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ORIGINAL RESEARCH

Characterisation of a Novel Hybrid IncFII$_{pHN7A8}$: IncR:IncN Plasmid Co-Harboring *bla*$_{NDM-5}$ and *bla*$_{KPC-2}$ from a Clinical ST11 Carbapenem-Resistant *Klebsiella pneumoniae* Strain

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Purpose: We aimed to characterize a novel *bla*$_{NDM-5}$ and *bla*$_{KPC-2}$ co-carrying hybrid plasmid from a clinical carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strain.

Methods: Antimicrobial susceptibility was determined by the broth microdilution method. Plasmid size and localization were estimated using S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting. Plasmid transfer ability was evaluated by conjugation experiments. Whole genome sequencing (WGS) was performed using Illumina NovaSeq6000 and Oxford Nanopore MinION platforms. Genomic characteristics were analyzed using bioinformatics methods.

Results: Strain ZY27320 was a multidrug-resistant (MDR) clinical ST11 *K. pneumoniae* strain that confers high-level resistance to carbapenems (meropenem, MIC 128 mg/L; imipenem, MIC 64 mg/L) and ceftazidime/avibactam (MIC >128/4 mg/L). Both S1-PFGE–Southern blotting and whole genome sequencing revealed that the carbapenemase genes *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$ were carried by the same IncFII$_{pHN7A8}$:IncR:IncN hybrid plasmid (pKPC2_NDM5). Conjugation experiments indicated that pKPC2_NDM5 was a non-conjugative plasmid.

Conclusion: This is the first report of a hybrid plasmid carrying both carbapenemase genes *bla*$_{NDM-5}$ and *bla*$_{KPC-2}$ in a clinical *K. pneumoniae* ST11 isolate that confers resistance to both ceftazidime/avibactam and carbapenems, thereby presenting a serious threat to treatment in clinical practice.

Keywords: *bla*$_{NDM-5}$, *bla*$_{KPC-2}$, *Klebsiella pneumoniae*, hybrid plasmid, IS*26*

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a global threat to clinical practice because of its ability to cause severe infections with high mortality rates.[1,2] Carbapenemase production is the dominant cause of carbapenem resistance in carbapenem-resistant *Enterobacteriaceae*, with KPC and NDM being the most common. KPC-2 carbapenemase, encoded by

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bla$_{KPC-2}$ is predominant in *Klebsiella pneumoniae* and can be potently inhibited by new β-lactamase inhibitors such as avibactam, vaborbactam, and relebactam. Thus, ceftazidime/avibactam is a valuable treatment option to combat CRKP-producing KPC carbapenemase.[3] However, metallo-β-lactamase NDM encoded by *bla*$_{NDM,}$ which is most common in *Escherichia coli* in China,[4,5] cannot be overcome using clinically available β-lactamase inhibitors, including ceftazidime/avibactam.[6] CRKP, which produces both KPC and NDM, confers resistance to nearly all β-lactams and β-lactamase inhibitors, and presents a significant challenge in clinical treatment owing to extremely limited options. In recent years, the co-production of KPC and NDM (mainly KPC-2 and NDM-1) has become a severe threat to public health, with increasing reports worldwide,[7–12] and has been documented in other *Enterobacteriaceae*.[13–15] A nationwide survey in China revealed an increased prevalence of CRKP co-producing KPC-2 and NDM-1 in recent years.[16] According to previous reports, *bla*$_{KPC}$ and *bla*$_{NDM}$ are usually located on separate plasmids that can be transferred to new recipient bacteria through conjugation. There are few reports on plasmids co-carrying *bla*$_{KPC}$ and *bla*$_{NDM}$. Recently, an IncR plasmid known as pCF2075-1 carrying both *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ was identified in *Citrobacter freundii*.[17] Additionally, an IncHI5 plasmid named pK254-KPC_NDM co-carrying *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ was discovered in *Klebsiella michiganensis*.[18] Furthermore, a conjugative hybrid plasmid containing *bla*$_{KPC-2}$ and *bla*$_{IMP-4}$ that co-expressed serine- and metallo-carbapenemases was documented in a clinical *Klebsiella quasipneumoniae* isolate.[19] Here, we describe the co-occurrence of NDM-5 as the most common metallo-carbapenemase and KPC-2 as the most prevalent serine β-lactamase on a chimeric plasmid from a clinical *K. pneumoniae* ST11 isolate.

Materials and Methods

Strain Identification and Antimicrobial Susceptibility Testing

Strain ZY27320 was obtained from an abdominal drainage sample of a man aged 61 with severe abdominal infection who was hospitalized in a tertiary hospital in February 2021. The isolate was identified as *K. pneumoniae* using a Bruker Microflex MALDI-TOF (Bruker, Germany).

The minimum inhibitory concentrations (MICs) of colistin, tigecycline, ceftazidime/avibactam, piperacillin/tazobactam, ceftolozane/tazobactam, imipenem/relebactam, meropenem/vaborbactam, meropenem, imipenem, ertapenem, levofloxacin, ciprofloxacin, amikacin, fosfomycin, and aztreonam were determined using the microbroth dilution method with *E. coli* ATCC 25922 as a quality control. The results were interpreted based on the MIC breakpoints of the Clinical and Laboratory Standards Institute (CLSI M100-S32, 2022). The MIC breakpoint of tigecycline was based on the standards of the US FDA and National Medical Products Administration (NMPA) in China.

S1 -PFGE and Southern Blot Hybridization

To clarify the plasmid location of *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$, we performed S1-PFGE and Southern hybridization as described previously[19] using digoxigenin-labelled *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$-specific probes with an NBT/BCIP color detection kit (Roche, Mannheim, Germany).

Conjugation Assay

To assess plasmid transferability, we performed conjugation experiments by filter mating and broth mating using different recipient bacteria (*E. coli* J53, *E. coli* C600, and induced rifampin-resistant *K. pneumoniae* ATCC13883). *E. coli* J53 transconjugants were selected on MH agar plates containing 100 mg/L of sodium azide and 4 mg/L of meropenem. *E. coli* EC600 transconjugants were selected on MH agar plates containing 600 mg/L of rifampicin and 4 mg/L of meropenem. *K. pneumoniae* ATCC13883 transconjugants were selected on MH agar plates containing 300 mg/L of rifampicin and 4 mg/L of meropenem.

Whole Genome Sequencing and Analysis

The whole genome of ZY27320 was sequenced using the Illumina NovaSeq6000 (Illumina, San Diego, CA, USA) and Oxford Nanopore MinION (Nanopore, Oxford, UK) platforms (Digital-Micro Biotech Co., Ltd, Hangzhou, China). The hybrid assembly of Illumina and Nanopore reads was performed using Unicycler v0.4.8.[20] Complete plasmid sequences were annotated using the RAST server.[21] Multilocus sequence typing (MLST), resistance genes, virulence genes, and plasmid replicons were performed using MLST 2.0, ResFinder 4.1, VirulenceFinder 2.0, and PlasmidFinder 2.1 web

databases (http://www.genomicepidemiology.org/services), respectively. Insertion sequence elements (ISs) were identified using the ISFinder.[22] The self-transmissible mobile genetic elements (MGE) and origin of the transfer site were identified using the OriTfinder web server.[23] Sequence alignment and visualization were performed using Proksee web server v1.1.4 and Easyfig v2.2.3.[24]

Accession Numbers

The complete chromosome and plasmid sequences of *Klebsiella pneumoniae* ZY27320 were deposited in GenBank under BioProject No. PRJNA975440; accession numbers CP128564-CP128565.

Results

Strain ZY27320 was resistant to all β-lactamase inhibitors (ceftazidime/avibactam, piperacillin/tazobactam, ceftolozane/tazobactam, imipenem/relebactam, and meropenem/vaborbactam), carbapenems (ertapenem, meropenem, and imipenem), quinolones (ciprofloxacin and levofloxacin), aminoglycosides (amikacin), fosfomycin, and aztreonam; it was susceptible only to tigecycline and colistin (Supplementary Table 1).

S1-PFGE detected one plasmid approximately 167 kb in size in ZY27320 cells. Southern blotting with KPC and NDM probes revealed bands at the same location, suggesting that $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$ were located on the same plasmid (Supplementary Figure 1).

To better understand the genetic environment of $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$, the complete genome of ZY27320 was successfully sequenced by Illumina and Nanopore sequencing. The hybrid assembly using Unicycler revealed a circular chromosome of 5,578,590 bp (accession no. CP128564) with a GC content of 57.3%, and one circular plasmid of 167,277bp, designated pKPC2-NDM5 (accession no. CP128565) with a GC content of 53.0%. MLST typing of the assembled genome showed that ZY27320 belonged to the ST11. The chromosome of ZY27320 carries three antimicrobial resistance (AMR) genes (conferring resistance to fosfomycin (*fosA*), β-lactams ($bla_{\text{SHV-182}}$), and aminoglycosides (*aadA2*)) and three virulence factors, including *fyuA* encoding a siderophore receptor, *iutA* encoding a ferric aerobactin receptor, and an *irp2* gene.

pKPC2_NDM5 is a multiple drug resistance (MDR) plasmid that carries multiple AMR genes conferring resistance to β-lactams ($bla_{\text{KPC-2}}$, $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-65}}$, and $bla_{\text{TEM-1B}}$), fosfomycin (*fosA3*), streptomycin (*aadA2*), sulfamethoxazole (*sul1*), aminoglycosides (*rmtB*), and trimethoprim (*dfrA12*). In addition, plasmid pKPC2_NDM5 showed no sequence similarity to the virulence plasmid pLVPK[25] and contained no virulence factors. We compared the sequence of pKPC2_NDM5 with the plasmid sequences co-carrying $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ registered in GenBank.[17,18] BLASTN results showed that pKPC2_NDM5 exhibited low coverage (20%) with an IncR plasmid pCF2075-1 (accession no. CP119166) from an *Citrobacter freundii* strain, and shared 24% coverage with an IncHI5 plasmid pK254-KPC_NDM (accession no. OM938013) from an *Klebsiella michiganensis* strain. Three replicons, $\text{IncFII}_{\text{pHN7A8}}$, IncR, and IncN, were identified in pKPC2_NDM5 by using PlasmidFinder. $\text{IncFII}_{\text{pHN7A8}}$:IncR bla_{KPC}-bearing plasmids are widely disseminated in *K. pneumoniae* strains from China and are typically associated with ST11 strains.[26] Plasmid pKPC2_NDM5 had high coverage (73–90%) and sequence similarity (99.93– 99.97%) with several $\text{IncFII}_{\text{pHN7A8}}$:IncR:IncN bla_{KPC2}-carrying plasmids of *K. pneumoniae* strain from Zhengzhou City, China, such as pKP18-41-KPC2 (accession no. CP082012) and pKP19-2196-KPC2 (accession no. CP082042) and pKP18-2172-KPC2 (accession no. CP082039) and pKP18-1-KPC2 (accession no. CP082003) and pKPN-hnqyy-kpc (accession no. CP074118).[27] Subsequently, a series of $bla_{\text{NDM-5}}$-carrying plasmids with high similarity (99.53–100%) appeared to carry complementary sequences to form the backbone of pKPC2_NDM5. A $bla_{\text{NDM-5}}$-carrying plasmid pKPN-hnqyy-ndm (accession no. CP074117) exhibited 33% coverage and 100% identity, coincidentally originating from the same NDM-5 and KPC-2 co-producing *K. pneumoniae* isolate K9 as the $bla_{\text{KPC-2}}$-carrying plasmid pKPN-hnqyy-kpc (accession no. CP074118).[27] Interestingly, these two plasmids from a single NDM-5 and KPC-2 coproducing *K. pneumoniae* appeared to integrate into the backbone of pKPC2_NDM5 (Figure 1). Detailed sequence analysis revealed that pKPC2_NDM5 was a cointegrate consisting of four regions: an ~116 kb $\text{IncFII}_{\text{pHN7A8}}$:IncR:IncN plasmid-derived KPC-module (Region-1), an ~ 2.4 kb *fosA* carrying region (Region-2), an ~ 43.5kb $bla_{\text{NDM-5}}$ carrying NDM-module (Region-3), and an ~ 4.6 kb $bla_{\text{TEM-1B}}$ *and rmtB* carrying region (Region-4). The Region-1 was perfectly aligned with the plasmid pKPN-hnqyy-kpc and Region-3 shares 100% sequence identity with that of plasmid pKPN-hnqyy-ndm. All four regions are bracketed by two IS*26* elements (Figure 2). The formation

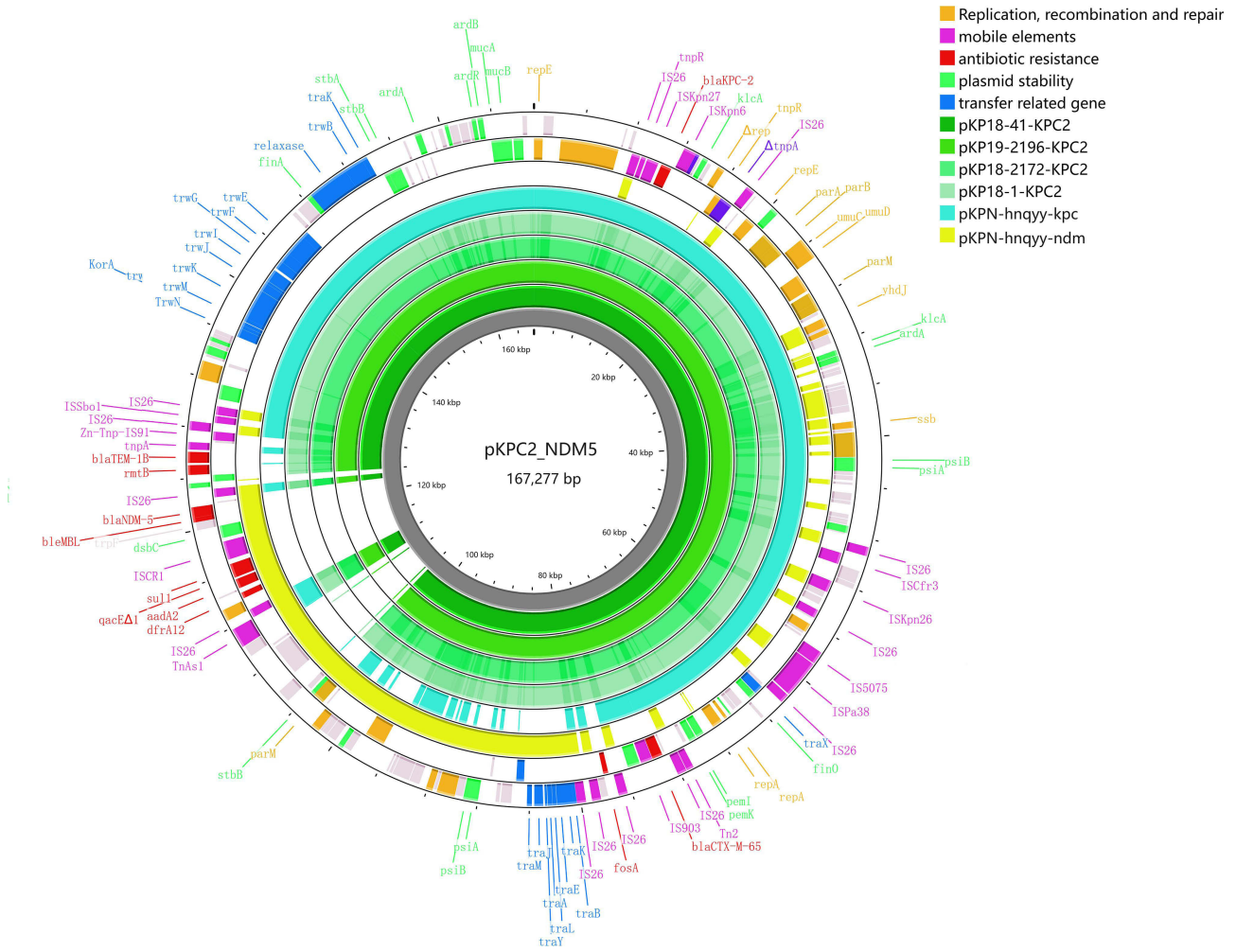

Figure 1 Schematic map of plasmid pKPC2_NDM5 and alignment with other similar plasmids from GenBank. The visualization map was generated by Proksee web server v1.1.4.

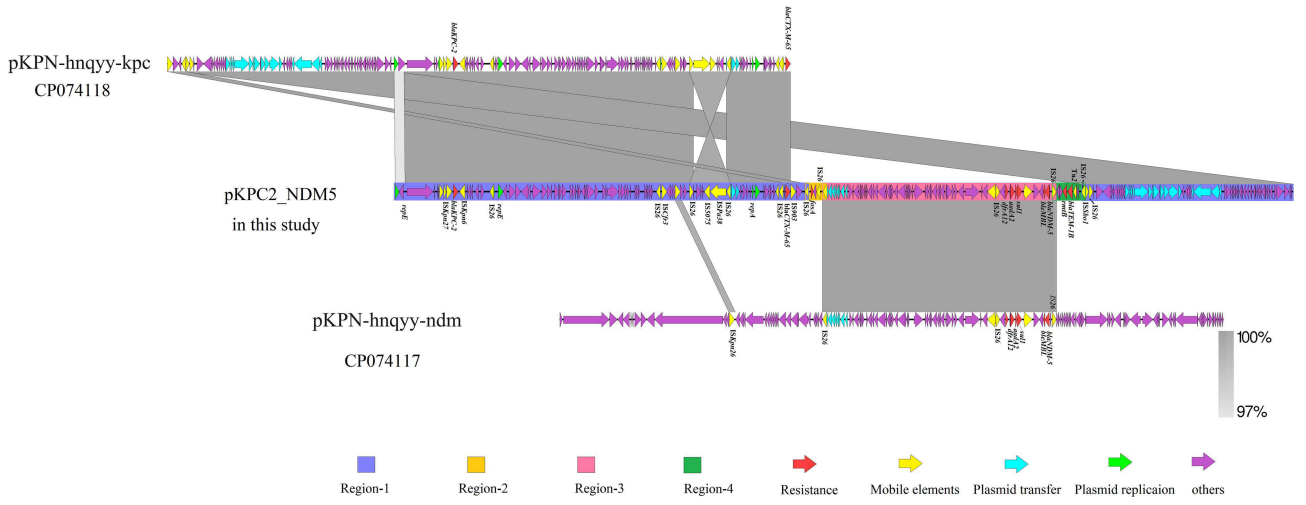

Figure 2 Sequence alignment of plasmid pKPC2_NDM5 with sequences of pKPN-hnqyy-ndm (GenBank accession no. CP074117), and pKPN-hnqyy-kpc (GenBank accession no. CP074118). The visualization map was created by EasyFig software.

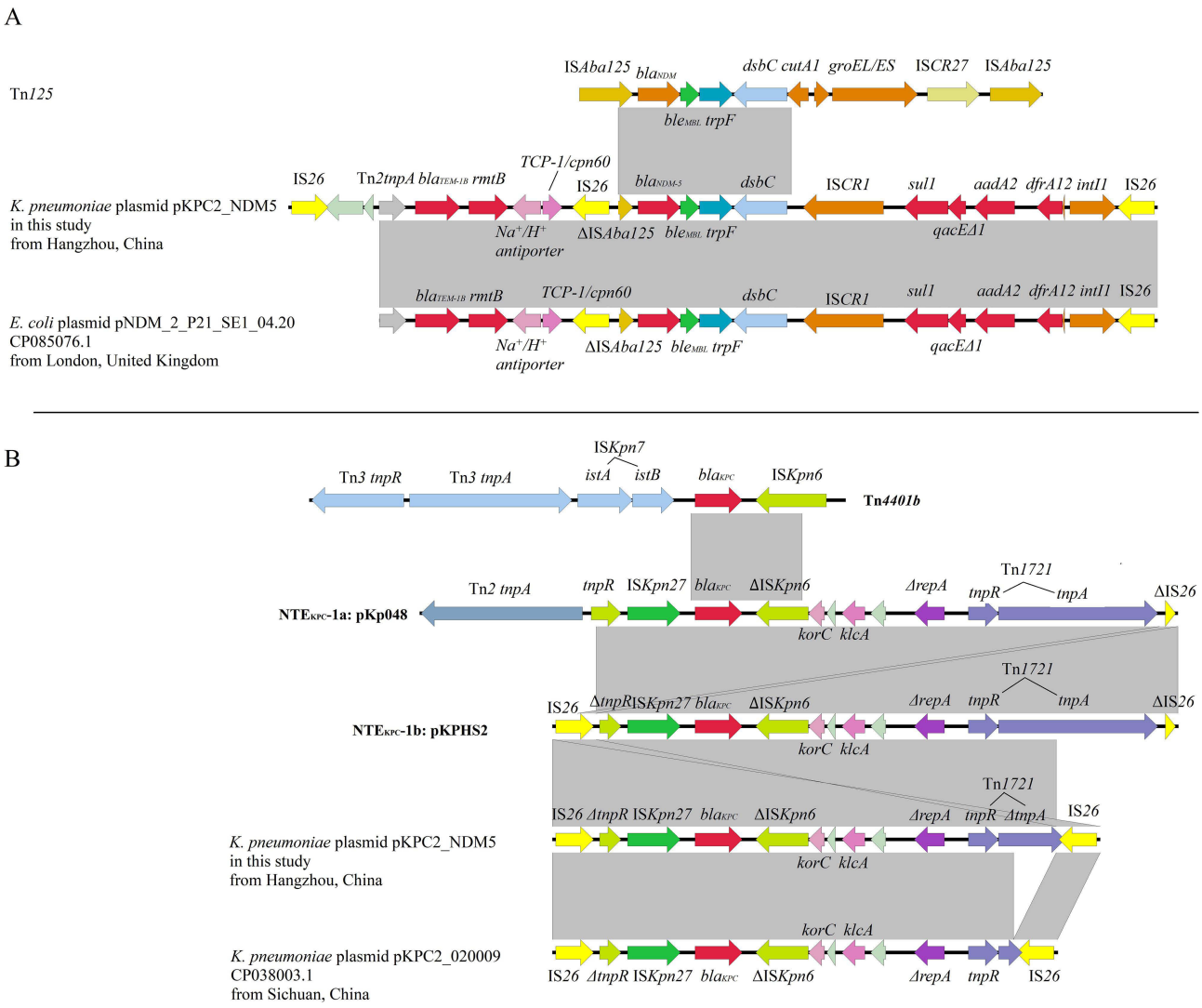

Figure 3 (A) Linear comparison of genetic environment of bla_{NDM-5} of pKPC2_NDM5 and that of pNDM_2_P21_SE1_04.20. (B) Linear comparison of the genetic environment of bla_{KPC-2} among pKP048, pKPHS2, pKPC2_NDM5, and pKPC2_020009. The plasmid comparison figure was generated by EasyFig software. Regions with identical sequences are shown in gray shadow between the different plasmids.

of pKPC2_NDM5 appeared to be a recombination event in which an IS*26*-flanked NDM-fragment, IS*26*-bounded *fosA*-fragment and an IS*26*-bounded bla_{TEM-1B} - *rmtB*-fragment were inserted into an $IncFII_{pHN7A8}$:IncR:IncN KPC-plasmid (plasmid pKPN-hnqyy-kpc) using IS*26* as the insertion site.

We further analyzed the environments of bla_{KPC-2} and bla_{NDM-5} (Figure 3). bla_{KPC-2} is located on a non-Tn*4401* element with the following structures: IS*26, ΔtnpR*, IS*Kpn27, bla*$_{KPC-2}$, and IS*Kpn6-korC-klcA-ΔrepA-tnpR-ΔtnpA*-IS*26*. The gene bla_{KPC} has typically been found in classical Tn*4401* elements,[26] as well as non-Tn*4401* elements (NTE_{KPC}), including NTE_{KPC}-I, NTE_{KPC}-II, and NTE_{KPC}-III.[28,29] The bla_{KPC}-bearing genetic elements in pKPC2-NDM5 were assigned to the NTE_{KPC}-I group because Δbla_{TEM} or Tn*5563*/IS*6100* were not inserted upstream of bla_{KPC-2}.[30] Compared with the bla_{KPC}-carrying fragment with the NTE_{KPC}-Ib structure in pKPHS2 (Accession no. CP003224), a large part of Tn*1721 tnpA* was absent and was replaced by an IS*26* element in the bla_{KPC-2} genetic context of pKPC2_NDM5. In addition, the genetic environment of bla_{KPC-2} in pKPC2_NDM5 had 92% query coverage compared to that of the plasmid pKPC2_020009 (accession no. CP038003) carrying a truncated *tnpA* of Tn*1721*.

The bla_{NDM-5} gene is located in a 16.7-kb multi-resistance module carrying *dfrA12, aadA2, sul1,* bla_{NDM-5}, *rmtB*, and bla_{TEM-1B} flanked by IS*26* elements. The bla_{NDM}-bearing element of pKPC2_NDM5 carries the common feature of ΔIS*Aba125-bla*$_{NDM}$-*ble*$_{MBL}$[6] and has the same genes as classical Tn*125* including *trpF* and *dsbC* downstream of ble_{MBL}.

In contrast to Tn*125*, the bla_{NDM}-carrying element in pKPC2_NDM5 lost several genes, including *cutA1, groES-groEL*, and IS*CR27*, but had one additional IS*26* element upstream of ΔIS*Aba125* and carried an additional AGR gene cluster (IS*CR1-sul1-aadA2-dfrA12-intI1*-IS*26*) downstream of *dsbC*. BLAST analysis revealed that the bla_{NDM}-bearing element in pKPC2_NDM5 was highly similar (99.99% identity and 94% coverage) to that of pNDM_2_P21_SE1_04.20 (accession no. CP085076.1) of one clinical *Escherichia coli* isolate from the United Kingdom (Figure 3) and many other plasmids, such as pM217_FII from Myanmar (not shown).

To understand the transmission capability of pKPC2_NDM5, we performed conjugation assays. We attempted filter mating and broth mating with different recipient bacteria (*K. pneumoniae* ATCC13883, *E. coli* strains EC600 and ECJ53) and tested various donor: recipient ratios. We did not obtain a transconjugant under any conditions. Plasmid extraction and electroporation were both unsuccessful. These results suggest that pKPC2_NDM5 is nontransmissible. To test this hypothesis, we submitted the plasmid sequence to the OriTfinder web server.[23,31] The plasmid pKPC2_NDM5 sequence contained an *oriT* region (153,968–154,068 bp) with a conserved nick region (154,025–154,034 bp), relaxase-encoding gene, type IV coupling protein-encoding gene *trwB*, and two T4SS gene clusters: *traB-traK-traE-traL-traA-traY-traM* (80,324–85,114 bp) and *trwN-trwM-trwK-trwJ-trwI-trwG-trwF-trwE* (135,941–153,401 bp). The conjugative region of the self-transmissible MGE seems to be intact, but pKPC2_NDM5 lacks transfer ability which still presents a puzzle.

Discussion

bla_{KPC-2} is the predominant carbapenemase gene widely spread among *Klebsiella pneumoniae* ST11 and is usually carried by IncFII-like plasmids in China.[32] In our study, the backbone of pKPC2-NDM5 displayed a multi-replicon $IncFII_{pHN7A8}$:IncR:IncN chimera structure, which may have evolved from several recombination events as described in previous study.[27] NDM-5, as a variant of NDM-1, was found to exhibit enhanced carbapenemase activity and hydrolysis ability of ceftazidime/avibactam,[33] and was carried by plasmids with diverse replicon types, including IncX3, IncFII, IncC, IncN, and IncR. Co-harboring bla_{NDM-5} and bla_{KPC-2} on a multidrug-resistant plasmid confers high-level resistance to both carbapenems and ceftazidime/avibactam and therefore becomes a significant challenge in clinical treatment. The plasmids co-carrying bla_{KPC-2} and bla_{NDM-1} were recently reported in *Citrobacter freundii* and *Klebsiella michiganensis*.[17,18] The emergence of this kind of plasmid co-carrying bla_{KPC-2} and bla_{NDM-5} in the most prevalent ST clone of *K. pneumoniae*, poses a significant public health threat and deserves significant attention.

Plasmid pKPC2-NDM5 appeared to be a hybrid plasmid generated by the movement of an IS*26*-associated translatable unit. The high copy number of IS*26* in pKPC2_NDM5 and its presence in each composition module suggest that IS*26* plays an essential role in the mobilization and recombination of AMR genes.[34,35] However, we did not recognize matching pairs of target site duplications (TSD) at the junction of each IS*26*, which are usually considered as the "tracers" of transposition. The lack of flanking TSDs was not uncommon, as reported previously, perhaps because of homologous recombination following IS*26* transposition.[36] Another explanation is that IS*26* transposes through replicative transposition, which can generate IS*26* copies without two-sided TSDs.[34]

pKPC2-NDM5 was non-conjugative, although it carried conjugal modules of the self-transmissible MGE. Interestingly, as the backbone of pKPC2_NDM5, the plasmid pKPN-hnqyy-kpc was proved to have high transfer ability to *E. coli* J53, however, plasmid pKPN-hnqyy-ndm was a non-conjugative plasmid.[27] Previous studies have shown that the expression of conjugative systems is tightly controlled, because the conjugation process is energetically expensive.[37] Although we could not measure the fitness cost of this plasmid, we suspected that the loss of horizontal transfer ability was a manifestation of fitness cost. Furthermore, although there have been many reports on the concurrence of the bla_{NDM}-plasmid and bla_{KPC}-plasmid in the same *K. pneumoniae* isolate,[9,16,38] the same plasmid co-harboring bla_{NDM} and bla_{KPC} in *K. pneumoniae* has not yet been reported. However, with the widespread use of ceftazidime/avibactam and frequent combined with carbapenems in clinical practice, plasmids co-carrying these two critical carbapenemase genes might gradually gain a selection advantage during continuous evolution. Therefore, KPC-NDM-plasmid could become a great challenge in the treatment of CRE infections and is worth strengthening monitoring in the future.

Conclusion

In this study, we characterized a novel hybrid MDR plasmid co-harboring bla_{KPC-2} and bla_{NDM-5} obtained from an ST11 clinical *K. pneumoniae* isolate. This chimeric plasmid is likely a consequence of a recombination event involving the insertion of a bla_{NDM-5} fragment into an $IncFII_{pHN7A8}$:IncR:IncN bla_{KPC2}-carrying plasmid, probably mediated by IS*26* elements. Despite the lack of plasmid self-transmissibility, integration of these two critical carbapenemase genes, bla_{NDM-5} and bla_{KPC-2}, into the same plasmid in *K. pneumoniae* presents great challenges for clinical treatment and requires stringent surveillance.

Ethical Approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine with the research ethics approval code of IIT20230943A. The study was conducted in accordance with the principles of the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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