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RAPID COMMUNICATION

Characterization of a Novel Sequence Type (ST) 6758 *Klebsiella Pneumoniae* and the Role of IncX3 Plasmid in the Transmission of bla_{NDM}

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Purpose: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has emerged as a significant public health threat, particularly as a superbug responsible for nosocomial infections. In this study, we report a novel sequence type 6758 of *K. pneumoniae* harboring the bla_{NDM-1} gene.

Material and Methods: Antimicrobial susceptibility testing was conducted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The complete genome sequence of the strain was determined using the Illumina NovaSeq 6000 platform and long-read MinION sequencer. Genomic features and resistance mechanisms of the strain were further comprehensively analysed using various bioinformatics approaches.

Results: Antimicrobial susceptibility testing revealed that this strain exhibited resistance to multiple antimicrobials, including ceftazidime, ceftriaxone, cefazolin, cefepime, imipenem, meropenem, ampicillin/sulbactam, and sulfamethoxazole/trimethoprim. The genome analysis identified sixteen resistance genes. The bla_{NDM-1} carbapenemase gene is located on a 47,823 bp IncX3-type plasmid (pNDM-CRKP331). A total of 41 *K. pneumoniae* strains carrying similar IncX3-type plasmids were retrieved from the NCBI database, representing 20 sequence types (STs) across 11 countries. The most common resistance gene carried by these IncX3-type plasmids is bla_{NDM}, and all these plasmids contain only the bla_{NDM} gene. The bla_{NDM}-carrying IncX3-type plasmids are widely prevalent in *K. pneumoniae* in China, spanning 15 STs.

Conclusion: In summary, our study reports the first genome sequence of an ST 6758 *K. pneumoniae* strain containing the class B β-lactamase bla_{NDM-1} isolated from a clinical sample. Given the global emergence of bla_{NDM}, measures should be taken to prevent the spread of these bla_{NDM}-carrying IncX3-type plasmids. Our findings contribute to the understanding of the transmission mechanisms of bla_{NDM} in *K. pneumoniae*.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, bla_{NDM-1}, whole-genome sequencing, IncX3 type plasmid, ST6758

Introduction

Klebsiella pneumoniae, as one of the most prevalent Gram-negative pathogens, is a significant source of both community-acquired and nosocomial infections.[1,2] The widespread use of antibiotics has led to the emergence of multidrug resistance (MDR) in *K. pneumoniae*, particularly the rise of carbapenem-resistant *K. pneumoniae* strains (CRKP). This trend poses a substantial threat to human health, as the options for treating CRKP infections dwindle, given the diminishing efficacy of available antibiotics.[3]

The first reported instances of CRKP date back to the 1990s.[4] Infection caused by carbapenem-resistant strains exhibit significantly higher mortality rates compared to those caused by carbapenem-susceptible strains.[5] In the United States and European countries, *K. pneumoniae* ST258 (sequence type) contributes significantly to the spread of carbapenem resistance, while ST11 is predominant in China.[6,7] The New Delhi metallo-β-lactamase-1 (NDM-1) was initially identified in *K. pneumoniae* and *Escherichia coli* in 2008 and has since become a global

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concern.[8] Due to the presence of mobile genetic elements such as transposons and plasmids, *K. pneumoniae* often plays a pivotal role in facilitating the transfer of antimicrobial resistance genes between environmental strains and clinical strains.[9] The IncX type plasmid is often associated with carbapenemase production, and the IncX3 plasmid is essential for the delivery of carbapenemase genes, especially bla_{NDM}.[10–13]

This study focuses on the genomic characterization of a new sequence type, ST6758 CRKP strain, isolated from an inpatient at a teaching hospital in China, which carries bla_{NDM-1} on an IncX3 type plasmid. The complete genome sequence of this strain was determined, and a comprehensive analysis was conducted to examine its genomic features, plasmid characteristics, and resistance mechanisms. Furthermore, the transmission mechanisms of the carbapenemase gene bla_{NDM-1} in *K. pneumoniae* were investigated.

Material and Methods

Patient and Isolate

On May 27, 2019, a 69-year-old male was admitted to a tertiary hospital in Zhejiang Province, China, presenting with dysuria. Clinical evaluations led to the diagnosis of prostatic hyperplasia and urinary retention. *K. pneumoniae* strain CRKP331 was isolated from the patient's urine sample on May 29. The strain was initially identified using the VITEK MS system (bioMérieux, France) and further validated by whole-genome sequencing. CRKP331 was susceptible to aminoglycoside antibiotics, and the aminoglycoside antibiotic isepamicin was used to treat the urinary tract infection. The patient eventually recovered.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was conducted using the VITEK 2 system (bioMérieux, France) with Gram-negative antimicrobial susceptibility testing cards (AST-GN13) and the standard broth microdilution test, adhering to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI). The antimicrobial agents tested in this study included amikacin, ceftazidime, ceftriaxone, ampicillin/sulbactam, sulfamethoxazole/trimethoprim, cefepime, gentamicin, imipenem, meropenem, levofloxacin, and tobramycin. Breakpoints were interpreted in accordance with the recommendations outlined in the CLSI guidelines.[14]

Whole-Genome Sequencing

The complete genome sequence of the strain was determined utilizing the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) in the 150-bp paired-end sequencing mode, with an average sequencing depth of ≥100×. Furthermore, long-read sequencing was conducted using a MinION sequencer (Nanopore, Oxford, UK). The short Illumina reads and long MinION reads were subjected to hybrid assembly using Unicycler (v0.4.7) in conservative mode. This resulted in the generation of complete circular contigs, which were then refined and corrected using Pilon with Illumina reads through multiple rounds of iteration until no further changes were detected. The resulting complete genome sequence was subsequently annotated automatically using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) server.

Genomic Analysis

A novel sequence type of *K. pneumoniae* was assigned utilizing the conserved segments of seven essential housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB* and *tonB*) based on the BIGSdb-Pasteur MLST (Multilocus Sequence Typing) analysis (https://bigsdb.pasteur.fr/klebsiella/). The BacWGSTdb server was employed to investigate the strain's antimicrobial resistance genes and plasmid replicons.[15,16] A comparative analysis was conducted using circular representations, depicted as concentric rings, to assess the bla_{NDM-1}-carrying plasmid and its resemblance to similar plasmids. This analysis was performed using the BLAST Ring Image Generator (BRIG).[17]

Phylogenetic Analysis

The phylogenetic tree was examined using CSI Phylogeny (version 1.4),[18] which is based on a core-genome single-nucleotide polymorphism (SNP) strategy. CSI Phylogeny was employed to identify and filter *K. pneumoniae* CRKP331

SNPs, validate the SNPs, and construct a phylogeny based on a concatenated alignment of the high-quality SNPs. The maximum parsimony algorithm was then used to generate a phylogenetic tree from the resulting SNPs, which was subsequently visualised on the iTOL webpage.[19]

Nucleotide Sequence Accession Numbers

The whole genome results for strain CRKP331 have been deposited in DDBJ/EMBL/GenBank, and the accession number is SAMN40739512.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Zhejiang Provincial People's Hospital (Ethics approval number 2019KY244). In this case, the Ethics Committee of Zhejiang Provincial People's Hospital granted an exemption from written informed consent because our study focused solely on bacteria. The clinical isolate *K. pneumoniae* CRKP331 was collected as part of the routine hospital laboratory procedures.

Results and Discussion

The minimum inhibitory concentrations (MICs) of the tested antibiotics are shown in Table S1. *K. pneumoniae* CRKP331 exhibited resistance to various antibiotics, including ceftazidime, ceftriaxone, cefazolin, cefepime, imipenem, meropenem, ampicillin/sulbactam, and sulfamethoxazole/trimethoprim. However, it remained susceptible to amikacin, gentamicin, and tobramycin, and was classified as intermediate to levofloxacin.

The genome sequence of *K. pneumoniae* CRKP331 consists of five contigs, with a total length of 5,765,149 bp. Of these, one is a chromosome, measuring 5,317,817 bp, while the remaining four are plasmids. Contig 2 is 206,059 bp, contig 3 is 111,193 bp, contig 4 is 82,257 bp, and pNDM-CRKP331 is 47,823 bp in length. The novel sequence type of CRKP331 was classified as ST6758 through MLST analysis. This classification was achieved by analyzing the conserved segments of seven essential housekeeping genes (*rpoB4, gapA2, mdh1, pgi1, phoE4, infB4 and tonB174*).

The antimicrobial resistance genes identified in the genome of the isolate are presented in Table 1. We identified the β-lactam resistance genes $bla_{\text{SHV-187}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{NDM-1}}$; the fosfomycin resistance gene *fosA*; the

Table 1 Antimicrobial Resistance Genes (ARGs) in Isolate K. Pneumoniae CRKP331

Antimicrobial Resistance Gene	Accession No. of Contig	Identity (%)	Position in Contig	Antimicrobial Resistance Category	Accession no. of Reference Gene
oqxB	1	99.02	1,177,000.1180152	Quinolone	EU370913
oqxA	1	99.15	1,180,176.1181351	Quinolone	EU370913
$bla_{\text{SHV-187}}$	1	99.08	2,632,897.2633763	Beta-lactam	LN515533
fosA	1	94.29	4,574,451.4574870	Fosfomycin	ACZD01000244
aac(6')-Ib-cr	2	100	112,203.112802	Quinolone	DQ303918
ARR-3	2	99.19	112,859.113351	Rifamycin	FM207631
dfrA27	2	100	113,484.113957	Trimethoprim	FJ459817
aadA16	2	99.64	114,138.114983	Aminoglycoside	EU675686
sul1	2	99.89	115,414.116280	Sulphonamide	EU780013
mph(A)	2	99.67	120,475.121396	Macrolide	U36578
tet(A)	2	100	123,553.124827	Tetracycline	AF534183
floR	2	98.19	125,428.126641	Phenicol	AF118107
$bla_{\text{TEM-1B}}$	2	100	131,497.132357	Beta-lactam	AY458016
$bla_{\text{CTX-M-3}}$	2	100	133,139.134014	Beta-lactam	Y10278
qnrS1	2	100	137,979.138635	Quinolone	AB187515
$bla_{\text{NDM-1}}$	pNDM-CRKP331	100	33,266.34078	Beta-lactam	FN396876

quinolone resistance genes *oqxB, oqxA, qnrS1* and *aac(6')-Ib-cr*; the trimethoprim resistance gene *dfrA27*; the rifamycin resistance gene *ARR-3*; the sulfonamide resistance gene *sul1*; the aminoglycoside resistance gene *addA16*; the tetracycline resistance gene *tet(A)*; the macrolide resistance gene *mph(A)* and the phenicol resistance gene *floR*.

With the exception of *oqxA, oqxB,* $bla_{\text{SHV-187}}$, and *fosA*, which are located on the chromosome, the remaining resistance genes are located on plasmids. A further analysis revealed the presence of *aac(6')-Ib-cr, ARR-3, dfrA27, aadA16, sul1, mph(A), tet(A), floR,* $bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-3}}$ and *qnrS1* on plasmid 2. In addition, the carbapenem resistance gene $bla_{\text{NDM-1}}$ was found to be located on plasmid pNDM-CRKP331. The bla_{NDM}-carrying plasmid pNDM-CRKP331 was designated as the IncX3-type plasmid. The similarity of pNDM-CRKP331 to other IncX3-type plasmids was analyzed using the basic local alignment search tool (Figure 1). These analogous plasmids were

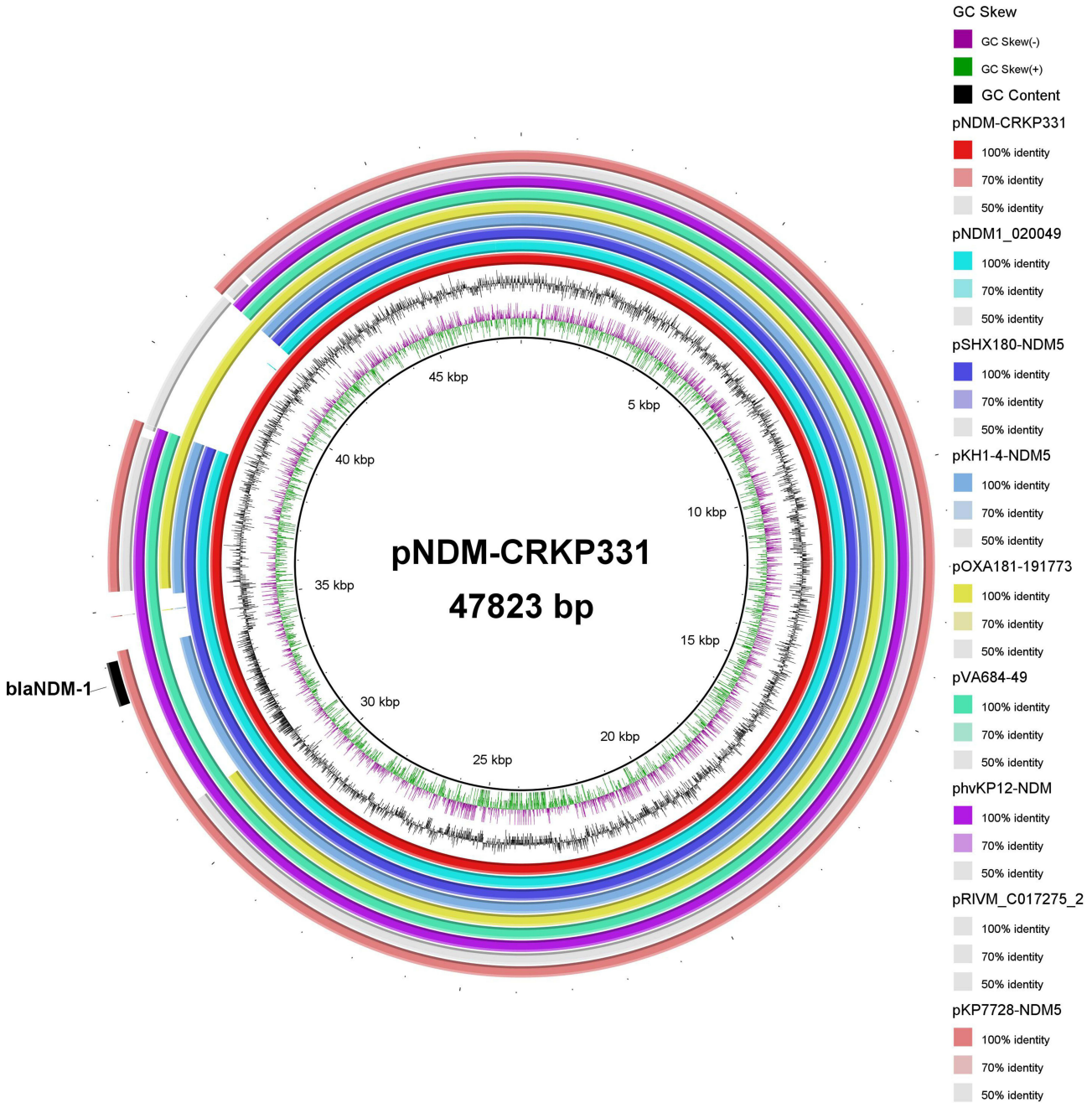

Figure 1 Circular comparison between the $bla_{\text{NDM-1}}$-carrying plasmid pNDM-CRKP331 and similar plasmids.

all derived from *K. pneumoniae*, including pNDM1_020049 (accession no. CP028786), pSHX180-NDM5 (accession no. CP094514), pKH1-4-NDM5 (accession no. CP102881), pOXA181-191773 (accession no. CP080367), pVA684-49 (accession no. CP093461), hvKP12-NDM (accession no. CP103320), pRIVM_C017275_2 (accession no. CP068868), pKP7728-NDM5 (accession no. CP092650). All the other plasmids, with the exception of pOXA181-191773 and pRIVM_C017275_2, carry the bla_{NDM} gene. This suggests that the IncX3 plasmid was an important vector for bla_{NDM} gene transfer in *K. pneumoniae*. A variety of bla_{NDM} sub-type were found on these IncX3 plasmid, which highlights the possibility that the IncX3 plasmid was one of the major platforms for bla_{NDM} gene evolution.[20]

A simple annotation of the pNDM-CRKP331 plasmid was performed using the RAST Server. This revealed the presence of IS*6* and IS*Kox3* upstream of bla_{NDM-1}, while IS*Aba125* and IS*3000* were identified just downstream of bla_{NDM-1}. The ISFinder database was utilized to identify the IS element of pNDM-CRKP331. A total of twenty-two IS elements were identified in the plasmid, belonging to five IS families, including IS*6*, Tn*3*, IS*Kra4*, IS*L3*, and IS*30*. Among these, IS*6* (19/46) and Tn*3* (9/46) were the most prevalent. This suggests that the bla_{NDM} gene may act as an external gene and recombine into the IncX3 plasmid via insertion or transposition of IS elements.

Using the Basic Local Alignment Search Tool (BLAST) with a threshold of 85% plasmid length and 80% coverage, 41 strains of *K. pneumoniae* were retrieved from the NCBI database, harboring IncX3-type plasmid similar to pNDM-CRKP331 (Table 2). Among these strains, twenty-two sequence types (STs) were identified, including ST1, ST11, ST14, ST15, ST16, ST17, ST23, ST35, ST43, ST258, ST307, ST340, ST427, ST437, ST485, ST505, ST512, ST656, ST766, ST1383, ST4523, and ST6758. The most common sequence types were ST11 (6/42), ST37 (6/42), and ST512 (5/41). These strains were distributed across 11 countries, with the highest prevalence in China (22 strains, 22/42), followed by the USA (5/42) and Italy (5/42). Phylogenetic analysis of the 42 strains is shown in Figure 2. The carbapenemase genes carried by these strains were predominantly bla_{NDM} (25/42). Additionally, bla_{KPC} was identified in 18 strains, including bla_{KPC-2} and bla_{KPC-3}. Notably, five strains carried both bla_{KPC} and bla_{NDM}. The CRKP331 strain demonstrates a unique spectrum of resistance genes. Nonetheless, it possesses a set of shared resistance genes with other strains, which confer resistance to β-lactams, aminoglycosides, fluoroquinolones, macrolides, tetracyclines, and sulfonamides. These observations highlight the imperative for customized therapeutic approaches and underscore the evolving and complex nature of antimicrobial resistance within *K. pneumoniae*.

We further investigated the IncX3-type plasmids in these strains. These plasmids predominantly carried single resistance genes (39/42), with a few (3/42) carrying two resistance genes. The resistance genes were primarily bla_{NDM} (25/42), encompassing three subtypes: bla_{NDM-1}, bla_{NDM-5}, and bla_{NDM-7}. Three strains carried bla_{KPC}, all of which carried two resistance genes (bla_{KPC} and bla_{SHV}). Additionally, three strains carried only $bla_{OXA-181}$. Some strains did not carry carbapenemase genes but instead carried an Extended Spectrum β-Lactamases (ESBLs) gene, such as bla_{SHV-12} (5/42) or $bla_{SHV-182}$ (5/42). Notably, the five strains from Italy (all ST512) carried an IncX3 plasmid with only $bla_{SHV-182}$, while the five strains from the USA (all ST258) carried an IncX3 plasmid, with four strains carrying only bla_{SHV-12} and one strain carrying both bla_{KPC-3} and bla_{SHV-12}. Of the 22 strains from China, all carried the IncX3 plasmid, with 20 carrying bla_{NDM} and the other two carrying $bla_{OXA-181}$. The IncX3 plasmid carrying the bla_{NDM} gene has been widely prevalent in *K. pneumoniae* in China, involving 15 ST types (ST1, ST11, ST14, ST15, ST17, ST23, ST35, ST340, ST485, ST505, ST656, ST766, ST1383, ST4523, ST6758).

In conclusion, we report a new ST-type CRKP strain, ST6758, which carries 16 antimicrobial resistance genes, including bla_{NDM}. These resistance genes are primarily located on plasmids, with the carbapenemase gene bla_{NDM-1} specifically located on an IncX3-type plasmid. A total of 41 *K. pneumoniae* strains carrying similar IncX3-type plasmids were retrieved from the NCBI database, representing 20 ST types across 11 countries. The most common resistance gene carried by these IncX3-type plasmids is bla_{NDM}, and all these plasmids carry only a single bla_{NDM} gene. IncX3-type

Table 2 Information of 41 Strains of K. Pneumoniae Retrieved from the NCBI Database That Harbouring Similar IncX3-Type Plasmid

Assembly Accession	Strain	Country	Collection date	Isolation Source	Query Coverage	Percent Identity	Plasmid Length	ST Type	Antimicrobial Resistance Genes
GCA 000814305.1	34618	USA	2011	Homo sapiens	82%	99.99%	43380bp	258	bla_{SHV-12}
GCA 001521895.1	NUHL24835	China	2014	Homo sapiens	94%	99.98%	46161bp	14	bla_{NDM-5}
GCA 001701845.2	20 GR 12	Greece	2012	Homo sapiens	82%	99.99%	43380bp	258	bla_{SHV-12}
GCA 001902215.1	MNCRE69	USA	2012	Homo sapiens	82%	99.99%	45288bp	258	bla_{SHV-12}
GCA 001902235.1	MNCRE53	USA	2012	Homo sapiens	82%	99.99%	45289bp	258	bla_{SHV-12}
GCA 002852995.3	SCKP020049	China	2016	Homo sapiens	94%	99.99%	54035bp	1	bla_{NDM-1}
GCA 003054385.1	SCM96	China	2017	Homo sapiens	94%	99.98%	46161bp	15	bla_{NDM-1}
GCA 011769805.1	50595	Czech Republic	2019	Homo sapiens	91%	99.98%	51140bp	11	$bla_{OXA-181}$
GCA 012970485.1	B16KP0226	South Korea	2016	Homo sapiens	81%	99.99%	46835bp	307	bla_{KPC-2}, $bla_{SHV-106}$
GCA 012971365.1	F16KP0075	South Korea	2016	Homo sapiens	81%	99.99%	46836bp	11	bla_{KPC-2}, $bla_{SHV-182}$
GCA 015353095.1	19110124	China	2019	Swine	94%	99.98%	46161bp	340	bla_{NDM-5}
GCA 015775135.1	ZG2017CW4-4-1-2	China	2017	River	94%	99.98%	49941bp	11	bla_{NDM-5}
GCA 016772555.1	45706	USA	2013	Homo sapiens	82%	99.99%	43379bp	258	bla_{SHV-12}
GCA 018623105.1	KP32558	China	2020	Homo sapiens	94%	99.97%	46161bp	656	bla_{NDM-5}
GCA 019443585.1	K191773	China	2019	Homo sapiens	91%	99.99%	51479bp	16	$bla_{OXA-181}$
GCA 019458505.1	1678	China	2021	Homo sapiens	94%	99.99%	46161bp	485	bla_{NDM-5}
GCA 020459305.1	C11	China	2018	Homo sapiens	94%	99.99%	54034bp	1383	bla_{NDM-1}
GCA 021390015.1	LZKP00001	China	2020	Homo sapiens	94%	99.98%	46162bp	35	bla_{NDM-5}
GCA 021397585.1	KP46	China	2017	Homo sapiens	92%	99.98%	53096bp	15	bla_{NDM-1}
GCA 022453565.1	KP7728	China	2021	Homo sapiens	91%	99.99%	54048bp	17	bla_{NDM-5}
GCA 022649725.1	WCHKP115038	China	2018	Homo sapiens	94%	99.99%	46161bp	11	bla_{NDM-5}
GCA 023612215.1	S01	Australia	2016	Homo sapiens	91%	99.99%	54048bp	15	bla_{NDM-7}
GCA 024226755.1	LH13-d	Switzerland	2022	Gallus gallus domesticus	80%	99.95%	46338bp	427	bla_{NDM-1}
GCA 024396875.1	KP82	China	2013	Homo sapiens	94%	99.99%	54035bp	437	bla_{NDM-5}
GCA 024637895.1	SHX180	China	2018	Homo sapiens	94%	99.99%	53065bp	4523	bla_{NDM-5}
GCA 024734155.1	KH1	China	2019	Homo sapiens	91%	99.98%	52085bp	37	bla_{NDM-5}
GCA 024734195.1	KW2	China	2019	Well water	94%	99.98%	46161bp	766	bla_{NDM-5}
GCA 024762135.1	hvKP12	China	2021	Homo sapiens	94%	99.99%	46161bp	11	bla_{NDM-5}
GCA 025677745.1	BSIKPN-11	China	2017	Homo sapiens	94%	99.98%	46161bp	11	bla_{NDM-5}

GCA 026247925.1	LC-1873/18	Italy	2018	Homo sapiens	82%	99.99%	43380bp	512	$bla_{SHV-182}$
GCA 026247945.1	LC-79/19	Italy	2019	Homo sapiens	82%	99.99%	43380bp	512	$bla_{SHV-182}$
GCA 026247965.1	LC-394/19	Italy	2019	Homo sapiens	82%	99.99%	43380bp	512	$bla_{SHV-182}$
GCA 026247985.1	LC-395/19	Italy	2019	Homo sapiens	82%	99.99%	43380bp	512	$bla_{SHV-182}$
GCA 026248005.1	LC-422/19	Italy	2019	Homo sapiens	82%	99.99%	43380bp	512	$bla_{SHV-182}$
GCA 028472725.1	SCKP090351	China	2019	Homo sapiens	94%	99.99%	54035bp	23	bla_{NDM-1}
GCA 028554845.1	S5 CRE5a	USA	2017	Homo sapiens	82%	99.99%	53321bp	258	bla_{KPC-3}, bla_{SHV-12}
GCA 029623595.1	GIMC1009:Kpn-52ICU-2H	Russia	2021	Homo sapiens	80%	99.99%	48359bp	307	bla_{NDM-1}
GCA 029834605.1	IITJ BC16	India	2021	Homo sapiens	87%	99.99%	46612bp	16	bla_{NDM-5}
GCA 030061645.1	VA684	Chile	2019	Homo sapiens	94%	99.99%	48523bp	505	bla_{NDM-7}
GCA 030544725.1	T877-PC	China	2021	Homo sapiens	91%	99.99%	51479bp	43	$bla_{OXA-181}$
GCA 030552975.1	KP9	China	2021	Homo sapiens	94%	99.98%	46162bp	37	bla_{NDM-5}

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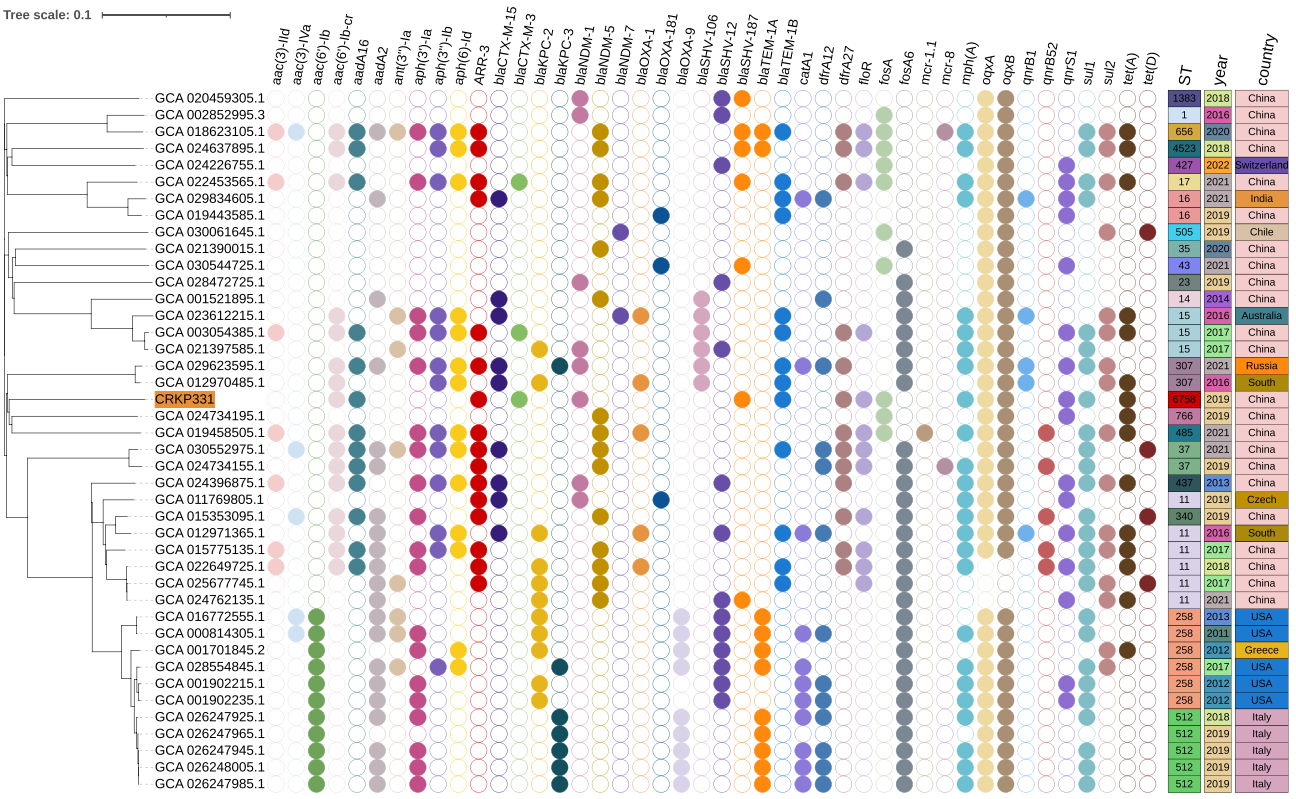

Figure 2 Phylogenetic analyses of CRKP331 and 41 *K. pneumoniae* strains carrying an IncX3-type plasmid similar to pNDM-CRKP331. The antimicrobial resistance genes are represented by different colours in the cells, whereas the gene is absent in the empty cells. Each square colour indicates a specific sequence type.

plasmids carrying the bla_{NDM} gene are widely prevalent in *K. pneumoniae* in China, spanning 15 ST types. Measures should be taken to prevent the spread of these bla_{NDM}-carrying IncX3-type plasmids. Our findings contribute to the understanding of the transmission mechanisms of carbapenemase genes in *K. pneumoniae*.

Acknowledgments

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Disclosure

The author reports no conflicts of interest in this work.

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