Genomic Characteristics of a Klebsiella pneumoniae Strain Coproducing Carbapenemases KPC-2 and NDM-5

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Abstract

The aim of this study was to characterize the resistance gene of a *Klebsiella pneumoniae* strain coproducing carbapenemases KPC-2 and NDM-5.*Klebsiella pneumoniae* KPN-hnqyy, which is separated from the stool specimen of a patient, is resistant to all carbapenem antibiotics. Its MLST type is ST11. The genes of carbapenemases bla_{KPC-2} and bla_{NDM-5} are positive. The gene of conjugant bla_{KPC-2} is positive and that of conjugant bla_{NDM-5} is negative. Whole-genome sequencing shows that the strain contains one chromosome and three plasmids. The chromosome genomes of *Klebsiella pneumonia* such as KP69 and KP19-2029 are more than 99.9% similar to the chromosome genome of the strain studied; also, a similar IncR and IncFII resistance gene fusion region is contained in the different types of plasmids they carry: the bla_{KPC-2} gene is located in a structure—which evolved from the Tn3-△Tn4401-Tn1721/Tn1722 sequence—inside this fusion region with its ends inserted into the transposase IS26 gene; the bla_{NDM-5} gene is located on an I-shaped transposon containing the special plasmids of the insertion fragment in phages, with its ends inserted into the transposase IS26 gene, too.The *Klebsiella pneumoniae* KPN-hnqyy that carries both bla_{KPC-2} and bla_{NDM-5} may be derived from the bla_{KPC-2} carrying *Klebsiella pneumoniae* KP69 and KP19-2029 strains accidentally obtain the bla_{NDM-5} gene by the transfer of IS26 transposable elements. The wide transmission of *Klebsiella pneumoniae* ST11 carrying the bla_{KPC-2} gene in China and its ability to obtain other carbapenemase genes through transposable element IS26 are well worth attention.

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a serious threat to human health and safety(1). In China, the detection rate of CRKP increased from 6.4% in 2014 to 10.9% in 2019, increasing year by year; especially in Henan Province, the detection rate of CRKP was 32.8% in 2019, ranking the first in China(http://www.carss.cn/Report/Details?aId=808). The main resistance mechanism of CRKP is carbapenemase(2), such as KPC- or NDM-like carbapenemases (referred to as KPC or NDM). KPC is the most globally distributed carbapenemase containing a variety of variants, for example, the most common variant KPC-2 can hydrolyze all β-lactam antibiotics, can be inhibited by avibactam, but cannot be inhibited by clavulanic acid(2, 3). NDM is also widely distributed in the world, for example, the most common variant NDM-1 can relieve all β-lactam antibiotics except aztreonam, but cannot be inhibited by avibactam(3). Compared with NDM-1, NDM-5 has two amino acid mutations, but has stronger hydrolytic activity(3-5). The ertapenem minimum inhibitory concentration (MIC) of NDM-5 producing strains is 4 ~ 8 times higher than that of NDM-1 producing strains(4). It's common for a strain of bacteria to produce multiple β-lactamases in gram-negative bacilli, but rare to produce multiple carbapenemases at the same time which is worth special attention. Studies have shown that in the combinations in which a strain of bacteria simultaneously produces multiple carbapenemases, NDM and OXA-48 combinations are the most common, accounting for 90%(3); KPC and VIM combinations are the second common, accounting for 75%, with most of the strains from the United States and Greece, and several strains from Spain(3); KPC-2 and NMD-1 combinations account for only 7%(3), while NDM-5 and KPC-2 double-positive *Klebsiella pneumoniae* are rarely reported in the literature. During our study, we found both NDM-5 and KPC-2 positive CRKP from stool samples, clinical samples, and hospital environment samples of a patient with hematological diseases, as well as his stool samples submitted at 6 months after his discharge from hospital, with special environmental characteristics of drug resistance genes.

Results

Strain identification and drug sensitivity

KPN-hnqyy was identified as CPKP, with minimum inhibitory concentrations of 64 μg/ml and 128 μg/ml for imipenem and meropenem, respectively, and was only sensitive to gentamicin, amikacin, chloramphenicol, polymyxin, and tigecycline, and were resistant to other antibiotics tested; specifically, ampicillin MIC > 16μg/ml, amoxicillin/clavulanic acid MIC > 16/8μg/ml, amikacin MIC ≤ 8μg/ml, aztreonam MIC > 16μg/ml, chloramphenicol MIC = 8μg/ml, ceftazidime MIC >

16μg/ml, ciprofloxacin MIC > 2μg/ml, polymyxin MIC = 1μg/ml, cefotaxime MIC > 32μg/ml, cefazolin MIC > 16μg/ml, cefepime MIC > 16μg/ml, gentamicin MIC ≤ 2μg/ml, levofloxacin MIC > 8μg/ml, ampicillin/sulbactam MIC > 16/8μg/ml, trimethoprim-sulfamethoxazole MIC > 2/38μg/ml, tigecycline MIC = 1.0μg/ml, and ceftazidime/avibactam MIC = 64μg/ml.

Results of carbapenemase phenotype and genotype determination

The results of inhibitor enhancement test showed that the difference in the inhibition zone diameter between the imipenem disk with EDTA or phenylboric acid and the inhibition zone diameter of the imipenem disk alone was less than 5 mm, and the difference in the inhibition zone diameter between the imipenem disk with EDTA and phenylboric acid and the inhibition zone diameter of the imipenem disk alone was more than 5 mm, so it was judged that the strains produced both class A and B carbapenemases (Fig. 1a); the enzyme immunochromatographic assay showed that both KPC and NMD were positive(Fig. 1b); the PCR amplification products were sequenced and compared, and it was verified that the KPN-hnqyy strain produced KPC-2 and NDM-5 carbapenemases.

Conjugation test results

Conjugons grew needle-like colonies after overnight culture on screening plates, and there were significant single colonies after 24 h. They were identified as Escherichia coli, and the imipenem and meropenem MICs were 64 μg/ml and 128 μg/ml, respectively. Enzyme immunochromatographic assays demonstrated that this conjugon produced KPC carbapenemase and was negative for NDM carbapenemase. Further amplification using PCR followed by sequencing confirmed *bla* $_{KPC-2}$ positive, but *bla* $_{NDM-5}$ negative.

Results of whole genome sequencing and bioinformatics analysis

After sequencing, it was found that strain KPN-hnqyy contained one chromosome and three plasmids, with respective length, GC content and drug resistance gene information shown in Table 1. MLST was classified as ST11. *Klebsiella pneumoniae* KP69 (gene registration number: CP025456) and KP19-2029 (gene registration number: CP047160) were very similar to this bacterium, with a coverage greater than 99% and similarity of 99.99% and 99.95%, respectively (Fig. 2).

Table 1
Whole genome sequencing data of *Klebsiella pneumoniae* KPN-hnqyy

Replicon	Name	Length (bp)	GC (%)	Inc Type	Carbapenemase gene	Other resistant genes	GenBank Accession
chromosome	KPN-hnqyy-chro	5531197	57.29	-	-	aadA2,oqxA,oqxB, *bla*$_{SHV-11}$	CP074116
Plasmid 1	pKPN-hnqyy-ndm	123557	52.94	-	*bla*$_{NDM-5}$	aadA2,dfrA12,sul1	CP074117
Plasmid 2	pKPN-hnqyy-kpc	116047	52.83	IncFII:IncR:IncN	*bla*$_{KPC-2}$	*bla*$_{CTX-M-65}$	CP074118
Plasmid 3	pKPN-hnqyy-3	10060	55.05	ColRNAI	-	-	CP074119

Plasmid pKPN-hnqyy-kpc was a fusion plasmid formed by three plasmid replicons, IncFII, IncR and IncN (Fig. 3), and each fusion site had a transposase gene of the IS26 family. IncR region was a *bla*$_{KPC-2}$ resistance gene region, IncFII region was a *bla*$_{CTX-M-65}$ resistance gene region, and the resistance gene region formed by them was also found in plasmid p69-2

(registration number: CP025458) and pKP19-2029-KPC2 (registration number: CP047161), with the consistency of 99.99% (Fig. 3). The IncN region mainly contained genes for functional proteins such as DNA polymerase with repair function, cobalamin synthesis, plasmid stability, transcriptional function, and functional protein type IV secretion system (VirB), which were the backbone and functional regions of the plasmid, and this structure was also found in the *Klebsiella pneumoniae* pL22-2 (registration number: CP031259) plasmid, with a consistency of 99.63%. The bla_{KPC-2} gene was located in a relatively conserved sequence, and ISkpn27 and ISkpn6 constitute the core region of drug resistance, with transposons of the Tn3 family upstream and Tn1721 downstream, and the IS26 family transposase genes were inserted at both ends (Fig. 4).

The pKPN-hnqyy-ndm plasmid was temporarily unclassifiable and has features typical of mosaic plasmids. IS26 transposable elements were inserted at both ends of the fusion region formed in the drug resistance gene region and transposable region (Fig. 5), and similar fragments (49853 bp ~ 92399 bp) were also present in plasmids such as pYJ6-NDM5 (registration number: AP023236) and pEM06-18-14_2 (registration number: CP063481), with similarities of 99.49% and 99.90%, respectively. The remaining part of the plasmid is a backbone region with two chromosomal sequences inserted in the middle, and upon comparision it was found that the inserted sequences were all phage-associated genes. Similar sequences containing phage-associated genes were also found in plasmid pIR5755(registration number: CP061971) and plasmid pJX2-3(registration number: CP064249), with a similarity of above 99%. The upstream of bla_{NDM-5} gene was the IS26 transposase and the IS30-like transposase gene of the △ISAba125 family, with sulfonamides, aminoglycosides, and trimethoprim-related resistance genes distributed downstream (Fig. 5).

Discussion

ST11 is the main clone of *Klebsiella pneumoniae* prevailing in China(10, 11). Many literatures have reported that ST11 *Klebsiella pneumoniae* carries bla_{KPC-2} gene(12, 13), and only a small number of reported strains carry both bla_{NDM-5} and bla_{KPC-2} genes(14, 15). Prof. Wang Hui pointed out that the Escherichia coli producing KPC-2 and NMD-1 at the same time is the KPC-2 producing Escherichia coli that accidentally obtained the plasmid of NDM-1(16). This article also partially supports this theory. *Klebsiella pneumoniae* KPN-hnqyy, KP69 and KP19-2029 have similar genomes (Fig. 2), but KPN-hnqyy produces both KPC-2 and NDM-5 carbapenemases, while KP69 and KP19-2029 only contains KPC-2 carbapenemase, so it is guessed that ST11 *Klebsiella pneumoniae* KPN-hnqyy that produces both KPC-2 and NDM-5 carbapenemase may be derived from similarly KPC-2 carrying *Klebsiella pneumoniae* KP69 and KP19-2029 that have obtained bla_{NDM-5} gene cluster accidentally. This was further verified by comparing the chromosomes of similar strains and their respective plasmids carrying bla_{KPC-2} gene cluster. The chromosomal genomes of *Klebsiella pneumoniae* KPN-hnqyy, KP69 and KP19-2029 were similar (Fig. 2), their plasmids of different types also contained similar IncFII and IncR drug resistance gene fusion regions (similarity > 99.9%) (Fig. 3), indicating that in such type ST11 *Klebsiella pneumoniae*, this drug resistance gene fusion region and chromosomal genome of the plasmid may be relatively stable, but there may be some recombination hotspots at both ends of the drug resistance gene region, resulting in the easy integration of different exogenous sequences and leading to a large range of recombination in the backbone region of the plasmid. In this study, the backbone IncN regions of plasmids pKPN-hnqyy-kpc and pL22-2 are similar (similarity > 99%) (Fig. 2), both ends are connected to the IS26 transposase gene, but the chromosomal genome similarity of their corresponding strains is 67.21% (Fig. 2), so it can be inferred that the IncN replicon region of plasmid pKPN-hnqyy-kpc may be derived from different strains by means of IS26-mediated gene recombination.

At present, it is reported abroad that the metastatic elements carrying the bla_{KPC-2} gene are mainly Tn4401 transposon(17), which contains two insertion sequences, ISKpn6 and ISKpn7, in their surrounding sequences (Fig. 4). However, it was found in China that the metastatic element carrying bla_{KPC-2} gene usually contained an insertion element of Tn3 upstream, and ISKpn7 was replaced with ISKpn27 downstream containing an insertion sequence of Tn1721/Tn1722 to form a Tn3-△Tn4401-Tn1721/Tn1722 complex transposable element carrying the bla_{KPC-2} gene(18). In this study, in the environment

of *bla*$_{KPC-2}$ gene, on the basis of the above complex transposable element, IS26 family transposase was inserted at both ends, and only a short sequence of Tn3 recombinase gene remained upstream, and the gene of some Tn1721/Tn1722 transposable elements was truncated downstream (Fig. 4), indicating that this sequence may evolve from a similar sequence of Tn3-△Tn4401-Tn1721/Tn1722. The effect of this transposable element sequence change on the efficiency of *bla*$_{KPC-2}$ gene transfer and the expression level of *bla*$_{KPC-2}$ gene is unknown.

The *bla*$_{NDM-5}$ gene is mostly carried by transferable plasmids such as IncX or IncF plasmids(19, 20). In this study, the pKPN-hnqyy-ndm plasmid is relatively special, temporarily unclassifiable, and the conjugation test is negative. Importantly, its plasmid backbone part contains two phage-associated insertion sequences, which are also found in the chromosomal genome of this strain. Phage is one of the important vectors for horizontal gene transfer and can mediate the transfer of various resistance genes such as *bla* $_{KPC}$ and *bla*$_{NDM}$(11). It has been documented that plasmids carrying the *bla*$_{KPC-2}$ gene can acquire phages and form a complex of plasmids and phages(21, 22). In this study, the relationship between *bla*$_{NDM-5}$ gene and phage and the effect of inserting phage fragments in the plasmid on the plasmid stability and conjugation efficiency remain unknown. The *bla*$_{NDM-5}$ gene environment contains a tandem resistance gene cassette composed of aadA2, dfrA12 and sul1 genes, and similar sequences are also found in plasmids of different species from a variety of bacteria(23), and it is typically characterized by IS26 insertion elements at both ends of the gene cassette. IS26, as one of the most important members of the IS6 insertion sequence family, can not only affect the horizontal transfer, integration, rearrangement, and expression of related genes within or between strains, but also affect the integration of plasmids(24, 25). Different from the viewpoint of Prof. Wang Hui et al.(16), the NDM-5 carbapenemase gene herein may not be obtained by plasmid conjugation, but by the transfer of IS26 transposable elements.

The *Klebsiella pneumoniae* KPN-hnqyy in this study was first found in stool screening of a child with hematological diseases, and was subsequently isolated from its pancreatic juice, blood, ascites, and stool samples 6 months after its discharge from hospital, indicating that this bacterium can colonize the patient's intestine for a long time and may become a pathogenic bacterium. Typically, in the absence of antibiotic pressure, if the plasmid of the strain contains a resistance gene, it allows the bacterium to pay an "adaptation price", resulting in the natural disappearance of the resistance plasmid at the end of treatment or after the colonizer is transferred to other hosts(26). However, the ST11 *Klebsiella pneumoniae* in this study colonized the patient's intestine for a long time, which may be due to mutations in the chromosome of the strain or the weakening of the "adaptation price" by the expression of plasmids. At present, ST11-type CRKP carrying *bla*$_{KPC-2}$is increasing at an alarming rate(27), especially the emergence of CRKP carrying both *bla*$_{KPC-2}$ and $_{NMD-5}$ genes in this study, and it can colonize the intestine for a long time; thereafter, it may disseminate into the surrounding environment through feces, which undoubtedly increases the difficulty of CRKP treatment, prevention and control.

Klebsiella pneumoniae positive for both KPC-2 and NDM-5 carbapenemases in this study is type ST11, and the *bla*$_{KPC-2}$ gene is located on the IncR/IncN/IncF fusion particle and is present in a structure evolved from Tn3-△Tn4401-Tn1721/Tn1722 with both ends inserted into the IS26 transposable element; the *bla*$_{NDM-5}$ gene is located on a special plasmid containing a phage insert and is present in a tandem resistance gene cassette containing multiple resistance genes composed of the IS26 transposable element at both ends. The *Klebsiella pneumoniae* KPN-hnqyy that carries both NDM-5 and KPC-2 may be derived from the KPC-2 carrying *Klebsiella pneumoniae* KP69 and KP19-2029 strains accidentally obtain the NDM-5 gene by the transfer of IS26 transposable elements. The wide transmission of *Klebsiella pneumoniae* ST11 carrying the blaKPC-2 gene in China and its ability to obtain other carbapenemase genes through transposable element IS26 are well worth attention.

Material And Methods

Source of strain

A patient, born in 2008, visited several hospitals in Nanjing and Zhengzhou, and was admitted to our hospital on December 31, 2020 with acute lymphoblastic leukemia and severe pancreatitis. Initially, a CRKP strain, named KPN-hnqyy, was screened from the drug-resistant strains in her stool, and then similar strains were detected in samples of her ascites, blood, pancreatic juice, and stool at 3 and 6 months after her discharge from hospital. In our hospital, the patient successively used cefoperazone/sulbactam, ceftazidime/avibactam combined with aztreonam, vancomycin and other drugs for treatment, all of which were less effective. Later, polymyxin B was used, along with adjunctive irrigation with gentamicin normal saline. The patient was cured of infection and discharged. The quality control strain was Escherichia coli ATCC25922, while NDM-1 and KPC-2 double-positive *Klebsiella pneumoniae* and sodium azide-resistant Escherichia coli J53 were preserved in our laboratory.

Instruments and reagents

Bacterial identification and drug sensitivity analyzer was BD Phoenix™ M50 instrument; carbapenemase genotype detection kit NG-Test® CARBA 5 was purchased from Shanghai Fosun Long March Medical Science Co., Ltd.; PCR amplification instrument was purchased from ABI; primer synthesis and product sequencing were completed by Shanghai Sangon Technology Co., Ltd.; sodium azide and meropenem were purchased from Sigma; whole genome sequencing was completed by BGI, and the other reagents, culture media, drug sensitivity disks and some E-test strips used in this study were purchased from Zhengzhou Auto Bioengineering Co., Ltd.

Strain identification and drug sensitivity

BD Phoenix™ M50 instrument was used to identify the strains and determine their MICs. The MIC determination of imipenem, meropenem, ciprofloxacin, levofloxacin and tigecycline was reviewed with E-test method. The tigecycline breakpoint was determined at the FDA standard (susceptible, ≤ 2 mg/L;intermediate, 4 mg/L; resistant, ≥ 8 mg/L), polymyxin breakpoint at EU 2021 drug sensitivity test standard (https://www.eucast.org), and the remaining breakpoints at the CLSI M100 requirements(6).

Carbapenemase phenotype and genotype confirmation test

This study carried out the enzyme inhibitor enhancement test to confirm the phenotype(7), simultaneously detected NDM, KPC, VIM, IMP, and OXA-48 carbapenemases with enzyme immunochromatographic assay, confirmed the variants of NDM and KPC using PCR method, sequenced the full-length target genes with universal primers, by reference to relevant literature(8, 9), and finally compared and determined with the BLAST online tool (https://blast.ncbi.nlm.nih.gov /Blast).

Conjugation test

In this study, strain KPN-hnqyy was the donor bacterium and the Escherichia coli J53 was the recipient bacterium. One milliliter of each of the two bacteria in the logarithmic growth phase was inoculated into 100 ml of nutrient broth, and after standing overnight at 37°C, 50 μl was spread on screening plates containing LB medium with 2 μg/mL meropenem and 100 μg/mL sodium azide, respectively, and after overnight culture, the conjugons were identified with the above method.

Plasmid genome sequencing, assembly, submission and gene environment analysis

KPN-hnqyy genomic DNA was extracted, broken into fragments of appropriate length by ultrasound and processed, and sequenced on PacBio RSII and Illumina platforms; the data were assembled with FalconV0.3.0 and Proovread 2.12 software, the data were subjected to quality control with FastQC software, and the assembled data were submitted to the Genebank to obtain sequence numbers and annotation information. The online tool (http://bacdb.cn/BacWGSTdb /index.php) was used to check the MLST typing, drug resistance genes, plasmid typing and other information of the strains. The software Easyfig2.2.3 was used to align genomic and open reading frame sequences, and the software BRIG v0.95 was used to generate visual plasmid circles.

Data Availability

The complete sequences of the KPN-hnqyy strain chromosome, pKPN-hnqyy-ndm, pKPN-hnqyy-kpc and pKPN-hnqyy-3 were submitted to GenBank(https://www.ncbi.nlm.nih.gov/nucleotide/) under accession numbers CP074116, CP074117 CP074118 and CP074119 respectively.

Declarations

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Contributions

Wei-qiang Xiao, conceptualization, experimental plan, project design, funding acquisition, bioinformatic and software analysis, manuscript preparation. Xiao-kun Wang and Yuan-ye Qu planned and executed experiments . Ming-yue Sun, Yan-min Chang and Qing-xia Xu provided logistical and technical support.

Ethics declarations

Competing interests

This study and protocol were carried out in accordance with the recommendations of the Ethics Committee of Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, Henan Province, China) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from the patients' guardians prior to the study.Authors declare no conflict of interest.

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Figures

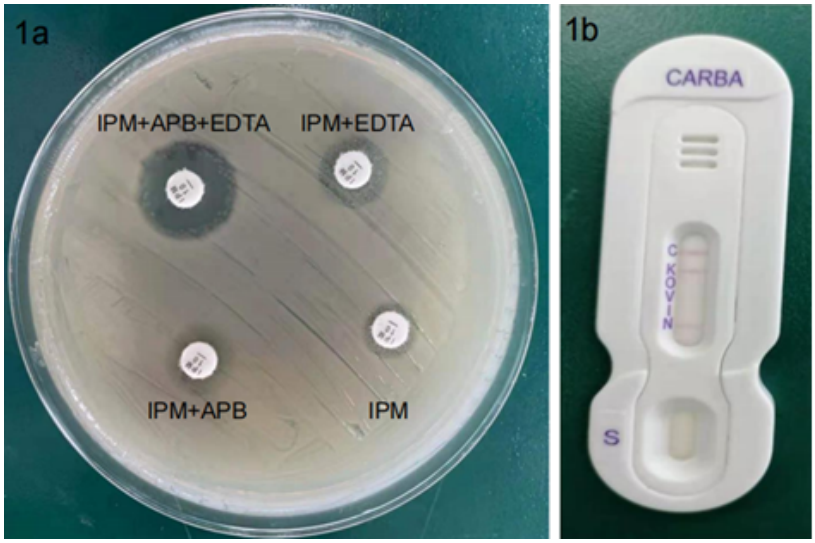

Figure 1

Carbapenemase Phenotype and Genotype Test. 1a. Enzyme inhibitor enhancement test to detect the phenotype of carbapenemase, IPM =imipenem, APB =3-Aminophenylboronic acid, imipenem disc inhibition zone has a diameter of 10.2mm, and imipenem disc +APB /EDTA inhibition zone has a diameter of 12.3mm or 13.6mm respectively. Imipenem discs +APB&EDTA inhibition zone has a diameter of 23.8mm; 1b. Immunochromatographic test of carbapenemase genotype, "C "= Quality Control, "K" = KPC, "N" = NDM, and Red Line = Positive.

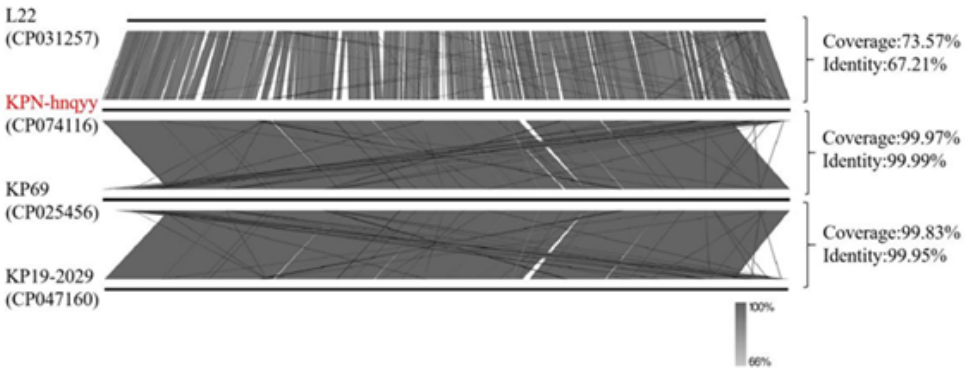

Figure 2

Genomic comparison of *Klebsiella pneumoniae* kpn-hnqyy with related strains. Gray represents the degree of similarity, and horizontal lines represent the length of the genome.

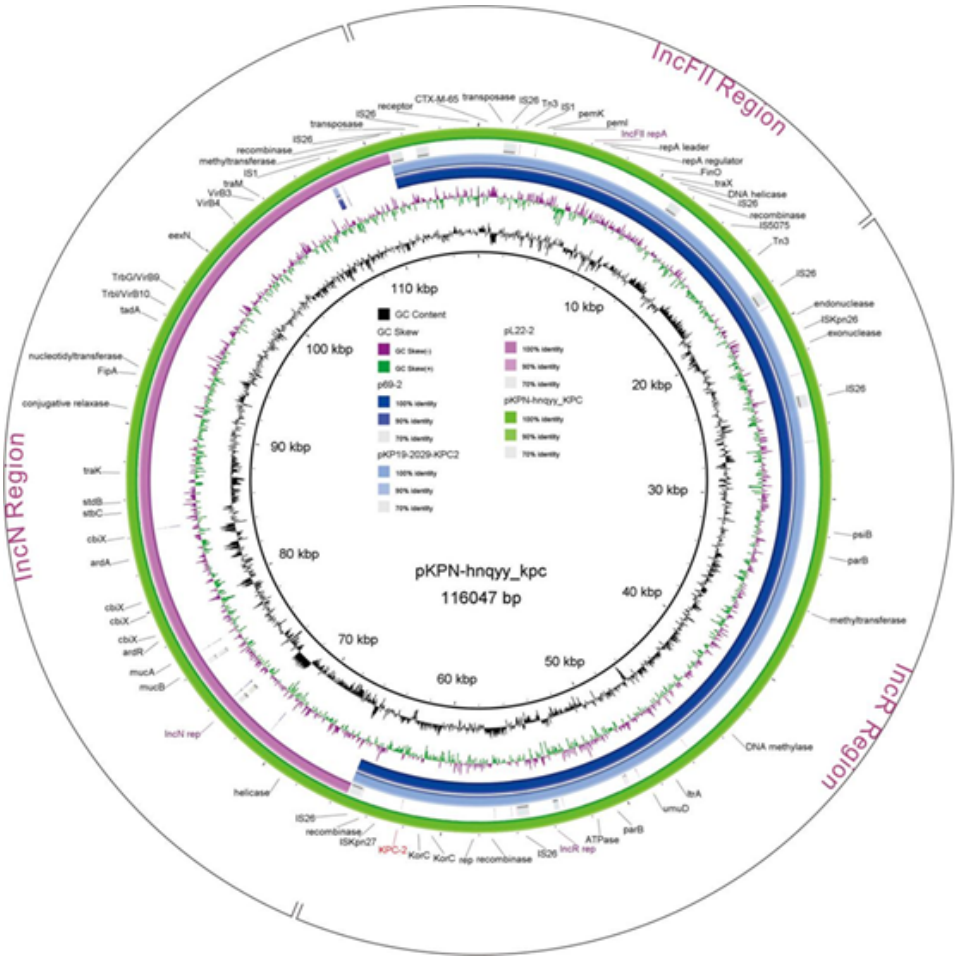

Figure 3

Sequence comparison of pKPN-hnqyy-kpc with three other plasmids. "[" represents the similarity sequences that can be transposed and appear in different plasmids; different colors represent different plasmids, and the depth of a plasmid color represents the similarity of the sequence; the scale indicates the length of the plasmid DNA sequence.

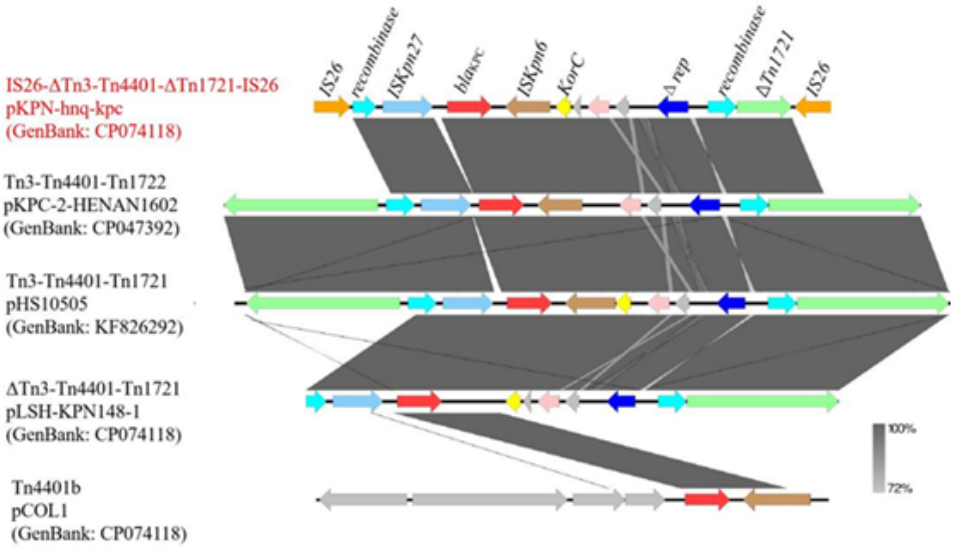

Figure 4

Comparison of *bla*$_{KPC-2}$ gene environment and similar sequences The gray level between different sequences represents similarity, the arrowed region indicates different open reading frames, and the arrow represents the direction of transcription.

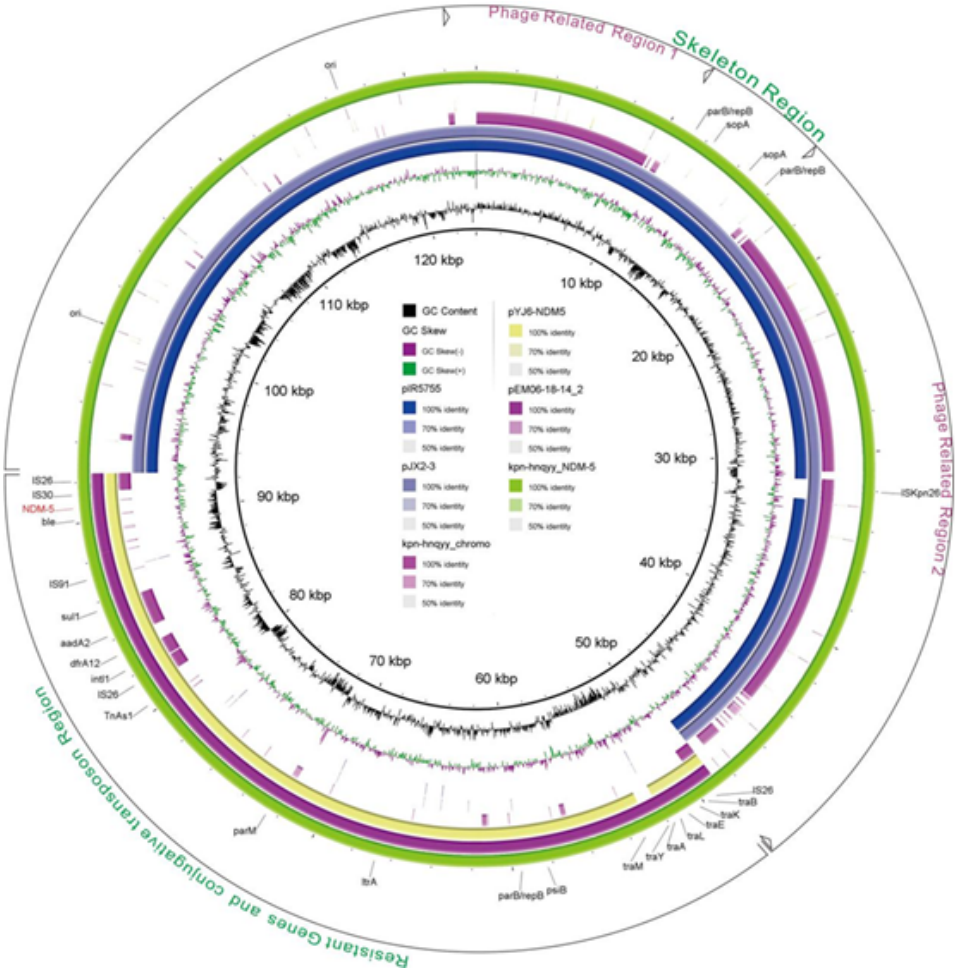

Figure 5

Comparison of pKPN-hnqyy-ndm with similar plasmid sequences. "[" represents the similarity sequences that can be transposed and appear in different plasmids; different colors represent different plasmids, and the depth of a plasmid color represents the similarity of the sequence; the scale indicates the length of the plasmid DNA sequence, and the range of "▷" and "◁" indicates the region where the main related genes exist.