Infection and Drug Resistance

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ORIGINAL RESEARCH

The Genomic Characterization of KPC-Producing *Klebsiella pneumoniae* from the ICU of a Teaching Hospital in Shanghai, China

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Purpose: This study retrospectively analyzed the genome characteristics of *blaKPC-2* in multidrug-resistant *Klebsiella pneumoniae* collected from the ICU of a teaching hospital in Shanghai, China.

Methods: From February 2018 to December 2019, 36 strains of multidrug-resistant *Klebsiella pneumoniae* were collected from the bronchoalveolar lavage fluid of critically ill patients. The genome of all isolates was obtained through the Illumina sequence, and single nucleotide polymorphisms of the *blaKPC-2* gene were analyzed to explore *blaKPC-2*'s evolutionary characteristics. Different strains' genetic relationships and homology were studied by constructing an evolutionary tree on a single copy orthologue. Pacbio combined Illumina sequence was conducted to evaluate the structure and potential mobility of drug-resistant plasmids of the strain KP-s26.

Results: The distribution of resistance and virulence genes had little difference, but most strains had significant differences in the plasmid-encoded region. Most strains (31/36) carried the carbapenemase gene *blaKPC-2*, with no single nucleotide polymorphism in different strains. Extended-spectrum β-lactamase resistance genes, such as *blaCTX-M* and *blaSHV*, were found in the isolates, but no metallo-β-lactamases were detected. All strains with *blaKPC-2* coexisted with chromosomal-associated fosfomycin resistance genes *fosA6*, and the coexistence of *blaKPC-2* and *blaCTX* variants (*blaCTX*-M-15, *blaCTX*-M-65, and *blaCTX*-M-27) was also detected in 29/31 strains. The isolate KP-s26 carried five circular plasmids. pA and pB were conjugate plasmids, as they carried drug resistance genes and contained a complete IV secretion system.

Conclusion: The *blaKPC-2* carbapenemase gene is relatively conservative in the process of evolution; drug-resistant plasmids containing conjugated transfer elements contribute to the spreading of drug resistance. The coexistence of *blaKPC-2* with *fosA6* or *blaCTX-M* variants was associated with increased fosfomycin resistance and broad-spectrum β-lactam resistance, respectively.

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Keywords: *Klebsiella pneumoniae*, *blaKPC-2*, whole-genome sequencing, single nucleotide polymorphism, drug-resistant plasmids

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Introduction

As one of the most common gram-negative bacteria, *Klebsiella pneumoniae* can cause various nosocomial infections.[1] Since the first strain of *Klebsiella pneumoniae* producing a class A carbapenem enzyme was found in the United States in 1996, the KPC enzyme has spread rapidly worldwide and aroused great public

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concern.[2,3] KPC is one of the main carbapenem enzymes encoded by *blaKPC* gene variants. The transmission of *blaKPC* involves multiple mechanisms of transfer from plasmid level to *blaKPC* gene clone transmission.[4] So far, 102 different variants have been reported in the KPC family (http://bldb.eu/), distributed in more than 115 ST-typed *Klebsiella pneumoniae*.[5] *BlaKPC-2* and *blaKPC-3* are the most common KPC gene variants globally, which has a high prevalence in Europe (Italy, Greece) and the Middle East (Israel).[6] In China, however, *blaKPC-2* is the main KPC variant, followed *by blaKPC-3*, KPC-33, and KPC-51 have also been reported.[7–9] To reduce the incidence of KPC-producing *Klebsiella pneumoniae*, it is crucial to reveal the evolution characteristics of the KPC gene during transmission.

However, few studies have described genomics' acquisition and transmission of mobile gene elements and drug-resistant plasmids. In this study, 36 strains of multidrug-resistant *Klebsiella pneumoniae* were collected, and whole-genome sequencing was used to clarify the strains' genomic diversity and the evolution characteristics of the KPC gene.

Methods

Collection and Identification of Bacterial Isolates

From February 2018 to December 2019, 36 strains of multidrug-resistant *Klebsiella pneumoniae* were isolated from the bronchoalveolar lavage fluid of ICU patients in a teaching hospital in Shanghai, China. The bacterial strain was identified as *Klebsiella pneumoniae* by VITEK-MS automatic microbiological analyzer (bioMérieux, Marcy l'Etoile, France), and *Escherichia coli* ATCC25922 was used for species identification control. This study was performed under the institutional guidelines for researching human beings, approved by the Human Ethics Committee of Shanghai Tenth People's Hospital (SHSY-IEC-4.1/18-74/01). Informed consent was signed at ICU admission.

Antibiotic Susceptibility Testing

The agar dilution method determined the minimum inhibitory concentrations (MICs) of 14 antibacterial drugs. The antibiotics involved in the susceptibility testing were as follows: piperacillin-tazobactam, ceftazidime, cefoperazone-sulbactam, cefepime, gentamicin, cefotaxime, imipenem, meropenem, amikacin, ciprofloxacin, levofloxacin, ampicillin, and ampicillin-sulbactam. In addition, the broth dilution method (DL, Zhuhai, China) was adopted to measure the MICs of tigecycline, polymyxins, and ceftazidime-avibactam. The drug susceptibility test results were interpreted according to the CLSI standards (https://clsi.org/). The agar dilution method determined the MIC to fosfomycin, and 25 μg/mL glucose 6-phosphate (G6P) (Aladdin, Shanghai, China) was added to the MH agar. The sensitivity breakpoints were set according to the European Committee for Antimicrobial Susceptibility Tests (EUCAST, 2018), and *Klebsiella pneumoniae* ATCC 700603 was chosen as controls.

Whole Genome Sequencing

The total DNA of 36 strains of multidrug-resistant *Klebsiella pneumoniae* was extracted by the TIANAmp MICRO DNA kit (TIANAmp, Tianjin Biotech, Tiangen, China). The whole genome was sequenced using the Illumina HiSeqTM X-10 platform (Illumina, San Diego, CA, USA). Paired-end reads of 150 bp were generated. After quality control, the clean data were assembled by SOAPdenovo V2. PacBio RSII single-molecule real-time (SMRT) sequencing platform (Pacific Biosciences, USA) combined with Illumina was used to obtain the complete whole replicon genome of strain KP-s26. Genomes were assembled by Canu and Spades simultaneously; the assembled sequence by each software was then analyzed mutually to ensure plasmid completeness and accuracy. Those were plasmid replicators identified by PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/),[10] antibiotic resistance genes annotated by CARD (https://card.mcmaster.ca/),[11] and the genomic sequence uploaded to the PubMLST database (http://pubmlst.org/) to obtain the ST typing of the strain. The genomes of 36 Strains of *Klebsiella pneumoniae* have been submitted to the Sequence Read Archive (SRA) with BioProject: PRJNA744405.

Construction of Phylogenetic Tree and Single Nucleotide Polymorphism

Prodigal V2.6.3 was used to predict all protein sequences of 36 isolates, orthofinderV2.5.2 calculated single-copy genes of the core genome according to protein sequences. Finally, Mafft V7.487 was used to compare sequences of 3827 core genes in 36 samples. Meanwhile, Gblocks Version 0.91b was used to shear the low-quality parts in the result. The phylogenetic tree was constructed by the maximum likelihood method from Version 2.1.10. In addition, the *blaKPC* gene of the isolated strain was analyzed by single nucleotide polymorphism (SNP) with BioEdit

7.2 to determine the single nucleotide polymorphism of *blaKPC-2* during the evolution of the strain.

Comparative Genome and Plasmid Analysis

The whole-genome sequence of KP-S26 as the reference genome to investigate each isolate's deletion and acquisition events, BRIG Version 0.95 was used to draw a circle map and mark the missing genes. Plasmids pA and pB were used as the reference genomes to conduct BLAST alignment with fasta sequences of pKP18069-CTX, pNMBU-W07E18_01, pC2601-2, pC2972_2, pC2974_2, p1_015093, and p17-16-KPC, respectively. The EVALue of BLAST alignment was 1E-5, the lower identity threshold was 50%, and the upper identity threshold was 70%. The circle graphs were annotated with comment files (GBK) of the pA and pB genomes.

Results

Antibiotic Susceptibility Results

Antibiotic sensitivity test showed that 36 isolates were resistant to piperacillin-tazobactam, ceftazidime, cefoperazone-sulbactam, ampicillin-sulbactam, cefepime, amikacin, gentamicin, fosfomycin, ciprofloxacin, levofloxacin, and ampicillin. Except for isolates KP-s18, KP-s29, and KP-s39, the other isolates were highly resistant to meropenem and imipenem. In addition, six strains of 36 *Klebsiella pneumoniae* were sensitive to amikacin, and all strains were sensitive to ceftazidime-avibactam, polymyxins B, and tigecycline. Drug sensitivity results showed that 36 strains of *Klebsiella pneumoniae* had multiple drug resistance; the drug-sensitive heat map is shown in (Figure 1).

Genome Sequence of 36 *Klebsiella pneumoniae*

According to short-reading sequencing, the genome size of the 36 isolates was 5.4–5.7 MB, which was composed of 5,707,982 ± 98,722 bp in 142 ± 34.98 scaffolds. The average GC content was 57 ± 0.12%, the length of genomic N50 was 185,802.9 ± 55,081.98. Thus, 5228–5772 genes encode open reading frames in the genomes of 36 *Klebsiella pneumoniae*. The results of genome assembly of the isolates are shown in (Table S1).

Comparative Genome Analysis of *Klebsiella pneumoniae*

The comparative genome circle map of all isolates is shown in Figure 2. Compared with the reference genome, other strains have little difference in the distribution of resistance genes and virulence genes, and most strains have apparent differences in the plasmid-encoded region. The strains with missing resistance and virulence genes compared with the reference genome were labeled in the outermost circle.

Drug Resistance Genes Carried by *Klebsiella pneumoniae*

Four ST types of *Klebsiella pneumoniae* were detected in our study, including ST11 (23/36), ST15 (11/36), ST37 (1/36), and ST65 (1/36). The annotation results of CARD resistance genes showed that carbapenem resistance genes were detected in 33 strains, two of them carried *blaKPC-24*, and the rest isolates carried *blaKPC-2*. Extended-spectrum β-lactamase resistance genes, such as *blaCTX-M* and *blaSHV*, were found in the isolates, and different variants of CTX-M, *blaCTX-M-15, blaCTX-M-65* and *blaCTX-M-27*, were also detected. The coexistence of *blaKPC* and *blaCTX* is shown in Figure 3. Five SHV variants of *blaSHV-11, blaSHV-28, blaSHV-52, blaSHV-134* and *blaSHV-165* were found in 12 isolates. Aminoglycosides (*AAC (3)-IIa,aadA*), fluoroquinolones (*qnrB4,emrR,patA*), macrolides (*mphA,Mrx*), sulfonamide (*sul1*) and fosfomycin (*fosA6*) were also found in *Klebsiella pneumoniae* isolates. No metallo-β-lactamases, including NDM, IMP, and VIM, were detected in all isolates. In this study, 36 strains of *Klebsiella pneumoniae* contained the same copy number of membrane porin Ompk37, by which antibiotics enter *Klebsiella pneumoniae*, and no mutation of membrane pore protein was detected by Ompk37 amino acid sequence analysis. The distribution of drug resistance genes is shown in (Table 1).

Sequence Analysis of *BlaKPC-2*

The KPC-24 gene carried by isolates KP-f04 and KP-s04 is located at the edge of the scaffold, so the prediction is incomplete. However, according to SNP analysis based on the *blaKPC-2* gene sequence in the other 31 strains, the gene had no single nucleotide polymorphism in different strains. Furthermore, the KPC-2 sequences of the other 31 strains were 100% the same, which meant no difference in

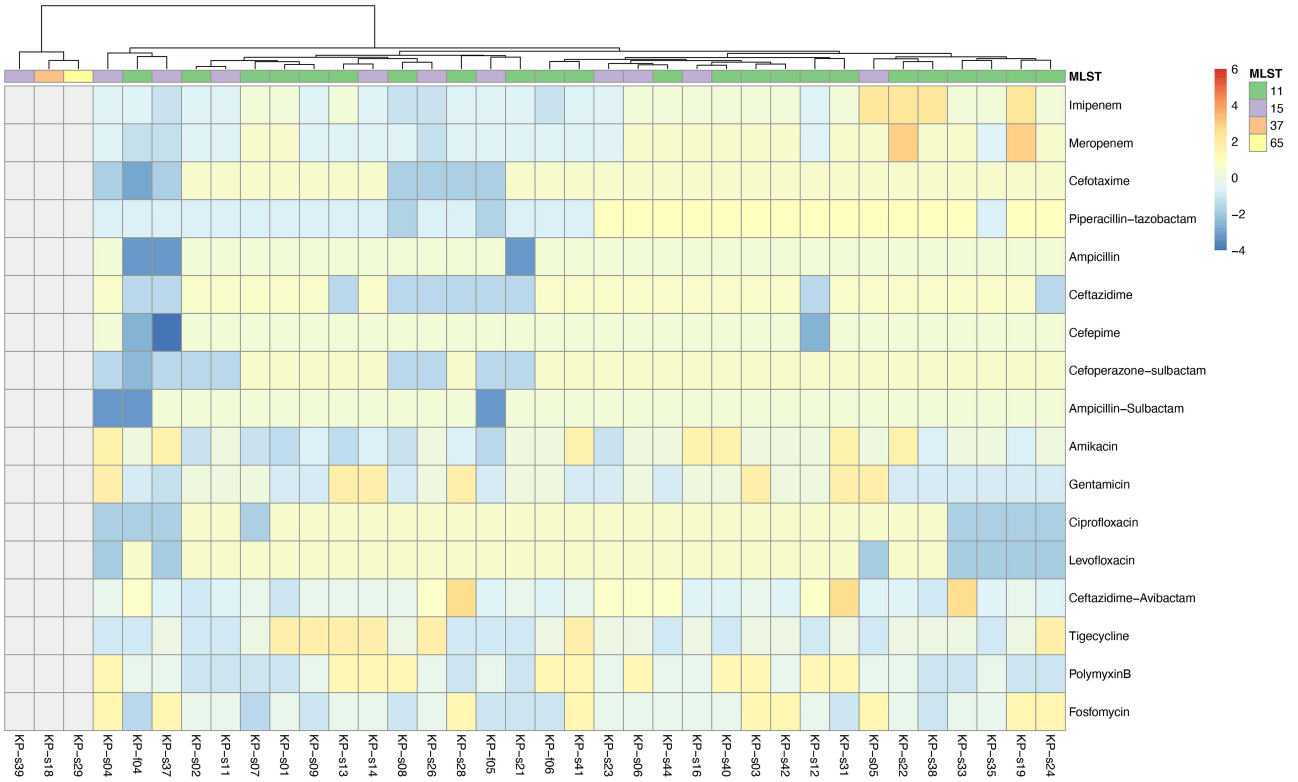

Figure 1 Drug susceptibility heat map of 33 KPC-producing *Klebsiella pneumoniae*. The minimum inhibitory concentrations (MICs) of 33 KPC-producing *Klebsiella pneumoniae* strains were homogenized against 17 antibiotics, and the colors in the legend indicate the change in susceptibility of the strains to the antibiotics,kP-s18, KP-s29 and KP-s39 without carbapenemase resistance genes were indicated in gray.

the KPC-2 gene sequences collected within a certain period (Figure 4). Thus, the results indicated that *blaKPC-2* was relatively conservative among these strains, and there was no base deletion, substitution, or mutation in the process of evolution or transmission.

Plasmid Structure and Potential Mobility of Isolate KP-S26

According to Pacbio combined Illumina sequencing data, KP-s26 contained two cyclic drug-resistant plasmids, pA and pB, and other plasmids do not contain drug resistance genes. Plasmid pA belongs to the IncF family and contains two replicators, IncFIB (K) and IncFII (K). The plasmid size and GC ratio were 202259bp and 52.61%, respectively. The genes for replication, transfer, drug resistance, and stability maintenance can be encoded by 210 open reading frames. The plasmid pA contained 15 drug resistance genes, such as *blaCTX-M-15, mphA, dfrA12, aadA2, sul1*, etc. There were 3 IS26 sequences near *blaCTX-M-15* and *drfA12*. The plasmid map was drawn to compare the genome of the pA plasmid with that of 6 other *Klebsiella pneumoniae* plasmids selected from NCBI. The homology between pA plasmid and pKP18069-CTX, pNMBU-W07E18_01, 203pC2601-2, C2972_2, pC2974_2, p1_015093 was 70%–100%. During plasmid pA formation, partial fragments of several different *Klebsiella pneumoniae* plasmids were fused to form a new heterozygous plasmid. The size of plasmid pB and ratio of G+C was 93992bp and 53.1%, respectively, containing 101 open reading frames. The pB plasmid contained KPC-2 and golS2 drug resistance genes. They showed 100% and 99.36% homology with the previously reported plasmids CP059891.1 and MK191023.1, respectively, and the query coverage was 92% and 100%, respectively, through BLAST analysis of the skeleton sequences of pA and pB. It indicated that the genome of plasmid pA was smaller than that of plasmid CP059891.1, and the two genomes were identical in 92% of the coverage (Figure 5).

There were genes encoding transfer site (oriT), relaxase, type IV coupling protein (T4CP), and type IV secretion system (T4SS) in plasmids pA and pB, which were classified as conjugate plasmids. The details of the five

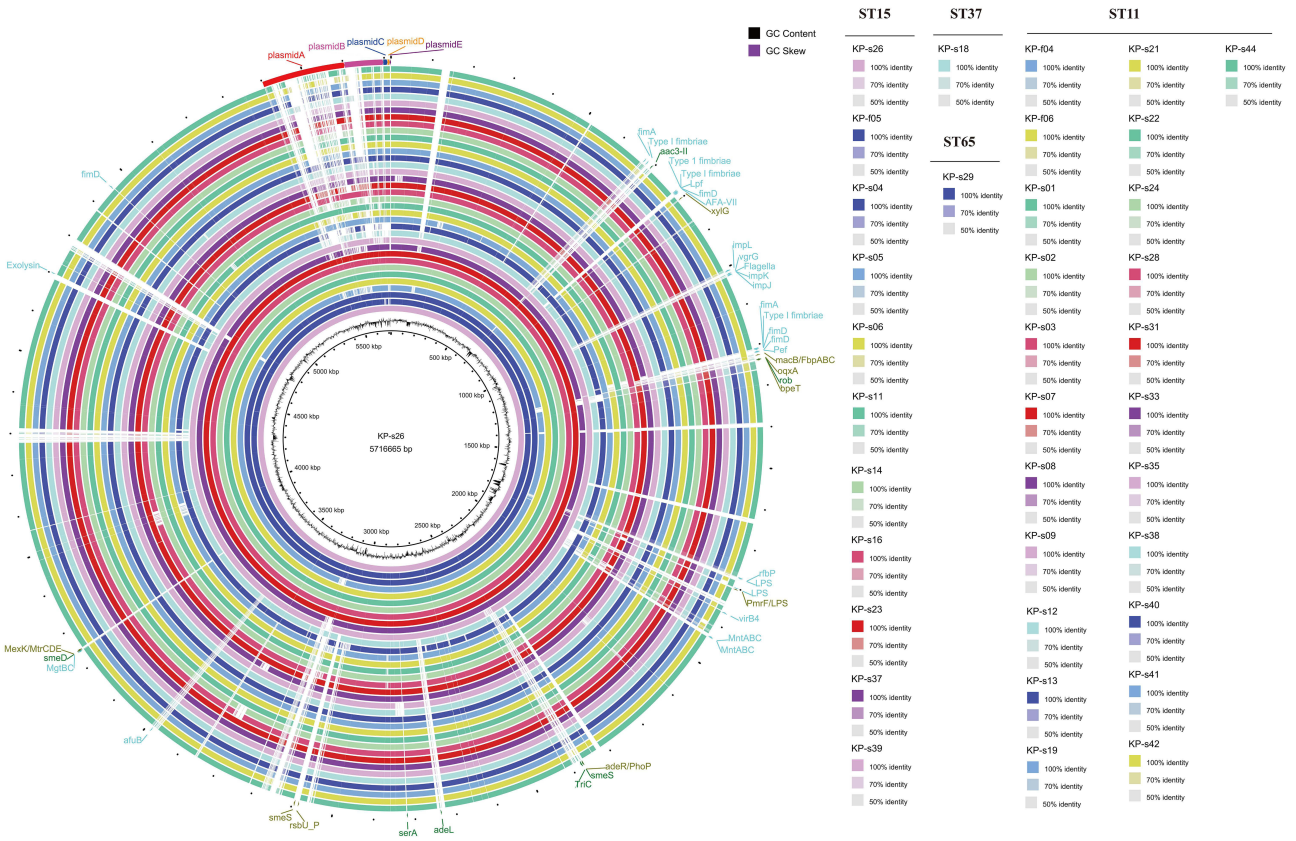

Figure 2 Comparative genomic circle map of 36 strains of *Klebsiella pneumoniae*. Taking KP-s26 as the reference genome, the remaining 35 *Klebsiella pneumoniae* were compared with KP-s26 for circle map.In the legend different colors represent different strains, black is GC content, green is GC offset of the leading chain, and purple is GC offset of lagging chain.Mark the missing genes on the circle map.

circular plasmids carried by KP-s26 are summarized in Table 2.

Discussion

In this study, whole-genome sequencing analysis found that most strains (31/36) carried the carbapenemase gene *blaKPC-2*, with no single nucleotide polymorphism in different strains. Furthermore, most strains had significant differences in the plasmid-encoded region, with little difference in the distribution of resistance and virulence genes. Pacbio combined Illumina sequence conducted in the isolate KP-s26 further demonstrated that it possessed conjugate plasmids pA and pB, which carried drug resistance genes and contained a complete IV secretion system. These results indicated that the *blaKPC-2* carbapenemase gene is relatively conservative in the process of evolution; drug-resistant plasmids containing conjugated transfer elements contribute to the spreading of drug resistance. Surprisingly, all strains with *blaKPC-2* coexisted with chromosomal-associated fosfomycin resistance genes *fosA6*, and the coexistence of *blaKPC-2* and *blaCTX* variants was also detected in 29/31 strains. Meanwhile, our drug sensitivity test has shown that all 36 strains were resistant to β-lactam antibiotics and fosfomycin, suggesting the coexistence of *blaKPC-2* with *fosA6* or *blaCTX-M* variants were associated with increased fosfomycin resistance and broad-spectrum β-lactam resistance, respectively.

Since multidrug-resistant (MDR) bacteria is considered as one of the greatest threats to human health by the World Health Organization, carbapenem-resistant enterobacterales (CRE), particularly carbapenem-resistant *Klebsiella pneumoniae* (CRKP), have spread substantially in recent years.[12,13] With the increasing resistance to other antibiotics, intravenous fosfomycin has been studied in the therapy of various CRKP infections, because it is active against many multidrug-resistant (MDR) pathogens and has a good safety profile and pharmacokinetics.[14,15] According to studies from the USA, the excellent efficacy for fosfomycin against KPC-producing *Klebsiella pneumoniae* was reported, even in colistin and tigecycline-resistant strains (susceptibility

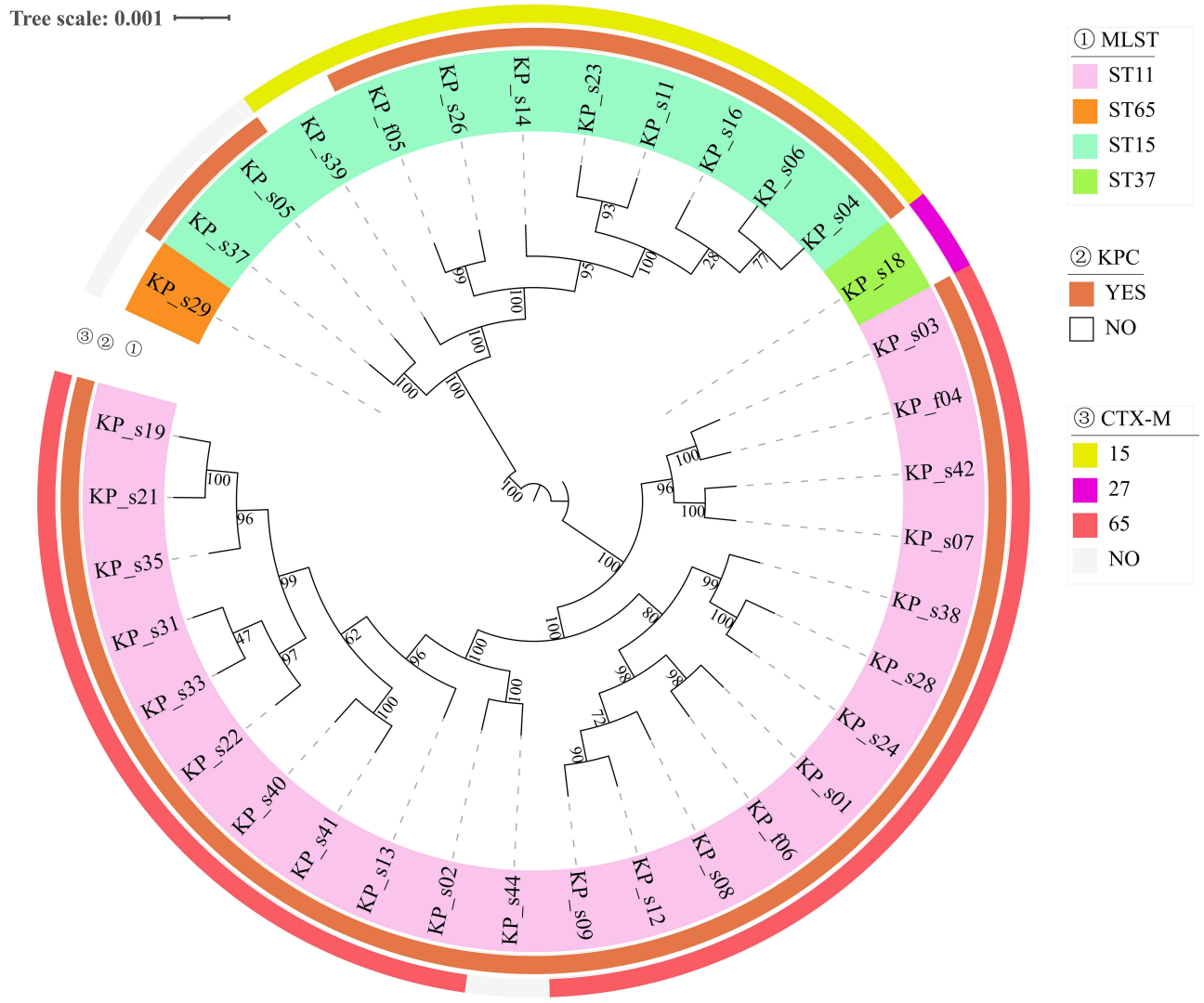

Figure 3 Phylogenetic tree of 36 *Klebsiella pneumoniae*. Construction Phylogenetic tree of 36 *Klebsiella pneumoniae* strains.In the legend different colors were used to represent the ST typing of strains, the expression of *blaKPC* and *blaCTX* in the second and third layers of the figure, respectively.

92% and 93%, respectively).[16,17] Low rates of fosfomycin resistance were also found in European countries, including a considerable proportion of CRE isolates.[18] However, the overall fosfomycin resistance in Asia, particularly China, was much higher than in other areas. Reports from Zhejiang Province, China, found that the fosfomycin resistance rates of CRKP isolates were 80.0% (64/80) and 48.5% (48/99), respectively.[19,20] In another study from China, *fosA3* was considered the primary mechanism responsible for fosfomycin resistance in CRKP isolates, as *fosA3* was responsible for resistance in more than 50% of cases in KPC-producing *Klebsiella pneumoniae*.[21] In our study, the drug resistance rate of fosfomycin reached an alarming 100%. Moreover, all strains had chromosomal-associated fosfomycin resistance genes *fosA6*, and most of them coexisted with *blaKPC-2*. A previous study from Ito et al[22] suggested that chromosomally located *fosA* genes represent a vast reservoir of fosfomycin resistance, and chromosomal *fosA* genes conferred high-level fosfomycin resistance when expressed. Thus, it could be inferred that the high-level fosfomycin resistance in this study was likely due to the high transferability of the chromosomal *fosA6* gene, accounting for intrinsic fosfomycin resistance. The *fosA3* and *blaKPC-2* genes have been reported to colocalize on the same transposon Tn1721,[23] but there is no study so far to report the coexistence of *fosA6* and *blaKPC-2*. More importantly, this raised our concern that the effectiveness of fosfomycin for CRKP isolates in Shanghai, even all over the country, is facing a more rigorous

Table 1 Distribution of Drug Resistance Genes in 36 Strains of *Klebsiella pneumoniae*

Isolates	MLST	β-Lactam Inhibitors	Fluoroquinolone	Macrolide	Aminoglycoside	Sulfonamide	Fosfomycin
KP-f04	ST11	*CTX-M-65 SHV-11 KPC-24*	*emrR,emrB,patA,QnrB4*	*Mrx,mphA,*	*aadA16,rmtB aac(6')-Iy, aac(6')-Ib8*	*sul4*	*fosA6*
KP-f05	ST15	*CTX-M-15 SHV-28 KPC-2*	*acrA,oqxB,oqxA,marA*	*Mrx,mphA,*	*acrD,aac(3)-IIa, aac(6')-Ib8*	*sul1*	*fosA6*
KP-f06	ST11	*CTX-M-65 KPC-2*	*QnrS1,acrB,acrA,marA*	*mphD*	*acrD,aadA3,rmtB, aac(6')-Ib8*	*sul2*	*fosA6*
KP-s01	ST11	*SHV-28 CTX-M-65 KPC-2*	*patA,emrB,QnrB4,acrA, acrB,oqxB,oqxA*	*mphD, mphA, Mrx,msrE*	*acrD, armA, aac(3)-IIa, aadA2, aph(3')-Ia*	*sul1*	*fosA6*
KP-s02	ST11	*CTX-M-65 KPC-2*	*emrR,emrB,patA,QnrS1,acrA, acrB,marA*	*msrE*	*acrD,rmtB,aadA3, aac(6')-Iy*	*sul1,sul2*	*fosA6*
KP-s03	ST11	*KPC-2 CTX-M-65*	*emrR,emrB,patA,QnrB4,acrA, acrB,oqxB,oqxA, marA*	*Mrx,mphA*	*acrD,aadA16, aph(3')-Ia*	*sul1*	*fosA6*
KP-s04	ST15	*SHV-28 CTX-M-15 KPC-2 SHV-28*	*patA,emrB,emrR,acrA, acrB,ramR,oqxB,oqxA, marA*	*Mrx,mphA*	*acrD,aac(3)-IIa, aadA2,aac(6')-Iy, aac(3)-IId*	*sul4*	*fosA6*
KP-s05	ST15	*SHV-165 KPC-2*	*patA,emrB,emrR,QnrB4,acrA, acrB,ramR,oqxB, oqxA,marA*	*mphD, mphA, Mrx,msrE*	*acrD,armA, aac(3)-IIa*	*sul1*	*fosA6*
KP-s06	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,acrA,acrB, oqxB,oqxA,marA*	*Mrx,mphA*	*acrD,aac(3)-IIa, aadA2*	*sul4*	*fosA3 fosA6*
KP-s07	ST11	*SHV-28 CTX-M-65 KPC-2*	*emrR,emrB,patA,acrA, acrB,marA*	*Mrx,mphA*	*acrD,rmtB, aac(6')-Iy*	-	*fosA3 fosA6*
KP-s08	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD, mphA*	*acrD,aadA3, aac(6')-Ib8*	*sul2, sul4*	*fosA6*
KP-s09	ST11	*SHV-134 CTX-M-65 KPC-2*	*patA,emrB,emrR,acrA, acrB*	*Mrx,mphA*	*acrD,baeR,rmtB, aadA2,mdtC, aac(6')-Ib*	*sul1*	*fosA6*
KP-s11	ST15	*SHV-28 CTX-M-15 KPC-2*	*patA,emrB,emrR,ramR, acrA,acrB,oqxB,oqxA, marA,adeF*	*Mrx,mphA*	*acrD,aac(3)-IIa, aadA2*	*sul1*	*fosA6*
KP-s12	ST11	*SHV-134 CTX-M-65 KPC-2*	*patA,emrB,emrR,acrA, acrB*	*Mrx,msrE*	*acrD,aadA2*	*sul1*	*fosA6*
KP-s13	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphA*	*acrD,rmtB,aadA3, baeR*	*sul1,sul2*	*fosA6*

(*Continued*)

Table 1 (Continued).

Isolates	MLST	β-Lactam Inhibitors	Fluoroquinolone	Macrolide	Aminoglycoside	Sulfonamide	Fosfomycin
KP-s14	ST15	*SHV-28 CTX-M-15 KPC-2*	*patA,emrB,emrR,acrA, acrB,oqxA,oqxB,marA*	*mphA,Mrx*	*acrD,aadA2, aac(3)-IIa*	*sul1*	*fosA6*
KP-s16	ST15	*SHV-28 CTX-M-15 KPC-2*	*patA,emrB,emrR,ramR, acrA,acrB*	*mphA,Mrx*	*acrD,aac(3)-IIa, aadA2*	*sul1*	*fosA6*
KP-s18	ST37	*SHV-134 CTX-M-27*	*patA,emrB,emrR,QnrB4,acrB, oqxA,oqxB,adeH*	*mphD,msrE*	*acrD,aadA2, aac(3)-IId*	*sul1,sul2, sul3*	*fosA6*
KP-s19	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,abeM, acrA,acrB,adeF*	*mphD*	*acrD,ant(3'')-IIa, aph(3'')-Ib,aph(6)*	*sul1,sul2*	*fosA6*
KP-s21	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA,adeG*	*msrE*	*acrD,rmtB,aadA3, armA,aac(6')-Ib9*	*sul1,sul2*	*fosA6*
KP-s22	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD, mphA, Mrx,msrE*	*mdtC,mdtB,acrD, rmtB,aadA3,baeR*	*sul1,sul2*	*fosA6*
KP-s23	ST15	*SHV-28 CTX-M-15 KPC-2*	*patA,emrB,emrR,acrA, acrB,oqxB,oqxA,ramR, marA*	*mphA,Mrx*	*acrD,aac(3)-IIa, aadA2*	*sul1*	*fosA6*
KP-s24	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD, mphA*	*acrD,rmtB,aadA3, aac(6')-Ib8*	*sul1,sul2*	*fosA6 fosA3*
KP-s26	ST15	*SHV-28 CTX-M-15 KPC-2*	*patA,emrB,emrR,ramR, oqxA,oqxB,acrA,acrB*	*mphA,Mrx*	*acrD,aadA2, aac(3)-IIa*	*sul1*	*fosA6*
KP-s28	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*Mrx*	*acrD,rmtB,aadA3, aac(6')-Iy*	*sul1,sul2*	*fosA6 fosA3*
KP-s29	ST65	*SHV-11*	*patA,emrB,emrR,acrA, acrB,oqxB,oqxA,marA*	*mphD, mphA, Mrx,msrE*	*acrD,aac(6')-Iy*	*sul4*	*fosA6*
KP-s31	ST15	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*Mrx,msrE*	*acrD,rmtB, aadA3,baeR*	*sul1,sul2*	*fosA6*
KP-s33	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD*	*acrD,rmtB,aadA3*	*sul1,sul2*	*fosA6*
KP-s35	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD,Mrx*	*acrD,aadA3*	*sul1,sul2*	*fosA6*
KP-s37	ST15	*SHV-28 KPC-2*	*patA,emrB,emrR,QnrB4,acrA, acrB,oqxA,oqxB*	*mphD,msrE*	*mdtC,mdtB,acrD, armA,baeR*	*sul1*	*fosA6*
KP-s38	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*msrE*	*acrD,rmtB, aac(6')-Iy*	*sul1*	*fosA6*
KP-s39	ST15	*SHV-28 CTX-M-15*	*patA,emrB,emrR,QnrS1,acrA, acrB*	*mphA,Mrx*	*acrD,aac(3)-IIa*	*sul4*	*fosA6*

(*Continued*)

Table 1 (Continued).

Isolates	MLST	β-Lactam Inhibitors	Fluoroquinolone	Macrolide	Aminoglycoside	Sulfonamide	Fosfomycin
KP-s40	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphA,Mrx*	*mdtC,mdtB,acrD, rmtB,aadA3,baeR*	*sul1,sul2*	*fosA6*
KP-s41	ST11	*CTX-M-65 KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*mphD*	*mdtC,mdtB,acrD, rmtB,aadA3,baeR*	*sul1,sul2*	*fosA6*
KP-s42	ST11	*CTX-M-65 KPC-24*	*patA,emrB,emrR,acrA, acrB,marA*	*mphA,Mrx*	*mdtC,mdtB,acrD, aac(6')-Iy*	*sul1*	*fosA6*
KP-s44	ST11	*KPC-2*	*patA,emrB,emrR,QnrS1,acrA, acrB,marA*	*Mrx*	*mdtC,mdtB,acrD, rmtB,baeR*	*sul2*	*fosA6*

challenge, as the transmission of such resistance will threaten two classes of last-line antibiotics.

All isolates in our study carried at least two or three ESBLs-producing genes (*blaCTX-M, blaSHV*), consistent with the broad-spectrum β-lactam resistance gene carried by carbapenem-resistant *Klebsiella pneumoniae* ST11 from China in recent years.[24] The *blaKPC* gene was found to coexist with *blaCTX-M* variants (*blaCTX-M-15, blaCTX-M-27, blaCTX-M-65*) in 30 strains of *Klebsiella pneumoniae*. Previous investigation has suggested that the coexistence of *blaKPC-2* and extended-spectrum β-lactamases leads to high resistance to β-lactam antibiotics.[25] Thus, it is easy to understand the high level of broad-spectrum β-lactam resistance in our antibiotic susceptibility test. The *blaKPC-2* gene has a complex and changeable genetic environment and can be located in different plasmids.[26] As the most common incompatible plasmid among Enterobacteriaceae, IncF plasmids are the most common type of *blaKPC-2* carrier. It is also the primary carrier of extended-spectrum β-lactamases, especially CTX-M-15.[27,28] Transposon Tn4401 is an active transposon carrying *blaKPC-2* in

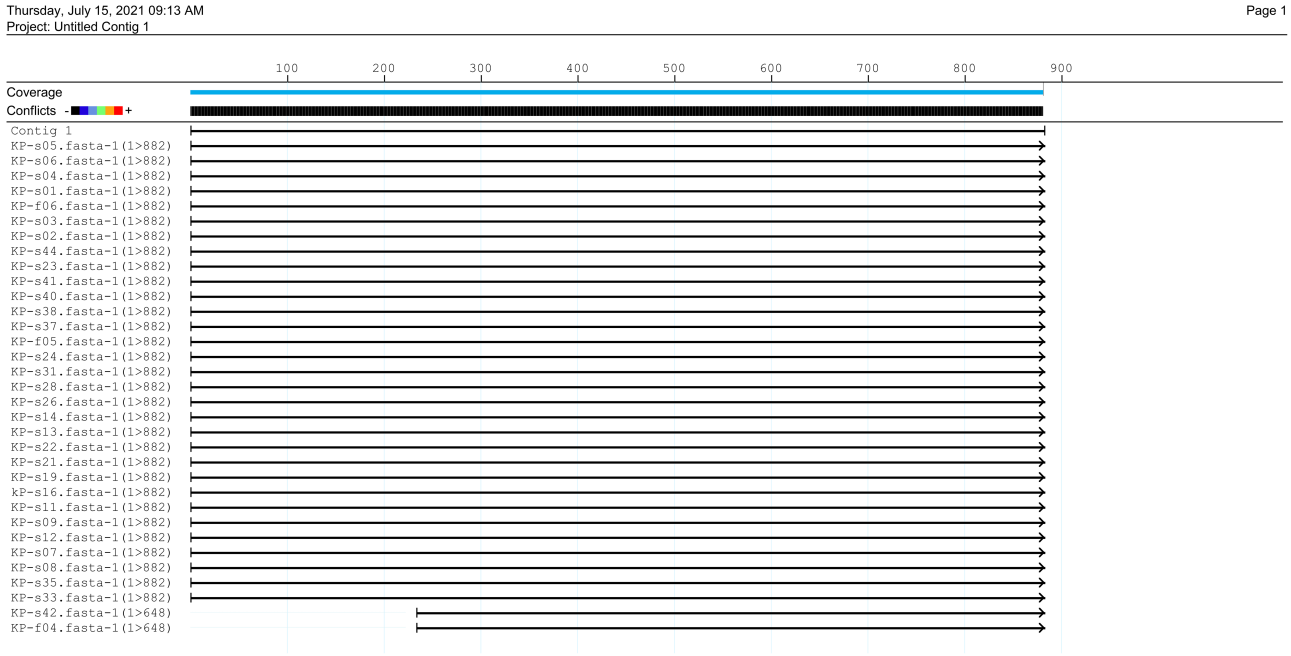

Figure 4 KPC gene sequence comparison of 33 KPC-producing *Klebsiella pneumoniae*. The figure shows the results of KPC gene sequence comparison among 33 KPC-producing *Klebsiella pneumoniae* strains. Different colors indicated the degree of sequence inconsistency, and black indicated that the sequence similarity was 100%. The KPC-24 gene carried by KP-s42 and KP-f04 is located at the edge of the scaffold, and the prediction is incomplete.

Klebsiella pneumoniae, which contains two insertion sequences ISKpn6 and ISKpn7 to mobilize *blaKPC-2* metastasis in the upstream and downstream of *blaKPC-2*.[29] Transposon Tn1721, located on the conjugate plasmid IncFII, is another transposon structure closely related to *blaKPC-2*.[30] Unlike Tn4401 and Tn1721, the KPC gene in ST11 CRKP is mainly located in non-Tn4401 element (NTEKPC). Our study found that the upstream and downstream *blakPC-2* in KP-s26 have no transposon and insertion sequence but contain a complete type IV secretion system. The type IV secretory system-mediated conjugation transfer is one of the crucial mechanisms of horizontal gene transfer.[31] Under the action of this system, *blakPC-2* in conjugated plasmids can be transferred, resulting in the transmission of drug-resistant genes.[32] By optimizing plasmid assembly results in Illumina sequencing of other KPC-producing *Klebsiella pneumoniae*, we demonstrated a complete type IV secretion system locating near the KPC gene. Thus, the results indicated that the conjugation of plasmids plays an important role in the spread of KPC-producing *Klebsiella pneumoniae*.

Indeed, a relatively small number of strains were collected from a single medical center in this study, which is insufficient to truly reflect the evolutionary characteristics of *blaKPC-2* in multidrug-resistant *Klebsiella pneumoniae* in Shanghai. In addition, although we reported the coexistence of the plasmid-encoded *blaKPC* gene and chromosomal fosfomycin resistance gene *fosA6* for the first time, its formation mechanism is not clarified. Therefore, further studies are needed to elucidate the molecular characteristics of *blaKPC* in the transmission process and the molecular mechanism of *blaKPC* and *fosA6* co-harboring.

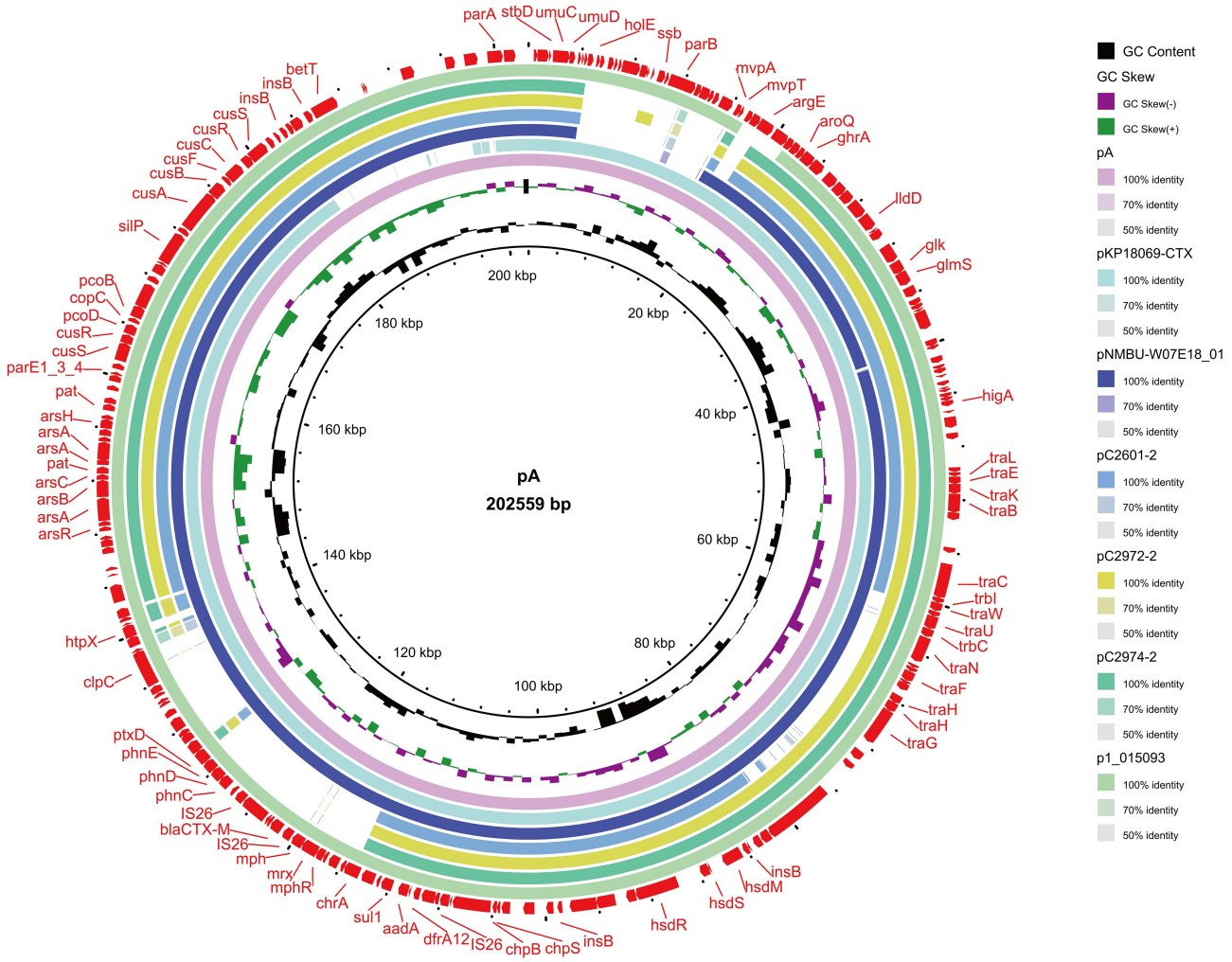

Figure 5 Continued.

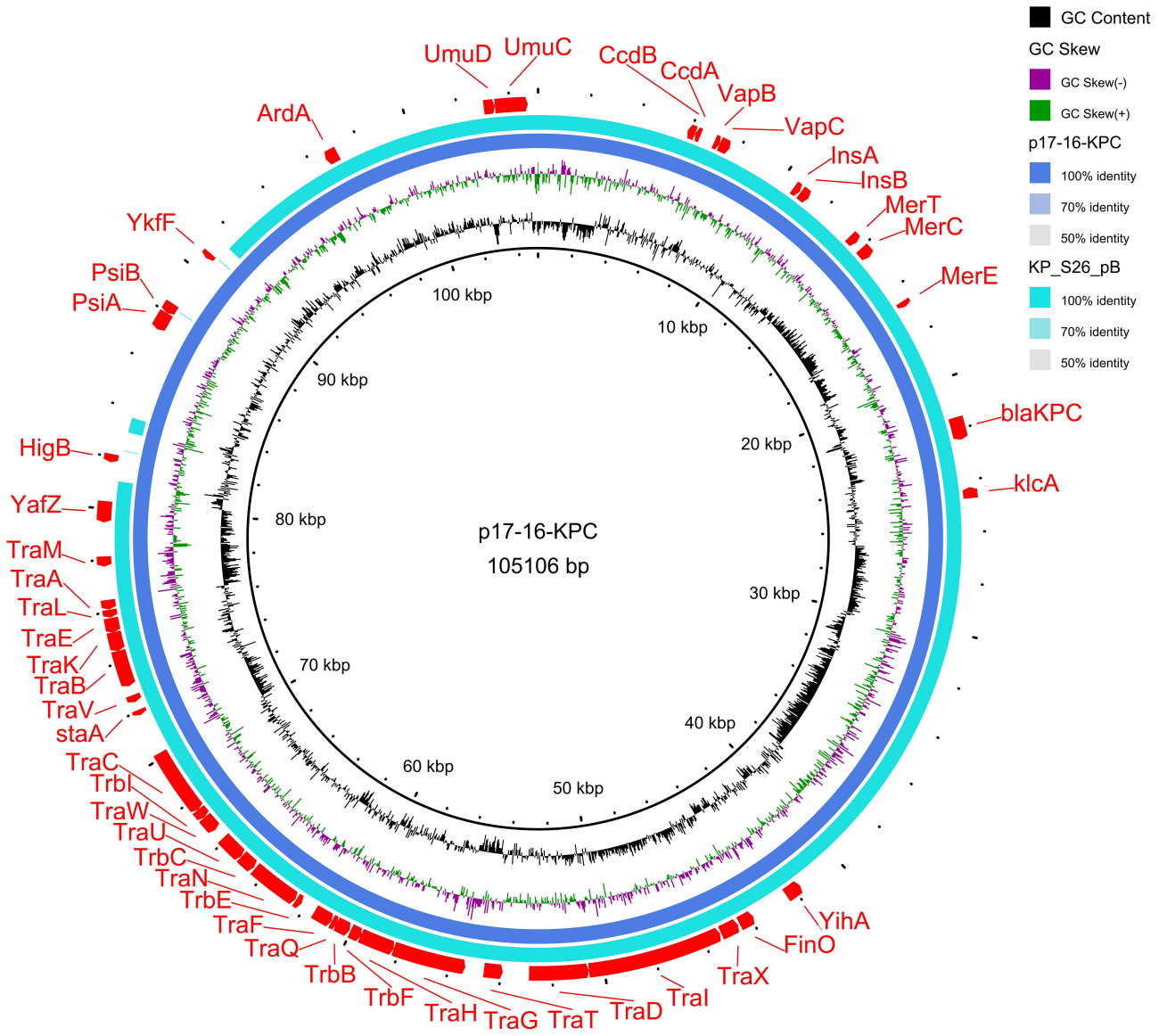

Figure 5 Plasmid map. Plasmid pA were compared with pKP18069-CTX, pNMBU-W07E18_01, pC2601-2, pC2972_2, pC2974_2, p1_015093, and plasmid pB uses MK191023.1 as the reference genome. In the outermost layer of the map, the drug resistance gene, movable element and type IV secretion system carried by the plasmid were marked. The missing portion indicates no expression or less than 50% genomic similarity in the strain genome compared to the reference genome.

In summary, the coexistence of plasmid-encoded *blaKPC* gene with chromosomal fosfomycin resistance gene *fosA6* or the ESBLs-producing genes *blaCTX-M* variants were reported in our study, indicating that the transmission of such resistance seriously affected the effectiveness of fosfomycin in the treatment of CRKP infection in Shanghai. Furthermore, the conjugation of plasmids played an essential role in the spread of KPC-producing *Klebsiella pneumoniae*, although the *blaKPC-2* carbapenemase gene is relatively conservative in the process of evolution.

Table 2 2 Conjugate Plasmids in *Klebsiella pneumoniae* KP-S26

Assembly ID	Length (bp)	Replicon	Plasmid Type	NCBI Blast	Length (bp)	G+C (%)	Identity (%)	Coverage (%)
PlasmidA	202559	IncFIB(K), IncFII(K)	Conjugative	CP059891.1	202,559	52.61	100	92
PlasmidB	93992	/	Conjugative	MK191023.1	93,992	53.1	99.36	100

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author YuHao Liu on reasonable request.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital with the ethics approval number of SHSY-IEC-4.1/18-74/01. All isolates in the study were cultured from bronchoalveolar lavage fluid from ICU patients who had signed informed consent for fiberoptic bronchoscopy and gave informed consent for the isolates to be used in this study. This study was carried out in accordance with the Declaration of Helsinki.

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Author Contributions

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.

Disclosure

The authors report no conflicts of interest in this work.

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