Infection and Drug Resistance

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ORIGINAL RESEARCH

Tandem Repeat of bla_{NDM-1} and Clonal Dissemination of a fosA3 and bla_{KPC-2} Co-Carrying IncR-F33: A–: B– Plasmid in *Klebsiella pneumoniae* Isolates Collected in a Southwest Hospital in China, 2010–2013

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Introduction: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has been widespread in coastal cities of eastern China since 2009. However, how CRKP spreads and evolves in southwest China is unclear.

Aim: We investigated the genetic characteristics and dissemination mechanisms of carbapenemase genes in forty-one non-repetitive CRKP isolates collected from a southwest hospital, Kunming, Yunnan, during 2010–2013.

Methodology: Drug susceptibilities were analyzed by using VITEK 2 compact system. Genetic relationships were ascertained based on multilocus sequence typing (MLST) and Pulsed-field gel electrophoresis (PFGE) analysis. Genetic backgrounds of bla_{KPC-2} and bla_{NDM-1} were revealed by DNA walking and high-throughput sequencing.

Results: All isolates were highly resistant to common antibiotics except for tigecycline. In total, 34 bla_{KPC-2}, 3 bla_{NDM-1}, 1 bla_{IMP-4} and 3 bla_{IMP-26} genes were identified and KP67 plasmid 1 co-harbored bla_{NDM-1} and bla_{IMP-26}. Five sequence types, namely ST11, ST290, ST340, ST395 and ST437, were recognized by MLST. Surprisingly, bla_{KPC-2} was only detected in ST11 strains. We described a clonal dissemination of *fosA3*-positive IncR-IncF33:A-:B- multireplicon plasmid carrying the gene cassettes IS*26*-ΔTn*3*-IS*Kpn27*-bla_{KPC-2}-ΔIS*Kpn6*-*korC*-*klcA*-Δ*repB*-Tn*1721* in all ST11 isolates. Three bla_{NDM-1} positive isolates belonged to three different ST types and their bla_{NDM-1} genetic backgrounds were also distinct. Interestingly, the flanking regions of bla_{NDM-1} in KP67 and KP72 were duplicated into one to five copies in a form of tandem repeat by the transposition of IS*91* like element. The bla_{NDM-1} of KP82 was carried on a common IncX3 plasmid.

Conclusion: This study described the early epidemiological characteristics of bla_{KPC-2}/bla_{NDM-1}-carrying CRKP, and reported a new tandem repeat pattern of bla_{NDM-1} cluster in Yunnan. These findings extend our knowledge on the carbapenemase gene evolutions.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, clonal dissemination, genetic backgrounds, IS*91* transposons, bla_{NDM-1} duplication

Introduction

Over the last fifty years, the development and the widespread use of antibacterial drugs has greatly helped people against bacterial infections. Clinically, the most commonly used antibacterial drugs are β-lactam antibiotics, such as

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cephalosporin and carbapenems, due to their broad-spectrum antimicrobial properties and strong activity against several extended-spectrum beta-lactamases.[1] Recently, many gram-negative Enterobacteriaceae strains have been reported resistant to carbapenems by producing carbapenemases, termed Carbapenems resistant Enterobacteriaceae (CRE).[2,3] Among CRE, the carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a taxon of considerable interest.[4,5] Three highly reactive carbapenemases have been described in CRKP, known as class A KPC-type, class B NDM-type, and class D OXA-48-like.[6,7] These carbapenemase-encoding genes are usually located on drug-resistant plasmids with the ability to transfer between bacterial cells through the concerted activities of mobile genetic elements.[8,9] Therefore, CRKP infections are recognized as a major challenge in health-care settings and are receiving growing concerns worldwide.[10,11]

The first $bla_{\text{KPC-2}}$ producing CRKP was identified in the United States in 1996, and subsequently spread worldwide,[12] whereas it was first reported in Zhejiang, a coastal province in southeast China, in 2004.[13] Although bla_{KPC} had been disseminated in diverse *K. pneumoniae* sequence types (STs), the vast majority of them were found to be restricted in a genetic related group, namely clone complex 258 (CC258).[14,15] This clone complex includes more than a thousand STs, of which the ST11 is the predominant clone in China.[4,16,17] With the continuous spread and evolution of $bla_{\text{KPC-2}}$ harboring plasmid in CRKP, the mobilization of different resistance gene cassettes facilitated the co-carriage of them on a single mosaic plasmid. Recently, a fosfomycin resistance gene, namely *fosA3*, encoded the FosA3 which inactivate fosfomycin by exerting glutathione-S-transferase activity, has been characterized on $bla_{\text{KPC-2}}$-carrying plasmids in China.[18–20] In those cases, researchers raised the possibility that *fosA3* may be transmitted to humans from animal sources and fosfomycin may not be recommended as an alternative option for treating CRKP infections. The more global concerned β-lactamase were $bla_{\text{NDM-1}}$ which first emerged in the Indian sub-continent in 2009.[21] Most previous studies have reported a single copy of the $bla_{\text{NDM-1}}$ gene encoded by diverse plasmids.[22–24] However, recent studies have demonstrated the existence of multiple copies of plasmid encoded $bla_{\text{NDM-1}}$.[25–27] These findings suggest a potential relationship between mobile elements and bla_{NDM} gene duplication, as well as future research trends on the interaction between gene replication and host phenotypic.

Occurrences of CRKP have been rising gradually in recent years in China, and have caused dissemination of nosocomial infections. The outbreaks of $bla_{\text{KPC-2/NDM-1}}$-carrying *K. pneumoniae* have been reported in some hospitals in eastern coastal cities of China, such as Shandong,[23] Shanghai,[28] Jiangsu[17,29] and Zhejiang.[30,31] Meanwhile, several surveys exhibited an increase in the prevalence of *K. pneumoniae* with decreasing susceptibility to Carbapenems in the southwest China since 2009.[32–34] However, few studies have focused on the molecular epidemiology of CRKP in Yunnan, a province of southwest China bordering the Indian sub-continent. In the present study, we aimed to describe the genetic characteristics and dissemination mechanisms of carbapenemase-encoding genes in CRKP isolates collected from hospitalized patients at the second affiliated hospital of Kunming medical university, Kunming, Yunnan, during 2010–2013.

Methods

Strains and Drug Susceptibilities

From 2010 to 2013, a total of 41 non-repetitive CRKP clinical isolates were collected from patients who had poor prognosis after carbapenem treatment during hospitalization (Table 1). Most of the samples were collected consecutively during February to August in 2013, while some isolates were collected intermittently before 2013. CRKP was defined as any *K. pneumoniae* isolate showing ertapenem and/or imipenem minimum inhibitory concentrations (MICs) of ≥2 mg/L or 4 mg/L, respectively. The minimal inhibitory concentrations (MICs) of several frequently used antibiotics (augmentin, piperacillin/tazobactam, cefuroxime axetil, cefuroxime sodium, cefoxitin, ceftazidime, ceftriaxone, cefoperazone sodium, cefepime, ertapenem, imipenem, amikacin, levofloxacin, tigecycline) were evaluated on the VITEK 2 compact system (bioMérieux, Craponne, France) and interpreted according to the 2021 Clinical and Laboratory Standards Institute (CLSI) guideline M100-S31 or Food and Drug Administration (U.S.).

MLST and PFGE Molecular Typing

Isolates were recovered on LB medium. Cells were harvested at the period of logarithmic growth. Genomic DNA was prepared using the DNA extraction kit (TIANamp Bacteria DNA Kit, Tiangen) following the manufacturer's protocol.

Table 1 Clinical Features, STs, Gene Identifications and Flanking Fragment Identifications Among 41 CRKP Isolates

Isolates	Clinical features					STs	bla_{KPC-2}	K-UP	K-DN	bla_{NDM-1}	bla_{IMP}
	Date (Year/Month)	Gender	Age	Source of Culture	Hospitalization Ward						
KP08	2010/08	F	78	Sputum	Respiratory Medicine	395	-	N/A	N/A	-	-
KP21	2013/04	M	66	Urine	Rehabilitation department	11	2	√	√	-	-
KP22	2013/04	M	44	Sputum	EICU	11	2	√	√	-	-
KP23	2013/04	F	72	Sputum	Emergency	11	2	√	√	-	-
KP24	2013/04	M	56	Secretion	EICU	11	2	√	√	-	-
KP27	2013/02	F	1	Secretion	Burns surgery	290	-	N/A	N/A	-	bla_{IMP-26}
KP29	2013/04	M	42	Secretion	Burns surgery	11	2	√	√	-	-
KP30	2013/04	M	48	Sputum	Urology	11	2	√	√	-	-
KP31	2013/05	M	84	Sputum	Respiratory Medicine	11	2	√	√	-	-
KP32	2013/05	M	75	Sputum	EICU	11	2	√	√	-	-
KP33	2013/05	M	97	Sputum	EICU	11	2	√	√	-	-
KP34	2013/05	M	91	Sputum	EICU	11	2	√	√	-	-
KP37	2013/05	M	97	Sputum	Emergency	11	2	√	√	-	-
KP38	2013/05	M	66	Urine	Urology	11	2	√	√	-	-
KP40	2013/05	F	71	Sputum	Emergency	11	2	√	√	-	-
KP42	2013/06	F	49	Sputum	SICU	11	2	√	√	-	-
KP43	2013/06	M	59	Blood	Hematology	11	2	√	√	-	-
KP46	2013/06	M	86	Sputum	EICU	11	2	√	√	-	-
KP50	2013/07	M	36	Sputum	Emergency	11	2	√	√	-	-
KP51	2013/08	M	42	Secretion	Burns surgery	290	-	N/A	N/A	-	bla_{IMP-26}
KP54	2013/06	M	73	Sputum	Cardiology Department	11	2	√	√	-	-
KP58	2013/07	F	42	Blood	Hematology	11	2	√	√	-	-
KP60	2013/07	M	82	Sputum	EICU	11	2	√	√	-	-
KP62	2013/07	F	44	Blood	Hematology	11	2	√	√	-	-
KP63	2013/04	M	72	Cerebrospinal fluid	General surgery	11	2	√	√	-	-
KP65	2013/04	M	50	Urine	Urology	11	2	√	√	-	-
KP66	2013/04	M	38	Blood	Burns surgery	11	2	√	√	-	-
KP67	2011/12	M	58	Sputum	ICU	290	-	N/A	N/A	√	bla_{IMP-26}
KP68	2013/03	F	71	Drainage fluid	Hepatobiliary surgery	11	2	√	√	-	-
KP69	2012/05	M	70	Blood	Urology	11	2	√	√	-	-
KP72	2013/03	F	66	Urine	Rehabilitation department	395	-	N/A	N/A	√	-
KP75	2013/02	M	67	Urine	Urology	11	2	√	√	-	-
KP76	2013/06	M	75	Urine	Urology	11	2	√	√	-	-
KP77	2011/12	M	65	Urine	Urology	11	2	√	√	-	-
KP78	2013/03	M	75	Urine	Burns surgery	11	2	√	√	-	-
KP79	2012/08	M	3	Urine	Pediatrics	340	-	N/A	N/A	-	bla_{IMP-4}
KP81	2013/02	M	31	Secretion	Burns surgery	11	2	√	√	-	-
KP82	2013/05	M	78	Sputum	Gastroenterology	437	-	N/A	N/A	√	-
KP83	2013/02	F	91	Sputum	Emergency	11	2	√	√	-	-
KP84	2013/02	F	74	Sputum	Emergency	11	2	√	√	-	-
KP85	2013/08	M	49	Drainage fluid	Hepatobiliary surgery	11	2	√	√	-	-

Abbreviations: F, female; M, male; ICU, intensive care unit; EICU, emergency intensive care unit; STs, sequence types; K-UP, upstream segment of bla_{KPC-2}; K-DN, downstream segment of bla_{KPC-2}; N/A, not applicable; -, negative.

Forty-one isolates were initially genotyped by multilocus sequence typing (MLST) as described previously.[35] The Nucleotide sequence of each locus was submitted to the *K. pneumoniae* online MLST database to obtain allele number. Allelic profile of each isolate was matched to the existing sequence types (STs) numbers (http://www.pasteur.fr). Additionally, pulsed-field gel electrophoresis (PFGE) genotyping was also performed.[36] Briefly, plugs were prepared in agarose blocks (1%) and digested with restriction enzyme *Xba*I. DNA fragments were electrophoresed for 24 h at 14 °C under the following conditions: 6V/cm voltage gradient, switch time from 4 to 40s, and with pulse angle of 120°. After separation, gels were stained with GelRed (Biotium, USA). The Cluster analysis was performed from the PFGE

data using the BioNumerics software v7.6 (Applied Maths Inc., Austin, Texas, USA). The similarity of PFGE patterns was calculated based upon Dice coefficients with a tolerance setting of 1.4% band tolerance and a 1.5% optimization setting for the whole profile, and the Dice similarity coefficient required to be >80% for the pattern to be considered as belonging to the same PFGE type. Dendrogram was constructed using the unweighted-pair group method with mathematical averaging (UPGMA).

Molecular Identification of Carbapenemase Genes

Five carbapenemase encoding genes (KPC, NDM, IMP, VIM and OXA-48) were detected by PCR.[37–41] The relevant primers are listed in Table S1. PCR amplification was performed in a 25 μL reaction containing 1 μL genomic DNA (about 200 ng), 22 μL Gold Mix TSE101 (TSINGKE, Beijing) and 1 μL of each primer (10 μM) under thermocycle conditions of 98 °C for 2 minutes, 35 × (98 °C for 10 sec, 15 sec at specific annealing temperature for each set of primers and 72 °C for 15 sec), and 72 °C for 5 minutes on a T100 Thermal Cycle (BIO-RAD, USA). Positive bands were cut out and purified, and then sequenced at TSINGKE Company (Kunming, China), bi-directionally. The sequencing results were checked and assembled using the ContigExpress (independent module from Vector NTI Advance V 11, Invitrogen, USA). Sequence comparison was performed using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).[42]

Bla$_{KPC-2}$ Genetic Background Analysis and Southern Blot

MLST analysis showed that most isolates belonged to ST11 type. Therefore, we randomly selected KP21 as a pioneer to identify flanking genes of the *bla*$_{KPC-2}$ gene using genome walking kit (TaKaRa, following the manufacturer's protocol). The flow diagram and walking primers were present in the Figure S1 and Table S2, respectively. After sequencing the flanking fragments, gene contents of the *bla*$_{KPC-2}$ genetic background were displayed. Specific scanning primers were designed based on the franking sequences (Table S3), and the corresponding flanking fragments of *bla*$_{KPC-2}$ positive isolates were amplified by *Takara LA Taq* Polymerase (TaKaRa) under the conditions of 94 °C for 2 minutes, 35 × (94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 3 or 7 min according to the flanking fragment size), and 72 °C for 5 minutes. Positive amplicons were further analyzed based on restriction fragment length polymorphism (PCR-RFLP). Briefly, PCR products were loaded on an agarose gel (1%) and separated by electrophoresis. Bands were excised from gels and purified using TIANgel Midi Purification Kit (Tiangen). The upstream and downstream fragments were digested by *Hae*II (TaKaRa) and *Eco*T14 (*sty*I, TaKaRa), respectively. All digestions were performed at 37°C overnight and then separated by agarose gel (2%) electrophoresis at 120 V for 70 min. The representative amplicons with different types of PCR-RFLP fingerprints were further sequenced bi-directionally. S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) and southern blotting hybridization were performed to determine the location of *bla*$_{KPC-2}$ gene. Briefly, the plugs were digested with S1 nuclease (TaKaRa) at 37°C for 30 min and then separated by electrophoresis. Southern blot hybridizations were performed by following the manufacturer's instructions of the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics) using the *bla*$_{KPC-2}$ digoxigenin-labeled specific probe. *Salmonella* serotype *Braenderup* strain H9812 was digested by *XbaI* and used for the molecular marker.[43] The gel bands of *bla*$_{KPC-2}$-carrying plasmids were cut out and purified. IncR replicon, IncFII replicon and *fosA3* genes were detected in these *bla*$_{KPC-2}$-carrying plasmids by PCR amplification as previously described.[44–46]

High-Throughput Sequencing

Three *bla*$_{NDM-1}$ positive isolates, two *bla*$_{KPC-2}$ positive isolates with different RFLP fingerprints together with KP21 were used for high-throughput sequencing. Briefly, genomic DNA was extracted as described above. Libraries for whole-genome sequencing were constructed and then sequenced on an Oxford Nanopore ONT platform (Shanghai Personal Biotechnology Co., Ltd., China). To correct sequencing errors, libraries (400 bp) for second-generation sequencing were also constructed and sequenced in 2×150 bp paired-end mode with a minimal coverage of 100 × on an Illumina NovaSeq sequencer. After quality control, the Oxford Nanopore data were assembled with HGAP4[47] and CANU (Version 1.6)[48] and then corrected by the second-generation sequencing data using pilon (Version 1.22)[49] under default parameter. These whole genome shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession PRJNA858206,

PRJNA858218, PRJNA742185 and the annotations were added by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (release 2021).[50]

Bioinformatics Analysis

To count the copy number of vital resistance genes in a certain plasmid, the full-length nucleotide sequences of bla_{KPC-2} and bla_{NDM-1} were BLAST searched against the Oxford Nanopore sequencing raw data using CLC Genomics Workbench v21.0.2. In brief, multi-BLAST tables were filtered with the following parameters: Number of HSPs≥1, Lowest E-value=0.00 and Greatest HSP length≥300 bp. The repeat level of each gene was ascertained by checking the high-scoring segment pairs (HSP) number in each multi-BLAST table. The entire nucleotide sequences of two bla_{NDM-1} repeat structures were imported and searched again by setting the Greatest HSP length≥7000 bp (for KP67) or 15,000 bp (for KP72). Genetic characteristic of each HSPs were checked manually. The representative bla_{KPC-2} and bla_{NDM-1}-carrying plasmids were compared with those available in NCBI nucleotide databases (https://ftp.ncbi.nlm.nih.gov/blast/db/) and NCBI plasmid reference database (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/plasmid/). The plasmid sequences of related hits were downloaded via NCBI accession number (Table S4). These plasmids were annotated with RAST 2.0 (https://rast.nmpdr.org/). ISFinder (https://www-is.biotoul.fr/) was used to determine the insertion sequence elements. Easyfig (v2.2.5) was used to map whole plasmid comparisons between closely related plasmids.[51]

Results

Antibiotic Susceptibilities and Carbapenemase Gene Detections

The minimum inhibitory concentrations (MICs) of 14 antimicrobial agents against 41 CRKP isolates showed multidrug resistance profiles, particularly to the tested β-lactamase inhibitor combinations, third-generation cephems and ertapenem (Table S5). Of the tested antimicrobial agents, levofloxacin (4.87%) and amikacin (31.70%) exhibited low sensitivity. In contrast, all isolates were susceptible to tigecycline (100%). In total, four types of carbapenemases were identified in these samples, including bla_{KPC-2} (n=34), bla_{NDM-1} (n=3), bla_{IMP-26} (n=3) and bla_{IMP-4} (n=1), as shown in Table 1. The bla_{VIM} and bla_{OXA-48} genes were not detected in any of the isolates, and the bla_{KPC-2}/bla_{NDM-1} co-harboring isolate was also not detected. Remarkably, KP67 was found to co-harbor bla_{NDM-1} and bla_{IMP-26}.

Distribution of MLST Sequence Types and PFGE Patterns

MLST analysis revealed 5 different sequence types (Table 1): ST11 (n=34), ST290 (n=3), ST340 (n=1), ST395 (n=2), and ST437 (n=1). All bla_{KPC-2} positive isolates belonged to ST11. Three bla_{NDM-1} positive isolates were ST290, ST395 and ST437, respectively. Three bla_{IMP-26} positive isolates were confined to ST290 type, whereas bla_{IMP-4} was only found in ST340 type. PFGE released five clades, consistent with the results of MLST (Figure 1). The genetic similarity of 34 isolates in the main clade of PFGE was 96.5%, and they all belonged to MLST genotype ST11, as shown by the red arrow in Figure 1. However, the genetic similarity of three isolates belonging to ST290 in PFGE was 82.7%, suggesting that their plasmids were quite different. In addition, only ST11 CRKP isolates can be detected in four wards (EICU, urology, emergency and hematology wards) and mostly from the sputum (n=13) and urine samples (n=5), suggestive of the likelihood of a clonal dissemination of ST11 CRKP among hospitalized patients after surgery or invasive therapy. We also noticed that two ST11 CRKP isolates from the urology wards could be dated back to early collecting years, one from May 2012 and the other from September 2011, indicating a long period spread of this clone.

Bla$_{KPC-2}$ Genetic Background Analysis and Southern Blot

After three-rounds of thermal asymmetric interlaced PCR (TAIL-PCR) on isolate KP21, three bands were sequenced, as shown in Figure S1. Sequence analysis showed that the assembled fragment was 11,275 bp length with a genetic background composed of IS*26*-Δ*tnpR*(Tn*3*)-IS*Kpn27*-bla_{KPC-2}-ΔIS*Kpn6*-*korC*-*klcA*-Δ*repB*-Tn*1721* (Figure 2). This genetic background exhibited a high degree of similarity to the previously published plasmids with 100% query coverage and 100% nucleotide identity.[19,52,53] Specific scanning primers (KPC-IS26F/U-SP2, KPC-D-SP2/Tn1771R) worked well and generated a 2880 bp PCR product for the upstream and an 8005 bp product for the downstream, as expected. As

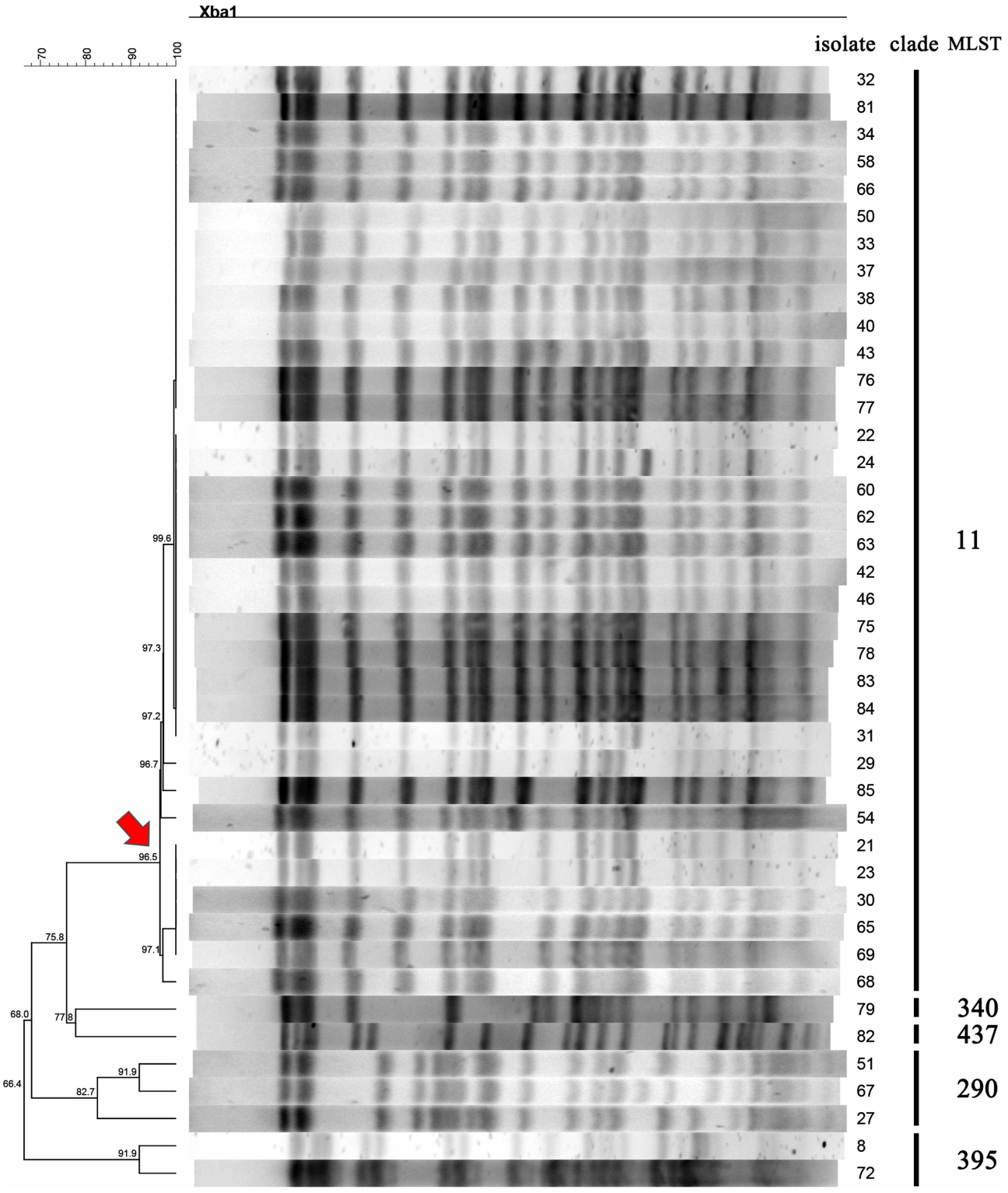

Figure 1 PFGE patterns and STs among 41 clinical CRKP isolates Each PFGE clade is defined based on the similarity and indicated by a black line. The strain number and MLST types are included along each PFGE lane. The red arrow indicates a primary branch that contains a number of isolates with close genetic relationship.

shown in Table 1, all 34 bla_{KPC-2} positive isolates successfully produced amplicons similar to that of KP21. PCR-RFLP showed a highly similar RFLP profiles among them, except for the downstream amplicon of KP29 (Figure 3). Further sequencing for this variant revealed an IS*26* element inserted into the *tnpA* (Tn*1721*) gene (Figure 2).

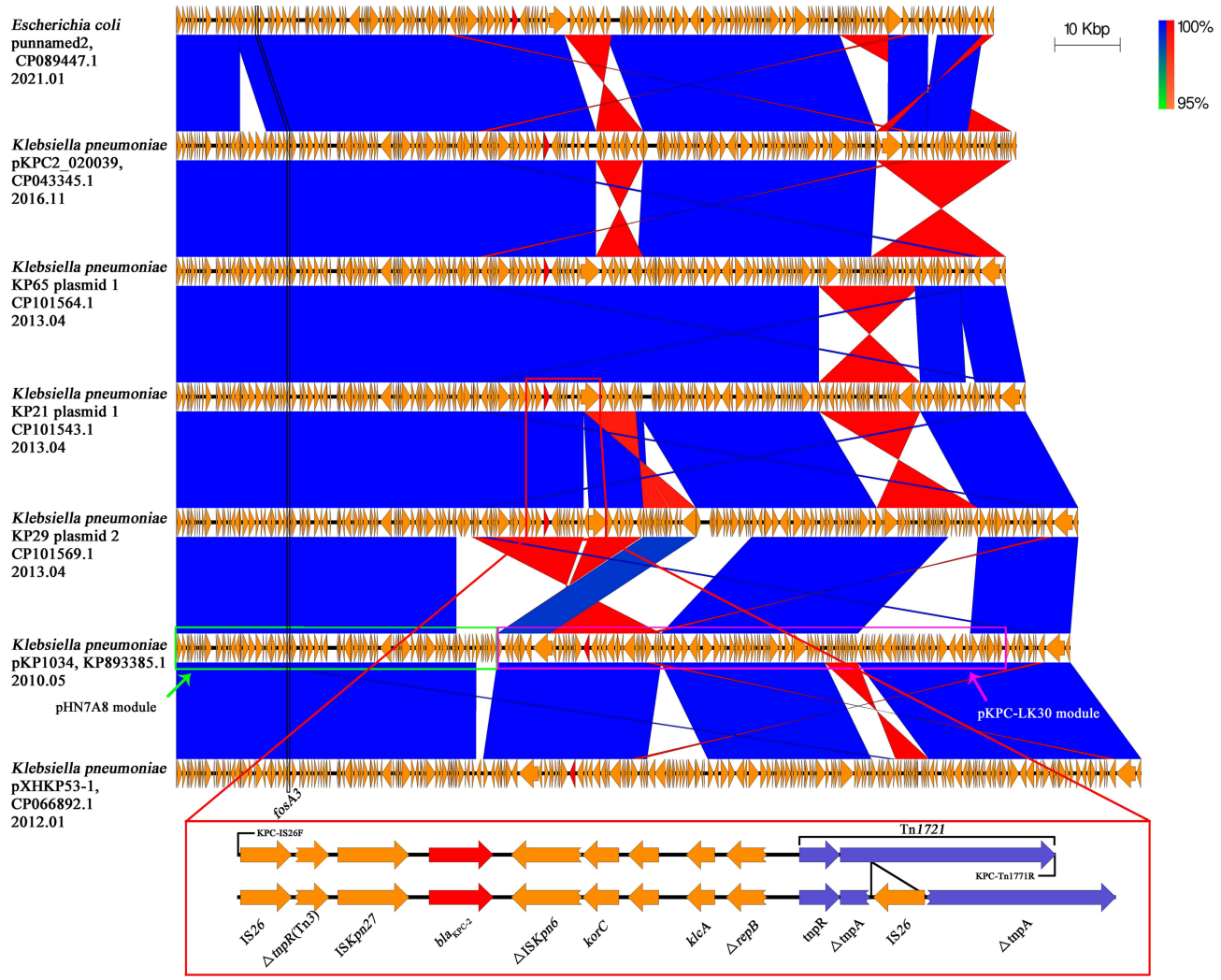

Figure 2 Comparison of bla_{KPC-2} carrying plasmids Linear comparison of gene contents of seven bla_{KPC-2} carrying IncR-IncF33:A-:B- plasmids. The ORF regions of bla_{KPC-2} genes are emphasized in red and other genes are shown in yellow. The pHN7A8 and pKPC-LK30 module regions are represented by green and red boxes. Detailed descriptions of the bla_{KPC-2} genetic backgrounds of KP21 plasmid 1 and KP29 plasmid 2 are shown in the red box at the bottom of the figure. Blue and red shadings indicate nucleotide identities among strains.

Based on PCR-RFLP results, we speculated that these bla_{KPC-2}-carrying plasmids shared a homologous backbone. S1-PFGE and southern blot results revealed that these bla_{KPC-2} positive CRKP isolates contained one or two large plasmids, and the bla_{KPC-2} was located on the smaller one of ~120 kb (Figure 4). Since the PCR-RFLP results of the bla_{KPC-2} downstream sequences of KP29 were different from those of other samples, KP21, KP29 and KP65 were selected for high-throughput sequencing to further compare the homologies among the three bla_{KPC-2} bearing plasmids. The annotation showed that bla_{KPC-2} was located on a *fosA3*-harboring plasmid with IncR-IncF33:A-:B- multireplicon. Then IncR FW/RV, IncFII FW/RV and *fosA3* specific primers were used to characterize replicons and fosfomycin resistance gene among bla_{KPC-2}-carrying plasmids. Results showed that all test samples produced positive bands for IncR and IncFII replicons. However, two isolates were negative for *fosA3* gene detection (data not show). The nucleotide sequence identity of two bla_{KPC-2}-carrying plasmids (KP29 plasmid 2 and KP21 plasmid 1) was 99.99%. The two plasmids differed in their DNA sequences by one insertion region and two inversion regions (Figure 2). High sequence similarities were also observed in the comparison of KP21 plasmid 1 and KP65 plasmid 1 (Figure 2). The KP21 plasmid 1 matched with several multireplicon plasmids with high gene arrangement similarity and gene nucleotide identity. By reviewing the information of their host bacteria (scientific names, collection locations and dates), it noticed that almost of them were *K. pneumoniae* collected in China and several strains were isolated within the same year or earlier than this

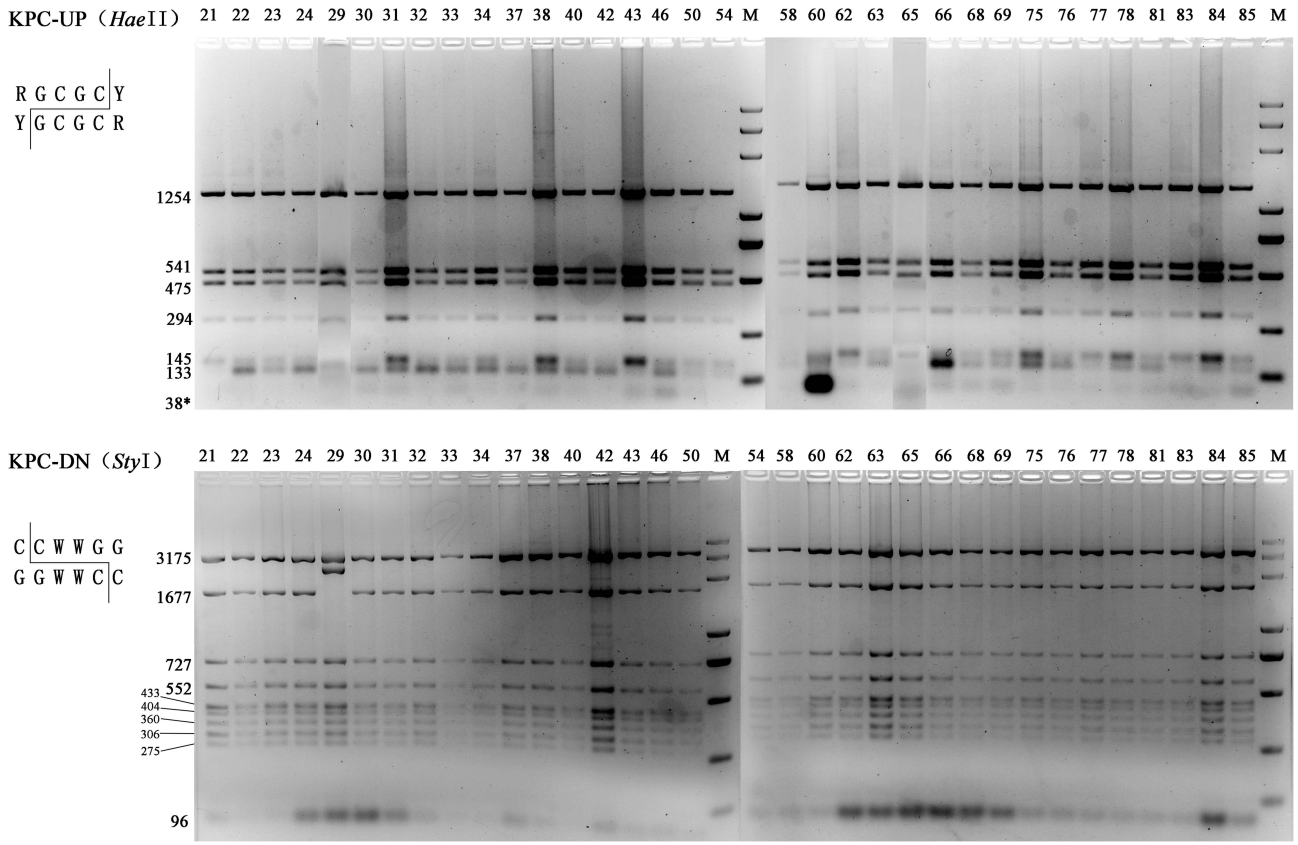

Figure 3 PCR-RFLP profiles of genetic background of bla_{KPC-2} Restriction endonucleases used to digest the PCR products of bla_{KPC-2} franking fragments are listed in the parentheses. The predicted sizes of digested fragments are marked beside the bands. Invisible predicted bands were marked with asterisks. M, DL5000 DNA marker (TSINGKE, China).

study, such as pXHKP6-1 (CP066888.1, 2010.09), pXHKP53-1 (CP066892.1, 2012.06), p1068-KPC (MF168402.1, 2012),[54] pKP1034 (KP893385.1, 2010.05).[19] These results suggest that this *fosA3* and bla_{KPC-2} co-carrying IncR-F33: A–: B– plasmid may have been circulating in China since 2010.

Copy Number Analyses of bla_{NDM-1} and bla_{KPC-2}

The coding sequences of bla_{NDM-1} and bla_{KPC-2} were respectively used as genetic markers to analysis their gene distributions in each Nanopore sequencing dataset. Multiple insertion events were discovered in KP67 plasmid 1 (replicon: IncHI2A) and KP72 plasmid 4 (replicon: IncFII(Yp)), resulting in the bla_{NDM-1} gene duplicated into one to five copies in the form of tandem repeats (Table 2). Results showed that the maximum number of bla_{NDM-1} HSPs found in a query read was up to 5 for KP72, while that was 4 for KP67. However, only one read with 2 HSPs of bla_{NDM-1} was detected in KP82. Similar result was noticed for bla_{KPC-2} gene in KP29. For KP21 and KP65, no more than one bla_{KPC-2} HSPs was found among their sequencing reads. Genetic analysis of the repeat unit of KP67 revealed a 10,844 bp length fragment composed of four elements, i e IS*91*-like insertion element (IS*91*-*sul1*-*qacE*), rifampicin and chloramphenicol resistance element (*arr*-*3*-*catB3*), carbapenem resistance core (ΔIS*aba125*-bla_{NDM-1}-*ble*-*prai*-Δ*dsbD*) and another IS*91*-like insertion element repeat, as shown in Figure 5. For KP72, the repeat unit was 23,853 bp length composed of IS*91*-like insertion element (ΔTn*3*-Δ*intI1*-*aadA1*-*qacE*-*sul1*-IS*91*), bla_{NDM-1} resistance core (*aphA*-*6*-IS*aba125*-bla_{NDM-1}-*prai*-*dsbD*-*cutA*-*groES*-Δ*groEL*) and another IS*91*-like insertion element repeat, as shown in Figure 6. The nucleic acid sequences of two IS*91*-like genes were identical and all belonged to ISCR1 family transposase. Considering, some multiple HSPs may reflect the gene arrangement in a part of the corresponding plasmid due to the limitation of read length; we further inspected the integrity of gene cassette in each repeat, manually. Results showed that KP67 contained one to three intact repeat units (Table 3). A variety of incomplete structures were also found and some of them contained

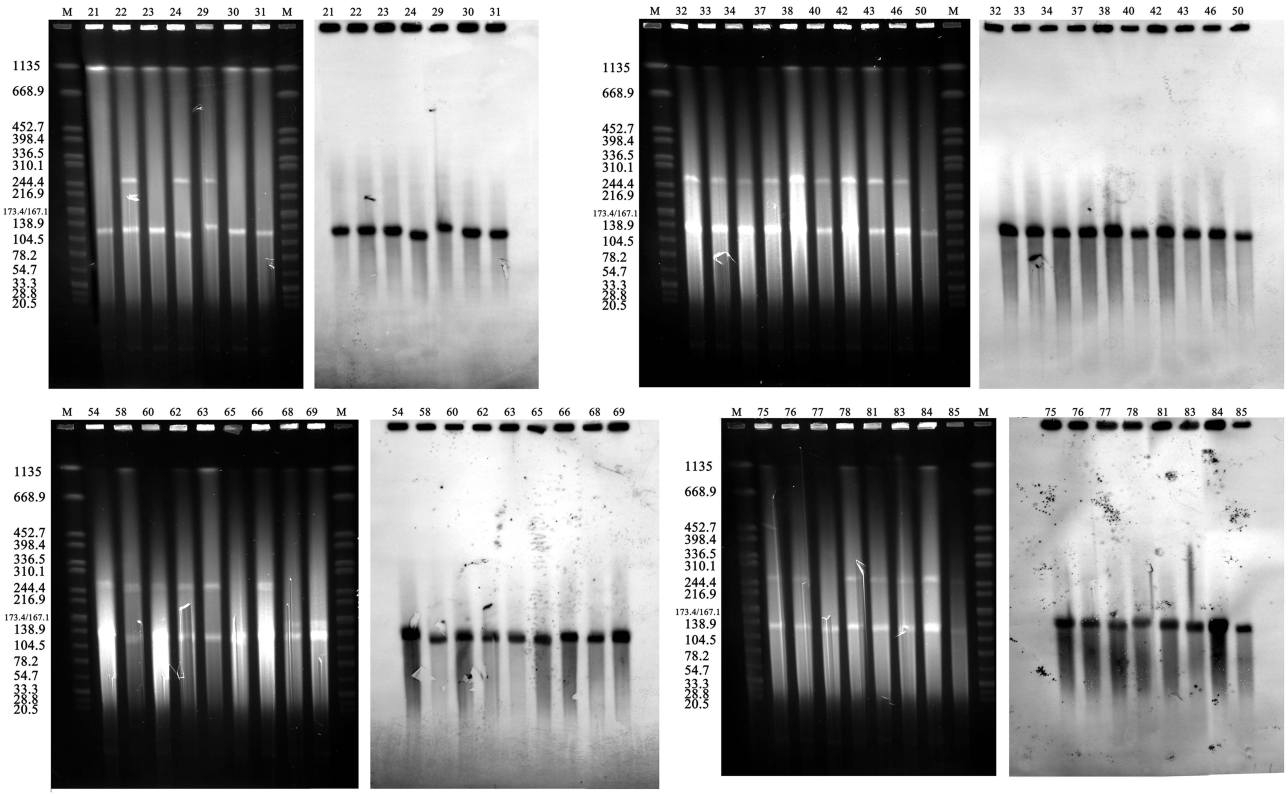

Figure 4 S1-PFGE pattern for ST11 isolates and Southern blot analysis of bla_{KPC-2} genes PFGE result of S1 digested plasmid DNA of ST11 isolates and the corresponding Southern hybridization using the bla_{KPC-2} specific probe. M, *Salmonella* serotype *Braenderup* strain H9812 was digested by *XbaI*.

more than three repeats. For KP72, only one intact repeat unit was detected and the remnant reads revealed one to six repeats (Table 3). Notably, we also detected bla_{NDM-1} repeat units of other genetic patterns, such as repeats with mobile elements inserted occasionally, or repeats translocating into chromosome or other plasmids. Together, these findings supported that the highly active IS*91*-like transposons mediated the tandem repeat of bla_{NDM-1} in this study.

High-Throughput Sequencing and Comparative Analysis

Overall, bla_{NDM-1} gene located on the resistance plasmids of 318,781 bp, 113,479 bp and 54,035 bp for KP67, KP72 and KP82, respectively. The gene organization of the repeat cassette identified from P67 plasmid 1 had been reported in two published plasmids, pTMTA97342 (*Phytobacter diazotrophicus*, AP025336.1) and pEC8-NDM-1 (*Escherichia coli*, CP060954.1, 2016.07), with 100% coverage and 99% identity (pEC8-NDM-1 plasmid was showed in Figure 5).

Table 2 Copy Number Analyses of bla_{NDM-1} and bla_{KPC-2} Genes

Genotype	Isolates	Length of Resistance Plasmid (bp)	Total Number Of Reads In Raw Data	Total Filtered HSPs	Number of Reads with Different HSPs of bla_{NDM-1} or bla_{KPC-2}				
					1	2	3	4	5
					HSP	HSPs	HSPs	HSPs	HSPs
bla_{NDM-1}	KP67	318,781	128,687	241	171	62	7	1	0
	KP72	113,479	200,432	2271	1880	319	58	12	2
	KP82	54,035	168,685	242	241	1	0	0	0
bla_{KPC-2}	KP21	129,998	239,076	207	207	0	0	0	0
	KP29	138,059	117,309	151	149	2	0	0	0
	KP65	126,958	116,921	122	122	0	0	0	0

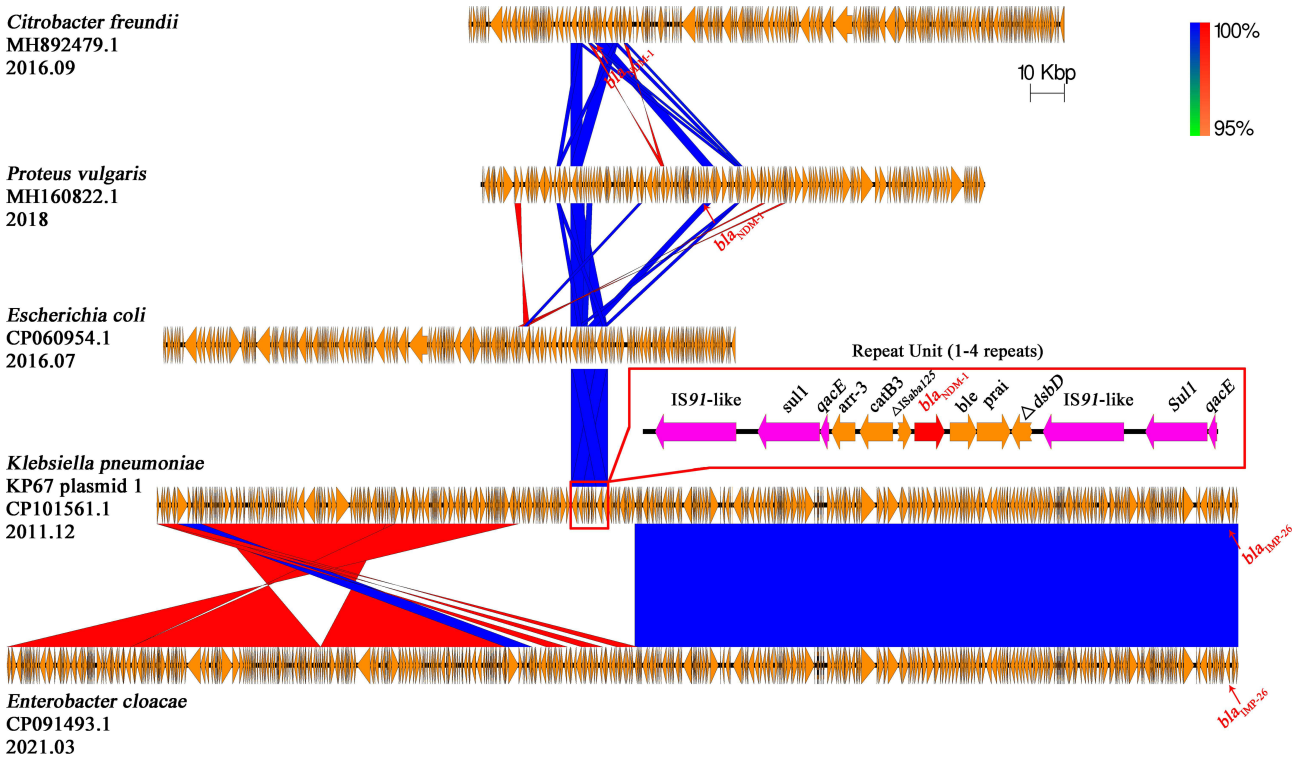

Figure 5 Comparison of the bla_{NDM-1} carrying KP67 plasmid 1 with other related plasmids The region spanning the bla_{NDM-1} on the KP67 plasmid 1 is detailed in the red box, and the repetitive elements are colored with purple. Other genes are depicted as arrows according to their direction of transcription and are shown in yellow. The bla_{NDM-1} and bla_{IMP-26} genes are emphasized by red arrows. Blue and red shadings indicate nucleotide identities among strains.

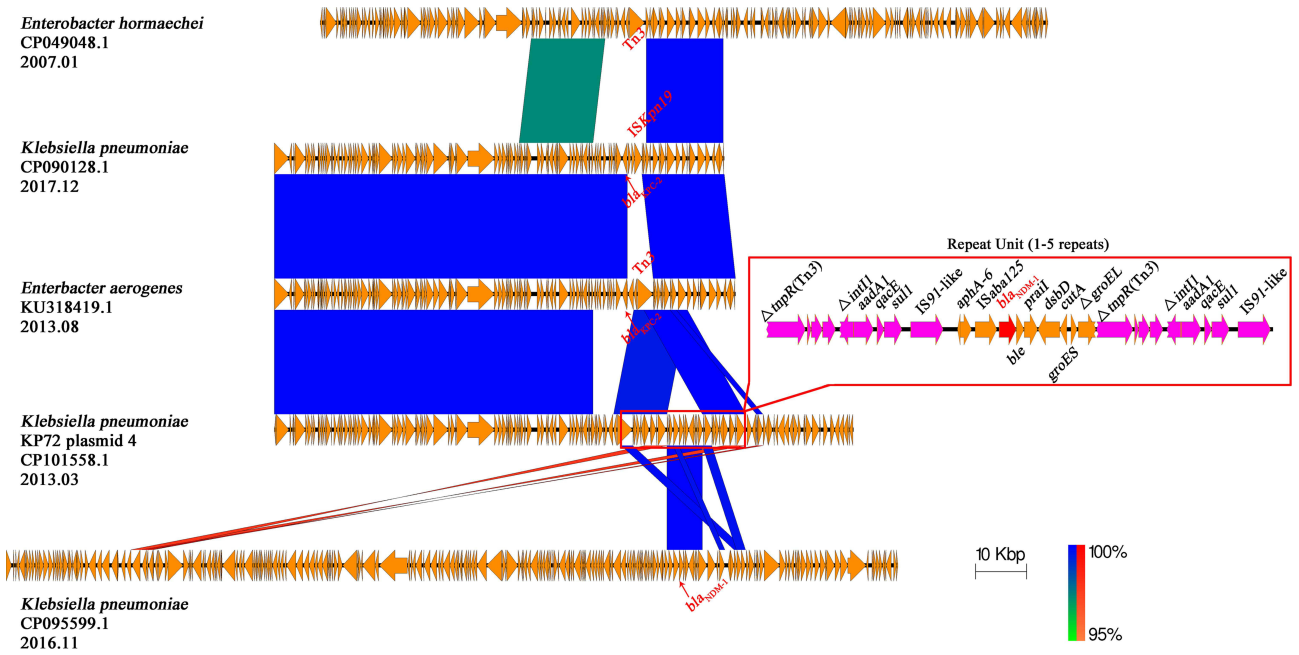

Figure 6 Comparison of the bla_{NDM-1} carrying KP72 plasmid 4 with other related plasmids The region spanning the bla_{NDM-1} on the KP72 plasmid 4 is detailed in the red box, and the repetitive elements are colored with purple. Other genes are depicted as arrows according to their direction of transcription and are shown in yellow. The bla_{NDM-1} and bla_{KPC-2} genes are emphasized by red arrows. Blue and red shadings indicate nucleotide identities among strains.

Table 3 Polymorphism Analyses of bla_{NDM-1} Genetic Background in KP67 and KP72

Isolates	Structure	Genetic Characteristic of bla_{NDM-1} Repeat Unit in HSPs Matching Reads	Number of Reads
KP67	Intact	Us+Rep^{67}+Ds	10
		Us+Rep^{67}+Rep^{67}+Ds	41
		Us+Rep^{67}+Rep^{67}+Rep^{67}+Ds	4
	Incomplete	Us+Rep^{67}	2
		Rep^{67}+Ds	4
		-Rep^{67}+Rep^{67}-	6
		Us+Rep^{67}+Rep^{67}	18
		-Rep^{67}+Rep^{67}+Ds	43
		-Rep^{67}+Rep^{67}+Rep^{67}-	9
		Us+Rep^{67}+Rep^{67}+Rep^{67}	4
		-Rep^{67}+Rep^{67}+Rep^{67}+Ds	12
		-Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}-	1
		-Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}+Ds	3
		-Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}-	1
		-Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}+Rep^{67}+Ds	1
		Other pattern	8
KP72	Intact	Us+Rep^{72}+Ds	1
	Incomplete	-Rep^{72}-	36
		Us+Rep^{72}-	43
		-Rep^{72}+Ds	6
		-Rep^{72}+Rep^{72}-	288
		Us+Rep^{72}+Rep^{72}-	33
		-Rep^{72}+Rep^{72}+Ds	47
		-Rep^{72}+Rep^{72}+Rep^{72}-	165
		Us+Rep^{72}+Rep^{72}+Rep^{72}-	18
		-Rep^{72}+Rep^{72}+Rep^{72}+Ds	11
		-Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}-	48
		Us+Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}-	2
		-Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Ds	5
		-Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}-	14
		-Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Ds	2
		-Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}+Rep^{72}-	2
		Other pattern	8

Notes: Rep=IS91+bla_{NDM-1}+IS91, Rep+Rep=IS91+bla_{NDM-1}+IS91+bla_{NDM-1}+IS91, ……. and so on. Us/Ds means the upstream/downstream fragments adjacent to the repeat unit. "–" indicates that the 5' or 3'end of a Rep is incomplete.

Interestingly, it is unusual to found a resistance element (*Arr-3-CatB3*) adjacent to the bla_{NDM-1}. To the best of our knowledge, we reported the earliest collection of strain with this trait. The three elements of the repeat unit in KP67 plasmid 1 were found to be scattered in some other plasmids, such as pNDM-2262 (*Citrobacter freundii*, MH892479.1, 2016.09) and pICEPvuBC22 (Proteus vulgaris, MH160822.1, 2018). The backbone of KP67 plasmid 1 shared a large region with high similarity to a plasmid (CP091493.1) in a strain of *Enterobacter cloacae* collected in 2021 (Figure 5). However, the bla_{NDM-1} coding sequence of the *Enterobacter cloacae* plasmid was absent. Interestingly, the bla_{IMP-26} coding sequence was found at the same locus in the two plasmids, both of which were identified as IncHI2A replicons, indicating a common origin.

Two plasmids were highly similar to KP72 plasmid 4 in gene content and nucleotide sequence, including pKP46_2_KPC (*Klebsiella pneumoniae*, CP090128.1, 2017.12) and pEA49-KPC (*Enterbacter aerogenes*, KU318419.1, 2013.08). The major differences between the above two plasmids, referring to the adjacent genes in the upstream of bla_{KPC-2}, are IS*Kpn19* and Tn*3* insertion element, respectively (Figure 6). However, bla_{KPC-2} and relevant

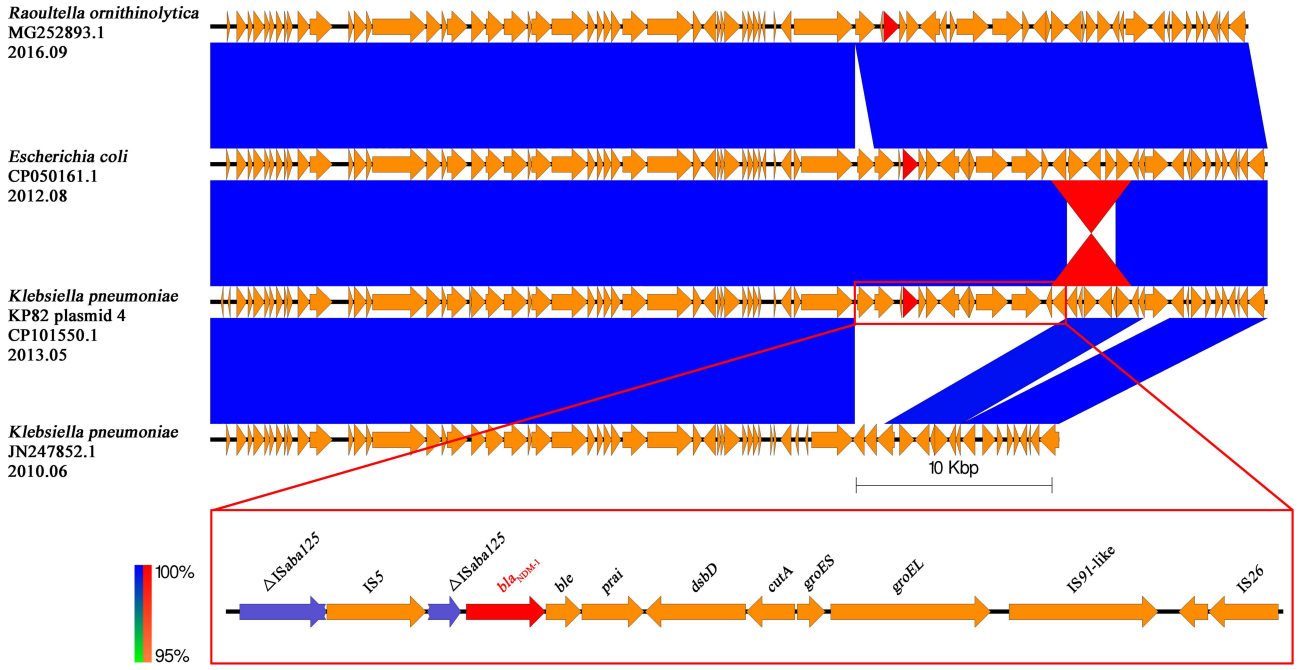

Figure 7 Comparison of the bla_{NDM-1} carrying KP82 plasmid 4 with other related plasmids The region spanning the bla_{NDM-1} on the KP82 plasmid 4 is detailed in the red box. The bla_{NDM-1} genes are emphasized in red. Other genes are depicted as arrows according to their direction of transcription and are shown in yellow. Blue and red shadings indicate nucleotide identities among strains.

upstream and downstream contents were absent in KP72 plasmid 4 which was introduced by a bla_{NDM-1} gene cluster instead. The bla_{NDM-1} gene in KP72 plasmid 4 formed a combination with a neighbored intact IS*aba125*, which was considered to originate from *Acinetobacter baumannii* according to previous studies.[55] This bla_{NDM-1} resistance core was usually found in many bla_{NDM-1}-carrying plasmids, such as pdm186b (CP095599.1, 2016.11), but the entire repeat unit has not been reported yet.

For KP82 plasmid 4 (replicon: IncX3), we identified an IS*Aba125* inserted by an IS*5* in the upstream of the bla_{NDM-1} gene (Figure 7). Besides, another IS91 family transposase, distinct from the former identified, was also found at the downstream of the bla_{NDM-1} gene cluster. The IS*91* family transposase was adjacent to an IS*26* element, an active transposon implicating resistance gene transposition and intermolecular replication.[56] This bla_{NDM-1} genetic background were generally found in many carbapenem-resistant Enterobacteriaceae plasmids, such as pRor-30818cz (*Raoultella ornithinolytica*, MG252893.1, 2016.09)[57] and pNDM-1_IncX3 (*Escherichia coli*, CP050161.1, 2012.08)[2] and always carried on an IncX3 plasmid which spread carbapenemase genes worldwide.[58]

Discussion

Carbapenem-resistant *K. pneumoniae* (CRKP) has emerged as a major source of antibiotic-resistant bacteria of global concern. Recently, the plasmid-mediated mobile tigecycline resistance gene *tet*(A) and its variants have been reported to decrease the tigecycline susceptibility in Enterobacteriaceae strains.[59,60] In our study, antimicrobial susceptibility test demonstrated highly resistant to common antibiotics other than tigecycline and the *tet*(A) gene was not found in our whole genome sequencing data. Therefore, tigecycline remains an important treatment option for CRKP infection. However, it is worth noting that under selective pressure, the coexistence of plasmid carrying bla_{KPC-2} and *tet*(A) gene in CRKP strains can develop a high-level tigecycline resistance.[61] We should continuously highlight the importance of monitoring the emergence of tigecycline-resistant in CRKP.

KPC was first reported in 2001 in the USA from a *K. pneumoniae* strain isolated in 1996.[12] In China, the first KPC-producing *K. pneumoniae* was isolated in Zhejiang Province in 2004.[13] Subsequently, some institutional outbreaks of this taxon were reported in central and eastern China with the ST11 type as the dominant clone.[16,28,33,62] In the present study, 34 out of 41 CRKP isolates were identified as ST11 type suggesting a rapid spread of bla_{KPC-2}-carrying *K. pneumoniae* in

Yunnan since 2011. Moreover, the 34 carriers are genetically closely related based on the results of bla_{KPC-2} genetic backgrounds analysis, southern blotting, plasmid replicon detection and *fosA3* gene detection. In general, these data suggested that the prevalence of CRKP in Yunnan may be caused by the clonal spread of ST11 strains associated with *fosA3* positive IncR-IncF33:A-:B- like drug-resistant plasmids. Our results were consistent with the prevalence of IncR-IncF33:A-:B- plasmid among CRKP in China.[18,19] However, sight but obvious genetic divergences were detectable in the PFGE pattern among these ST11 strains and in the schematic representation of the three representative IncR-IncF33:A-:B- plasmids. More than that, the majority of these strains were collected within a six-month period (February to July in 2013), with the earliest samples dating back to December 2011. The above findings suggested that these ST11 isolates may be prevalent in our hospital earlier than our primary sampling period and could have developed genetic mutations after prolonged dissemination.

The bla_{KPC-2} genetic background described here was structurally identical to that in many previously reported strains which dated back to 2010 and sustained transmission ever since. Interestingly, those strains were mostly originated in China and seemed to be associated with *K. pneumoniae* ST11 type.[4,19,54,63–69] These data implied that plasmids carrying this genetic background have been confined to *K. pneumoniae* ST11 so far and circulated since 2010 in mainland China. This resistant genetic background usually integrated into a *fosA3*-positive IncR-IncFII plasmid which was composed of three genetically and physically distinct modules: a pHN7A8-derived module which contains fosfomycin resistance gene *fosA3* and IncFII replicon, a pKPC-LK30-derived core module which harbors bla_{KPC-2} gene and IncR replicon, and another 10 kb module.[19] Comparative analysis showed that the core module tested in this study was significantly different from that of pKP1034, but more similar to those of p5-1 (isolated from Kunming) and pWCHKP020039 (collected from Chengdu, which is close to Kunming). These results indicated that the origin of plasmids with this core module in Kunming has a different evolutionary history from that of pKPC-LK30 and may have been widely spread in southwest China.

Interestingly, investigators reported that the *fosA3*-positive IncR-IncFII like plasmid was unsuccessfully transferred to *E. coli* J53Azi by conjugation experiments.[19,54,63] Despite their limitation in conjugation transfer, nontransmissible IncR plasmids can serve as a reservoir by fusion with other types of resistance plasmids or directly cotransfer with the helper self-transferable IncN3 plasmid, thereby enhancing its ability to transmit to other *K. pneumoniae* lineage or Enterobacteriaceae species.[70,71] More importantly, recent studies reminded the public that strains with this plasmid became high virulence and multidrug resistances.[61,66,72–74] In this study, the *tra* regions in the three sequenced plasmids were incomplete compared with that in pHN7A8 and the bla_{KPC-2} gene was only detected in ST11, suggesting a limited horizontal transmission of this plasmid.

NDM was first reported in *K. pneumoniae* and *E. coli* in 2009 and subsequently spread worldwide via vertical and horizontal transmission.[21,75] In China, the earliest NDM positive strains can be traced to *Acinetobacter baumannii* isolated in 2009 and then successively identified in other species, including *E. coli* and *K. pneumoniae*.[22,76–78] *Acinetobacter baumannii* has been suggested as an intermediate in the transmission of the bla_{NDM-1} gene from environmental pathogens to Enterobacteriaceae species.[55] Fu et al found that bla_{NDM-1} positive *Acinetobacter* spp. (represented by strain ABC7926, accession number JQ080305) shared a similar genetic structure in which bla_{NDM-1} genetic background made up of IS*Aba125*-bla_{NDM-1}-*ble*-Δ*trpF*-Δ*groES-groEL-InsE*-IS*Aba125*. In our study, fragment deletions were found in the region downstream of the bla_{NDM-1} in the three tested isolates resulting in the missing of the downstream IS*Aba125*. The IS*Aba125* upstream of the bla_{NDM-1} in KP82 was also truncated by IS*5*, while only a small residue of the IS*Aba125* was retained in KP67. The three bla_{NDM} positive isolates belonged to three different ST types, and each had its unique bla_{NDM-1} genetic backgrounds, providing evidences on the complexity and diversity of genetic features associated with the bla_{NDM-1} gene. The comparative analyses showed that the sequence of KP82 plasmid 4 can be found in many other species in China with more than 99% identity and 99% coverage, such as *Escherichia coli* (KX094555.1), *Enterobacter cloacae* (KF976405.1),[79] *Citrobacter freundii* (JX254913.2), *Enterobacter cloacae* (KY296103.1),[80] *Enterobacter hormaechei* (MF344560.1) and *Enterobacter kobei* (CP088232.1). This transmissibility and plasticity implies an alarming potential of KP82-like bla_{NDM-1}-carrying plasmid to spread and diversify among bacterial populations. It is also worth noting that two IS*91*-like elements with identical nucleotide sequence mediated bla_{NDM-1} flanking region duplicated into one to five copies in KP67 and KP72. This rather unusual IS element IS*91*

differs from the insertion sequence paradigm in that they lack conventional terminal inverted repeats (IRs) and can mobilize adjacent DNA sequences via a process called rolling circle replication.[8,81] Recently, multiple copies of bla_{NDM-1}-encoding plasmids have been reported with increasing frequency.[25–27,82–84] In those cases, diverse mechanisms mediated the generation of tandem repeat of bla_{NDM-1} gene by different IS elements, such as Tn*3*-like,[27] IS*aba1*25 [83] and IS*CR1*.[25,82] Importantly, there are different conclusions on whether repeat sequences affect the resistance phenotype in those strains.[25,27,82] Our findings emphasized the necessity for monitoring the prevalence of multicopy bla_{NDM-1} gene mediated by IS*91*-like IS element. Further research is warranted to clarify its potential role in the impact of host phenotype.

Conclusions

In summary, the clonal dissemination of CRKP ST11 clone harbors a *fosA3* and bla_{KPC-2} co-carrying IncR-F33: A–: B– plasmid was identified in multiple departments in our hospital, indicating an extensive cross-transmission of CRKP isolates among high-risk departments. In addition, we reported tandem repeat of bla_{NDM-1} gene mediated by the transposition of IS*91* element on two plasmids with different replicons, providing data on the complexity and diversity of genetic features associated with the bla_{NDM-1} gene. These findings help to better understand the spread history of CRKP in southwest China, and will provide reference for relevant studies in other regions or different periods in China.

Abbreviations

CRE, Carbapenems resistant Enterobacteriaceae; CRKP, Carbapenem-Resistant *Klebsiella pneumoniae*; NDM, New Delhi metallo-β-lactamase; UP, upstream; DN, downstream; ESBL, extended-spectrum β-lactamase; MIC, Minimal inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MLST, multilocus sequence typing; STs, sequence types; PFGE, pulsed-field gel electrophoresis; PCR, Polymerase chain reaction; TAIL-PCR, thermal asymmetric interlaced PCR; NCBI, National Center for Biotechnology Information; BLAST, Basic Local Alignment Search Tool; RFLP, Restriction fragment length polymorphism; ICU, intensive care unit; SICU, surgical intensive care unit; EICU, Emergency Intensive Care Unit; AMC, amoxicillin-clavulanate; PIP/T, piperacillin/tazobactam; CXM, cefuroxime axetil; CXM-S, cefuroxime sodium; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; SCF, cefoperazone/sulbactam; FEP, cefepime; ETP, ertapenem; IMP, imipenem; AMK, amikacin; LVX, levofloxacin; TGC, tigecycline.

Ethical Approval

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Kunming Medical University. Patients participating in the study were anonymous, as a result of the retrospective study, so informed consent was not obtained.

Authors and Contributors

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest.

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