* CG258 strains from at least nine distinct hospitals in eight different cities of China.

Materials and methods

Identification of bacterial strains

The use of human specimens and all related experimental protocols were approved by the Committee on Human Research of Jinling Hospital and carried out in accordance with the approved guidelines. The selected patients signed the written informed consent. All *K. pneumoniae* strains, including the 1068, 20049, 12139, and 64917 isolates, were subjected to species identification using 16S rRNA gene sequencing¹⁵ and PCR detection of *K. pneumoniae*-specific gene *khe*.¹⁶ The MLST scheme was derived from the Pasteur *Klebsiella* MLST database (<http://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>).

Plasmid conjugal transfer

Plasmid conjugal transfer experiments were carried out with the rifampin-resistant *Escherichia coli* EC600 being used as recipient and each of the 1068, 20049, 12139, and 64917 isolates as donor. Three milliliters of overnight cultures of each of donor and recipient bacteria were mixed together, harvested, and resuspended in 80 μ L of brain heart infusion (BHI) broth (BD Biosciences, San Jose, CA, USA). The mixture was spotted on a 1-cm² hydrophilic nylon membrane filter with a 0.45- μ m pore size (EMD Millipore, Billerica, MA, USA) that was placed on BHI agar (BD Biosciences) plate and then incubated for mating at 37°C for 12–18 hours. Bacteria were washed from filter membrane and spotted on Muller–Hinton (MH) agar (BD Biosciences) plates containing 1,000 μ g/mL rifampin together with 4 μ g/mL meropenem for selecting an *E. coli* transconjugant-carrying *bla*_{KPC}.

Plasmid electroporation

To prepare competent cells for plasmid electroporation, 200 mL of overnight culture of *E. coli* TOP10 in super optimal broth (SOB) at an OD₆₀₀ of 0.4–0.6 was washed three times with electroporation buffer (0.5 M mannitol and 10% glycerol) and concentrated into a final volume of 2 mL. One microgram of plasmid DNA, which was isolated from each of the 1068, 20049, 12139, and 64917 strains using a Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany), was mixed

with 100 µL of competent cells for electroporation at 25 µF, 200 Ω, and 2.5 kV. The resulting cells were suspended in 500 µL of SOB, and an appropriate aliquot was spotted on SOB agar plates containing 4 µg/mL meropenem for selecting an *E. coli* electroporant-carrying *bla*_{KPC}.

DNA sequencing and sequences assemble

Genomic DNA was isolated from the 1068 or 20049 isolate using a Qiagen Blood & Cell Culture DNA Maxi Kit (Qiagen). Plasmid DNA was isolated from the *E. coli* electroporant, containing p12139-KPC or p64917-KPC, with a Qiagen Large Construct Kit (Qiagen).

For the 1068 isolate, genome sequencing was performed with a sheared DNA library with an average size of 15 kb (ranged from 10 to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA), as well as a paired-end library with an average insert size of 400 bp (ranged from 150 to 600 kb) on a HiSeq sequencer (Illumina, CA, USA). The paired-end short Illumina reads were used to correct the long PacBio reads utilizing *proovread*,¹⁷ and then the corrected PacBio reads were assembled de novo utilizing SMARTdenovo (available from <https://github.com/ruanjue/smartdenovo>).

Genomic DNA of 20049, or plasmid DNA of p12139-KPC or p64917-KPC, was sequenced from a mate pair library with an average insert size of 5 kb (ranged from 2 to 10 kb) using a MiSeq sequencer (Illumina). DNA contigs that were not matching with the corresponding chromosome sequences were assembled based on their contig coverage values using Newbler 2.6.¹⁸ Gaps between contigs were filled using a combination of PCR and Sanger sequencing using an ABI 3730 Sequencer.

Sequence annotation and genome comparison

Open reading frames (ORFs) and pseudogenes were predicted using RAST 2.0¹⁹ combined with BLASTP/BLASTN searches²⁰ against the UniProtKB/Swiss-Prot²¹ and RefSeq²² databases. Annotation of resistance genes, mobile elements, and other features was carried out using the online databases including CARD,²³ ResFinder,²⁴ ISfinder,²⁵ and INTEGRALL.²⁶ Multiple and pairwise sequence comparisons were performed using MUSCLE 3.8.31²⁷ and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape 0.48.1 (<https://inkscape.org/en/>).

Phylogenetic analysis

The backbone regions of six plasmids p675920-1, p1068-KPC, p20049-KPC, p12139-KPC, p64917-KPC, and pCT-

KPC were compared with that of concatenated pHN7A8+ pKPC-LK30 by MUMmer 3.0.²⁸ Inference of homologous recombination was performed using ClonalFrameML²⁹ to remove recombination-associated single-nucleotide polymorphisms (SNPs). A maximum-likelihood tree was constructed from recombination-free SNPs using MEGA7³⁰ with a bootstrap iteration of 1000, using concatenated pHN7A8+ pKPC-LK30 as outgroup.

Detection of carbapenemase activity

The activity of class A/B/D carbapenemases in bacterial cell extracts was determined via a modified CarbaNP test.³¹ Overnight bacterial cell culture in MH broth was diluted 1:100 into 3 mL of fresh MH broth, and bacteria were allowed to grow at 37°C with shaking at 200 rpm to reach an OD₆₀₀ of 1.0–1.4. If required, ampicillin was used at 200 µg/mL. Bacterial cells were harvested from 2 mL of the above culture and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were resuspended in 500 µL of 20 mM Tris-HCl (pH 7.8) and lysed by sonication, followed by centrifugation at 10,000× *g* at 4°C for 5 minutes. Fifty microliters of the supernatant (the enzymatic bacterial suspension) were mixed with 50 µL of substrate I–V, respectively, followed by incubation at 37°C for 2 hours: substrate I: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), substrate II: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8) and 3 µg/µL imipenem, substrate III: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 3 µg/µL imipenem, and 4 µg/µL tazobactam, substrate IV: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 3 µg/µL imipenem, and 3 mM EDTA (pH 7.8), substrate V: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 3 µg/µL imipenem, 4 µg/µL tazobactam, and 3 mM EDTA (pH 7.8).

Bacterial antimicrobial susceptibility test

Bacterial antimicrobial susceptibility was tested by BioMérieux VITEK 2 and interpreted using Clinical and Laboratory Standards Institute guidelines.³²

Nucleotide sequence accession numbers

The complete sequences of plasmids p1068-KPC, p20049-KPC, p12139-KPC, and p64917-KPC were submitted to GenBank under accession numbers MF168402, MF168404, MF168403, and MF168405, respectively.

Results and discussion

Clonal *bla*_{KPC}-carrying *K. pneumoniae* isolates

During 2012–2016, 51 *bla*_{KPC}-positive *K. pneumoniae* isolates were recovered from five types of specimens (sputum, urine,

blood, pus, and peritoneal lavage fluids) of the patients from eight different departments (Intensive Care Units, Department of Respiratory Medicine, Department of Neurosurgery, Department of Neurology, Department of Emergency, Department of Endocrinology, Department of General Surgery, and Department of Hematology) in five distinct public hospitals from Beijing City, Nanjing City, Shijiazhuang City, and Lianyungang City in China (Table S1). These patients were admitted with primary diseases to the hospitals and suffered from different kinds of hospital-acquired infections during hospitalization. They received intravenous treatment of susceptible antimicrobial agents based on the antimicrobial susceptibility test results, and their infection symptoms were progressively improved.

As determined by MLST, these 51 isolates could be assigned into three different STs, namely ST11 (49 strains), ST1711 (one strain), and ST2040 (one strain), all of which belonged to the CG258. The above results indicated the clonal dissemination of *bla*_{KPC}-carrying *K. pneumoniae* in these hospitals.

pCT-KPC-like IncFII plasmids in *K. pneumoniae* isolates

The previously sequenced *bla*_{KPC-2}-carrying p675920-1 was recovered from *K. pneumoniae* 675920 from one of the above hospitals.¹³ In this study, one *K. pneumoniae* isolate

with the widest drug-resistance spectrum was selected from each of the remaining four hospitals to characterize the *bla*_{KPC}-carrying plasmids in these strains. Repeated attempts through conjugation failed to transfer the *bla*_{KPC} markers of the four selected strains (1068, 20049, 12139, and 64917) into *E. coli* EC600. However, these *bla*_{KPC} markers could be transferred into *E. coli* TOP10 through electroporation, yielding four corresponding *bla*_{KPC}-positive *E. coli* electroporants. All these wild-type and electroporant strains had the Ambler class A carbapenemase activity (data not shown) and were resistant to ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, cefazolin, cefuroxime, cefuroxime axetil, ceftazidime, ceftriaxone, aztreonam, imipenem, and meropenem (Table 1); the production of KPC enzymes accounted for the above phenotypes of these strains.

High-throughput sequencing with indicated wild-type and electroporant strains showed that the 1068, 20049, 12139, and 64917 isolates contained the *bla*_{KPC-2}-carrying plasmids p1068-KPC, p20049-KPC, p12139-KPC, and p64917-KPC, respectively. These four plasmids have circularly closed sequences, 146,103, 151,653, 169,424, and 169,419 bp in length, with mean G+C contents of 53.7%, 53.5%, 53.6%, and 53.5%, and contained 208, 212, 221, and 219 predicted ORFs in total, respectively (Table 2).

Table 1 Antimicrobial drug susceptibility profiles

Category	Antibiotics	MIC (mg/L)/antimicrobial susceptibility								
		1068	1068-KPC-TOPI0	20049	20049-KPC-TOPI0	12139	12139-KPC-TOPI0	64917	64917-KPC-TOPI0	TOPI0
Penicillins	Ampicillin	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	4/S
	Ampicillin/sulbactam	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	≥32/R	4/S
	Piperacillin	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≤4/S
	Piperacillin/tazobactam	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≤4/S
Cephalosporins	Cefazolin	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤4/S
	Cefuroxime	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	8/S
	Cefuroxime axetil	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	4/S
	Ceftazidime	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	16/R	≥64/R	16/R	≤1/S
	Ceftriaxone	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S
	Cefepime	≥64/R	≥64/R	≥64/R	8/I	≥64/R	8/I	≥64/R	8/I	≤1/S
Monobactam	Aztreonam	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S
Carbapenems	Imipenem	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≤1/S
	Meropenem	≥16/R	≥16/R	≥16/R	8/R	≥16/R	8/R	≥16/R	8/R	≤0.25/S
Aminoglycosides	Amikacin	≥64/R	≥64/R	≥64/R	≤2/S	≥64/R	≥64/R	≥64/R	≥64/R	≤2/S
	Gentamicin	≥16/R	≥16/R	≥16/R	≤1/S	≥16/R	≥16/R	≥16/R	≥16/R	≤1/S
	Tobramycin	≥16/R	≥16/R	≥16/R	≤1/S	≥16/R	≥16/R	≥16/R	≥16/R	≤1/S
Fluoroquinolones	Ciprofloxacin	≥4/R	≤0.25/S	≥4/R	≤0.25/S	≥4/R	≤0.25/S	≥4/R	≤0.25/S	≤0.25/S
	Levofloxacin	≥8/R	≤0.25/S	≥8/R	≤0.25/S	≥8/R	≤0.25/S	≥8/R	≤0.25/S	≤0.25/S
Furane	Nitrofurantoin	≥512/R	≤16/S	256/R	≤16/S	≥512/R	≤16/S	≥512/R	≤16/S	≤16/S
Sulfanilamides	Trimethoprim/sulfamethoxazole	≤20/S	≤20/S	≤20/S	≤20/S	≥320/R	≤20/S	≥320/R	≤20/S	≤20/S

Abbreviations: I, intermediately resistant; MIC, minimum inhibitory concentration; R, resistant; S, sensitive.

BLAST analysis indicated a total of nine sequenced plasmids (Table 3; including the above five plasmids sequenced in our laboratory) constituted a novel group of plasmids, which had pHN7A8/pKPC-LK30 hybrid structures as characterized previously.^{13,33} Notably, all of these nine plasmids were recovered from clinical *K. pneumoniae* isolates from China. The five plasmids p675920-1, p1068-KPC, p20049-KPC, p12139-KPC, and p64917-KPC sequenced in our laboratory together with the first sequenced pCT-KPC were included in a genomic comparison herein. p675920-1 was used as the reference for genomic comparison because its hybrid structure had been characterized in great detail.¹³

General genomic diversity of pCT-KPC-like plasmids

The molecular structure of each of p675920-1, p1068-KPC, p20049-KPC, p12139-KPC, p64917-KPC, and pCT-KPC

was divided into the backbone, and several accessory modules that were defined as acquired DNA regions associated with and bordered with mobile elements and inserted at different sites of the backbone (Figure S1). These six plasmids were highly similar (>82% BLAST query coverage and >99% maximum nucleotide identity) to one another across whole genomes (Table S2).

There were totally 67 SNPs (among them 44 were recombination free) presented within the common (core) backbone regions of these six plasmids. A maximum likelihood phylogenetic tree (Figure S2) was constructed based on the recombination-free SNPs, and accordingly these six plasmids could be assigned into two clades I (p20049-KPC, pCT-KPC, p1068-KPC, and p675920-1) and II (p12139-KPC and p64917-KPC).

The backbone gene contents of these six plasmids displayed four major modular differences (Figure 1): 1)

Table 2 Major features of plasmids analyzed

Category	IncFII plasmids					
	pCT-KPC	p1068-KPC	p20049-KPC	p12139-KPC	p64917-KPC	p675920-1
Total length (bp)	151,466	146,103	151,653	169,424	169,419	163,995
Total number of ORFs	209	207	211	220	219	216
Mean G+C content (%)	53.8	53.7	53.6	53.5	53.5	53.6
Length of the backbone (bp)	103,892	98,528	110,596	122,370	120,628	121,755
Accessory modules	<i>bla</i> _{KPC-2} region ^a , MDR region ^a , Tn6346-associated region, and IS26	<i>bla</i> _{KPC-2} region ^a , MDR region ^a , Tn6346-associated region, and IS26	<i>bla</i> _{KPC-2} region ^a , <i>bla</i> _{CTX-M-65} region ^a , <i>rmtB</i> region ^a , and IS26	<i>bla</i> _{KPC-2} region ^a , MDR region ^a , <i>catA2</i> region ^a , and Tn6346-associated region	<i>bla</i> _{KPC-2} region ^a , MDR region ^a , <i>catA2</i> region ^a , and Tn6346-associated region	<i>bla</i> _{KPC-2} region ^a , MDR region ^a , Tn6346-associated region, and IS26

Note: ^aThese accessory modules contained resistance genes.

Abbreviations: MDR, multidrug resistance; ORF, open reading frame.

Table 3 Plasmids with pHN7A8/pKPC-LK30 hybrid structures

Plasmids	Accession number	Reference	BLAST coverage+identity			
			A	B	C	D
p1068-KPC ^{§,¶}	MF168402	This study	100%+100%	100%+100%	89%+99%	78%+99%
p20049-KPC ^{§,¶}	MF168404	This study	100%+100%	100%+99%	97%+99%	78%+99%
p12139-KPC ^{§,¶}	MF168403	This study	100%+100%	100%+100%	100%+99%	93%+99%
p64917-KPC ^{§,¶}	MF168405	This study	100%+100%	100%+100%	100%+99%	93%+99%
p675920-1 ^{§,¶,¶}	MF133495	¹³	100%+100%	100%+99%	98%+99%	90%+99%
pCT-KPC [§]	KT185451		100%+100%	100%+99%	96%+99%	78%+99%
pKP1034 [#]	KP893385	³³	100%+100%	100%+99%	62%+99%	89%+99%
pCP018455	CP018455	³⁹	100%+100%	100%+99%	94%+99%	91%+99%
pKPC-CR-HvKP4	MF437312	⁴⁰	100%+100%	100%+99%	100%+99%	86%+99%

Notes: All these plasmids are recovered from clinical *Klebsiella pneumoniae* isolates from China. The nucleotide sequences of A (*repA* of pHN7A8), B (*repB* of pKPC-LK30), C (whole genome of pHN7A8), and D (whole genome of pKPC-LK30) were BLASTed against the complete nucleotide sequences of the plasmids listed in the table. [§]Fully sequenced in our laboratory. [¶]Included in comparative genomics analysis in the present work. [#]pHN7A8/pKPC-LK30 hybrid structures have been previously characterized. pCP018455 was designated herein.

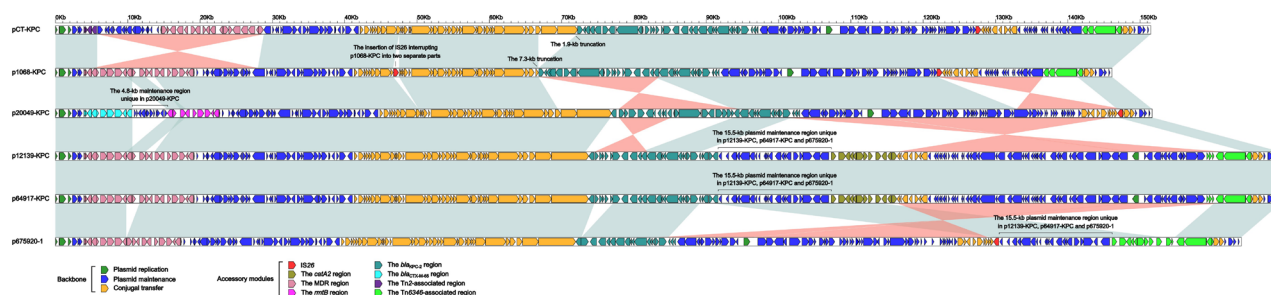


Figure 1 Linear comparison of plasmid genome sequences.

Notes: Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on the function classification. Shading denotes the regions of homology (>95% nucleotide identity). Accession numbers of pCT-KPC and p675920-1 for reference are KT185451 and MF133495, respectively.

p20049-KPC contained a unique 4.8-kb maintenance region (from *relB* to *mobB*) relative to the other five plasmids; 2) a 15.5-kb plasmid maintenance region (from *orf375* to *parA*) was found in p12139-KPC, p64917-KPC, and p675920-1 but not pCT-KPC, p1068-KPC, and p20049-KPC; 3) 1.9- and 7.3-kb truncations occurred in the conjugal transfer regions of pCT-KPC and p1068-KPC, respectively, which resulted from insertion of the *bla*_{KPC-2} region; and 4) the conjugal transfer region of p1068-KPC was interrupted into two separate parts due to insertion of IS26. The recombination of pKPC-LK30 (containing no conjugation transfer genes) with pHN7A8 demolished the conjugation transfer gene organization of pHN7A8, resulting in all these six plasmids to be nonconjugative.

These six plasmids contained several accessory modules (Figure 1): 1) the *bla*_{KPC-2} regions were found in all these plasmids and slightly differed from one another; 2) the MDR regions were observed in all these plasmids and displayed considerable modular diversity; notably, the MDR regions in pCT-KPC, p1068-KPC, and p64917-KPC/p12139-KPC were disrupted into two separated parts (probably due to IS26-mediated homologous recombination), which were located at two different sites of the backbones; and 3) the Tn6346-associated regions were presented in five plasmids except for p20049-KPC, but their gene contents display significant variations. These six plasmids carried eight resistance markers (*bla*_{KPC-2}, *rmtB*, *fosA3*, *catA2*, *bla*_{CTX-M-65}, *bla*_{TEM-1B}, *bla*_{SHV-12}, and *mer*) in total, all of which were located in the accessory modules (Table S3).

The *bla*_{KPC-2} regions

The *bla*_{KPC-2} regions of the seven plasmids pKPC-LK30, p1068-KPC, pCT-KPC, p12139-KPC, p64917-KPC, p675920-1, and p20049-KPC exhibited four stages of evolution (Figure 2) and carried three close Tn6296³⁴ derivatives (ΔTn6296-1 to ΔTn6296-3) severing as the core genetic

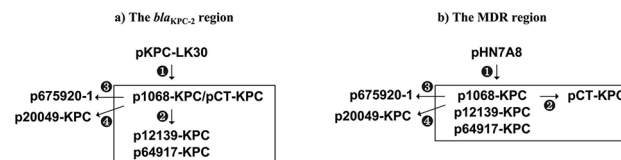


Figure 2 Deduced evolutionary history of major accessory resistance regions.

Notes: Shown are the deduced evolutionary history of the *bla*_{KPC-2} regions (a) and the MDR regions (b) of indicated plasmids. Circled numbers indicate different stages of evolution.

environments of *bla*_{KPC-2} (Figure 3). First, the *bla*_{KPC-2} region of pKPC-LK30 was organized in order of a Tn3-family transposon remnant, a truncated IS26–*bla*_{SHV-12}–IS26 unit, ΔTn6296-1 generated from Tn6296 after the loss of ΔTn1722-3' and truncation of Tn6376, ΔTn21 with insertion of IS5075 into its inverted repeat right (IRR), an IS903B remnant, and ISKpn14, and it evolved into the counterpart of p1068-KPC/pCT-KPC after the loss of the Tn3-family transposon remnant. Second, the deletion of the truncated IS26–*bla*_{SHV-12}–IS26 unit, the truncation of ΔTn6296-1 into ΔTn6296-2 with deletion of ΔTn1722-5', and the replacement of IS903B remnant–ISKpn14 by Δint–*orf225*–*parA*–*orf492*–IS26 were observed in p12139-KPC/p64917-KPC relative to p1068-KPC. Third, the evolution of p675920-1 from p1068-KPC involved the deletion of the truncated IS26–*bla*_{SHV-12}–IS26 unit as well as the truncation of ΔTn6296-1 into ΔTn6296-3 with deletion of ΔTn1722-5'–Δ*repB*–*klcA*–*orf6*–*korC*. Fourth, compared to p1068-KPC, p20049-KPC underwent the inversion of a large region composed of ΔTn6296-1 and the truncated IS26–*bla*_{SHV-12}–IS26 unit.

The MDR regions

Similarly, the MDR regions of the seven plasmids pHN7A8,⁹ p1068-KPC, p12139-KPC, p64917-KPC, pCT-KPC, p675920-1, and p20049-KPC displayed four stages of evolution (Figures 2 and 4). First, the MDR region of pHN7A8 was generated from insertion of the Tn2–*rmtB* element at a

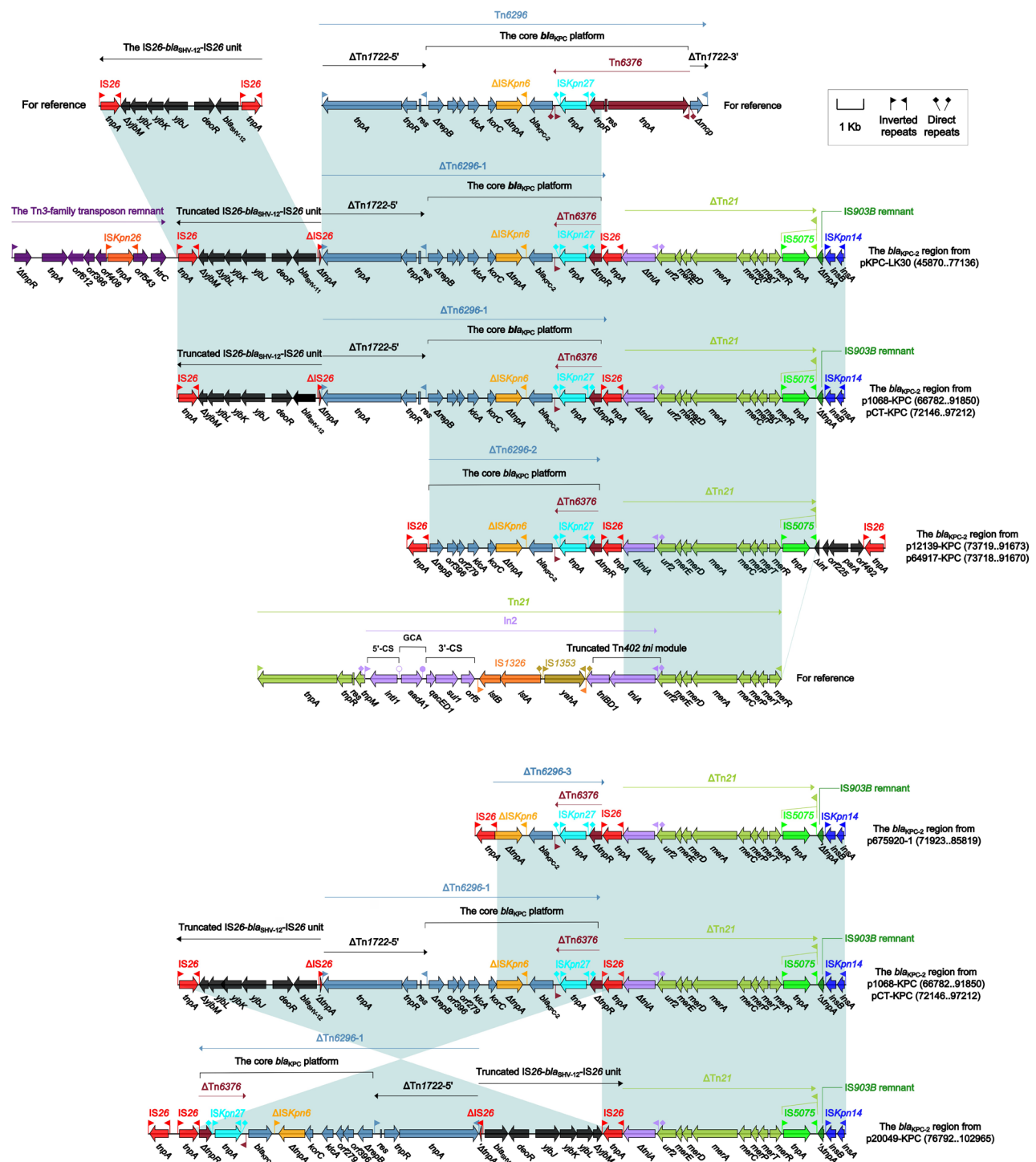


Figure 3 The *bla*_{KPC-2} regions and comparison with related regions.

Notes: Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on the function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids. Accession numbers of the IS26-*bla*_{KPC-2}-IS26 unit, Tn6296, and Tn21 for reference are CP003684, FJ628167, and AF071413, respectively.

site within *insB* of IS1R (making interruption and further truncation of *insB*), followed by that of a large region (organized sequentially as IS26, Δ Tn6377,³⁵ the IS26-*fosA3*-IS26 unit,³⁶ and IS1294) at a site between *tnpA* and *tnpR* of Tn2

(leading to truncation of both *tnpA* and *tnpR* and loss of *res*); it evolved into the counterpart of p1068-KPC/p12139-KPC/p64917-KPC after fragmentation of IS1294 into Δ IS1294-5' and Δ IS1294-3' as well as inversion of IS26-*fosA3*-IS26-

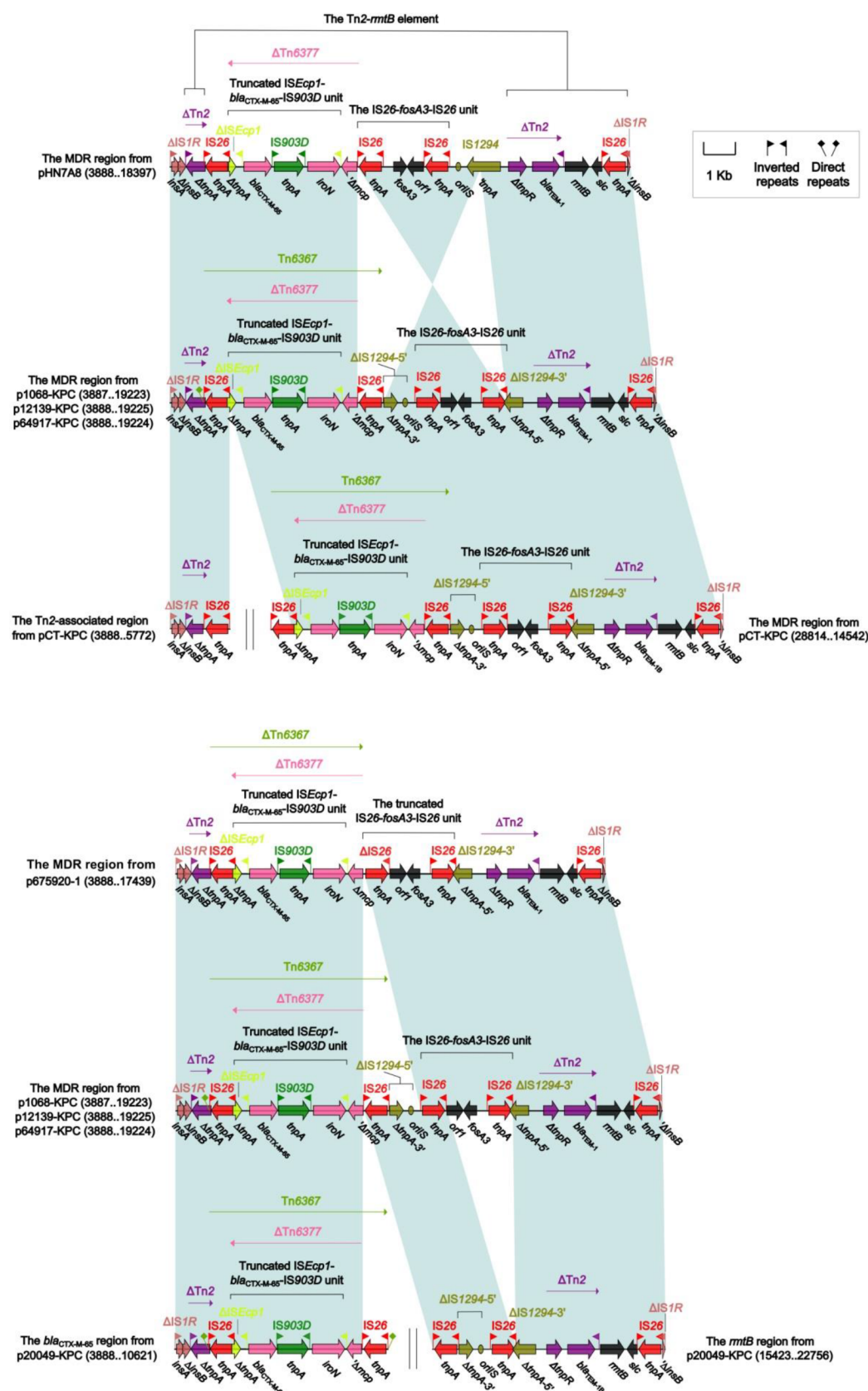


Figure 4 The MDR regions and comparison with related regions.

Notes: Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on the function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids.

$\Delta IS1294-5'$. Second, compared to p1068-KPC, the counterpart of pCT-KPC was split into two separate regions, namely the Tn2-associated region and the MDR region. Third, the evolution from p1068-KPC to p675920-1 involved deletion of IRL_{IS26}- $\Delta IS1294-5'$. Fourth, the split of the MDR region into the two separate parts (ie, the *bla*_{CTX-M-65} region and the *rmtB* region) and the deletion of the IS26-*fosA3*-IS26 unit were observed in p20019-KPC relative to p1068-KPC.

The Tn6346-associated regions and the *catA2* regions

The Tn6346-associated region of p675920-1 could be divided sequentially as a Tn3-family transposon remnant, IS26, a small *GlSul2* remnant containing no resistance genes, $\Delta Tn6346-1$ and IS26 (Figure 5). The counterparts of p12139-KPC and p64917-KPC split into two separate parts: 1) the *catA2* region characteristic of the connection of the above Tn3-family transposon remnant with the IS26-*catA2*-IS26 unit as observed in p112298-*catA* (accession number, KY270851)³⁷; and 2) the residue Tn6346-associated region composed in order of ISKpn14, a 288-bp IS903B remnant, $\Delta Tn6346-1$ and IS26. The residue Tn6346-associated regions of pCT-KPC and p1068-KPC could be modularly divided into IS26, $\Delta Tn6346-2$, and IS26. $\Delta Tn6346-1$ and $\Delta Tn6346-2$ ¹³ were the 5'-terminal remnants (containing no resistance genes) of the Tn3-family unit transposon Tn6346.³⁸

Prevalence of pCT-KPC-related plasmids

To illustrate the prevalence of pCT-KPC-like plasmids in the 51 strains, a total of 10 backbone genes or gene loci and two accessory resistance genes across the pCT-KPC genome were selected for PCR detection followed by amplicon sequencing. Ten selected backbone makers could be assigned into three origins: 1) *repA1* (IncFII-family replication initiation protein), *pemIK* (toxin-antitoxin system), *stbA* (mediator of plasmid stability), and *traC* and *traX* (conjugal transfer proteins) from pHN7A8; 2) *repB* (IncR-type replication initiation protein), *parA* (partition protein), *vagD* (toxin of the toxin-antitoxin system), and *relEB* (toxin/antitoxin system) from pKPC-LK30; and 3) *traA* (conjugal transfer protein) from a 5.1-kb conjugal transfer region found in pCT-KPC rather than pHN7A8 and pKPC-LK30. The two selected resistance genes *bla*_{KPC-2} and *rmtB* were found in the accessory resistance regions of pKPC-LK30 and pHN7A8, respectively. Of these 51 strains, 49 harbored all 10 backbone markers, while the remaining two contained nine backbone markers except *traC*. *bla*_{KPC-2} and *rmtB* were detected in all these 51 strains. These results denoted that *bla*_{KPC-2} and *rmtB*-carrying pCT-KPC-like plasmids were present in all these 51 *K. pneumoniae* strains. In conclusion, the dissemination of *bla*_{KPC-2} and *rmtB* in these hospitals were associated with the spread of genetically closed related pCT-KPC-like plasmids among clonal *K. pneumoniae* CG258 strains.

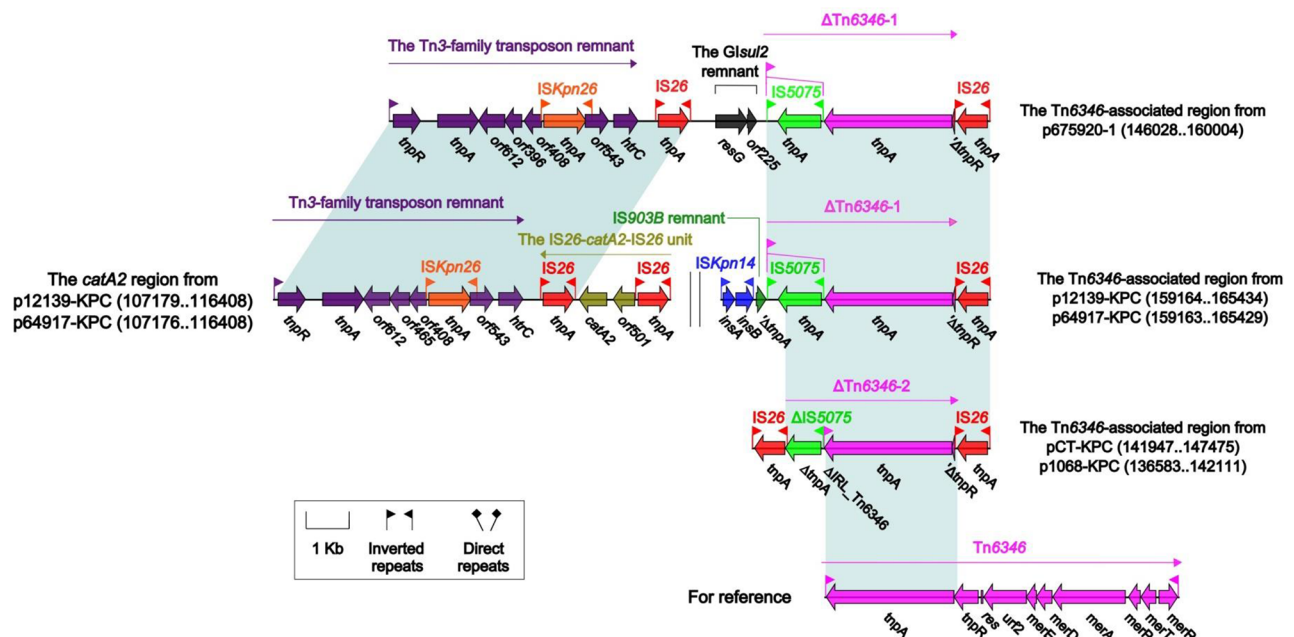


Figure 5 The Tn6346-associated regions and the *catA2* regions and comparison with related regions.

Notes: Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on the function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids. Accession number of Tn6346 for reference is EU696790.

Concluding remarks

The six IncFII-family plasmids p675920-1, pCT-KPC, p1068-KPC, p20049-KPC, p12139-KPC, and p64917-KPC displayed similar pHN7A8/pKPC-LK30 hybrid structures but slightly differed from one another due to deletion and acquisition of various backbone and accessory regions. At least seven antibiotic resistance genes, including *bla*_{KPC-2}, *rmtB*, *fosA3*, *bla*_{CTX-M-65}, *bla*_{TEM-1B}, and *bla*_{SHV-12}, were present in each of these six plasmids, mediating MDR of these isolates. pCT-KPC-like plasmids were detected herein in a collection of 51 KPC-positive *K. pneumoniae* CG258 isolates in five Chinese hospitals. In addition, there were still three available sequenced plasmids, namely pKP1034, pCP018455, and pKPC-CR-HvKP4 (Table 3), which also displayed pHN7A8/pKPC-LK30 hybrid structures and were recovered from *K. pneumoniae* CG258 isolates from three additional Chinese hospitals.^{33,39,40} These denoted clonal dissemination of *K. pneumoniae* CG258 strains, harboring *bla*_{KPC-2} and *rmtB*-carrying IncFII-family pKPC-LK30/pHN7A8 hybrid plasmids, in multiple Chinese hospitals. At least nine copies of IS26 were found in each of these six plasmids and would act as the common regions (ie, adaptors) to mediate homologous recombination, facilitating assembly and mobilization of accessory resistance regions within and among these highly mosaic plasmids.^{41–43} There were four IS26-flanking mobile elements in total, including Tn6367, the IS26-*bla*_{SHV-12}-IS26 unit, the IS26-*fosA3*-IS26 unit, and the IS26-*catA2*-IS26 unit, and the last three lacked paired short DR sequences (target site duplication signals for transposition) at their both ends and therefore could be annotated as typical IS26-composite transposons.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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