Preplanned Studies

Co-harboring *bla*$_{KPC-2}$ and *bla*$_{IMP-4}$ on an IncP Plasmid in A Clinical Isolate of *Klebsiella pneumoniae* — Shanghai Municipality, China, 2023

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Summary

What is already known about this topic?

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a major threat to global health. The co-production of multiple carbapenemases has emerged as a critical concern, further limiting the effectiveness of last-resort antibiotics such as ceftazidime-avibactam.

What is added by this report?

This study identifies an IncP6 plasmid co-harboring both *bla*$_{KPC-2}$ and *bla*$_{IMP-4}$ in a clinical isolate of *K. pneumoniae*. Comprehensive genomic analysis reveals a complex plasmid structure shaped by recombination events and highlights its potential for mobilization, underscoring the heightened risk of carbapenem resistance.

What are the implications for public health practice?

The emergence and diversification of plasmids co-harboring distinct carbapenemase genes highlight the urgent need for comprehensive genomic surveillance, stringent infection control protocols, and judicious antimicrobial management. These measures are essential to curtail the spread and evolution of multidrug-resistant organisms, which pose a substantial threat to public health globally.

ABSTRACT

Introduction: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a significant global public health threat. The dissemination of resistance is accelerated by plasmids harboring multiple carbapenemase genes, posing a particular challenge to the limited treatment options, including ceftazidime-avibactam.

Methods: In this study, a CRKP strain, KpBSI024, was isolated from a patient with bloodstream infection in the intensive care unit of a tertiary hospital in China. The whole-genome sequencing combined with bioinformatic analysis was used to investigate the structural features of plasmids and associated resistance genes. In addition, conjugation experiments were conducted to assess the transferability of the resistance plasmid.

Results: KpBSI024 exhibited resistance to carbapenems and ceftazidime-avibactam and was identified as sequence type ST1514. Whole-genome sequencing revealed that two carbapenemase genes, *bla*$_{KPC-2}$ and *bla*$_{IMP-4}$, coexisted on a 53 kb IncP6-type plasmid. This plasmid exhibited a complex structure, likely formed through multiple recombination events mediated by IS*26* between plasmids of different Inc types. Although the resistance plasmid encodes a type IV secretion system, it lacks a relaxase gene and is therefore non-self-transmissible; however, it could be transferred at low frequency to *Escherichia coli* with the assistance of a conjugative plasmid. The growth of the transconjugants was not affected by the acquisition of the resistance plasmid, and they displayed resistance profiles to carbapenems and ceftazidime-avibactam similar to the donor strain.

Conclusions: The coexistence of *bla*$_{KPC-2}$ and *bla*$_{IMP-4}$ on an IncP-type plasmid in a clinical *K. pneumoniae* isolate highlights the critical role of recombination events in the dissemination of resistance genes. The emergence of such multidrug-resistant plasmids underscores the urgent need for genomic surveillance and the development of innovative antimicrobial strategies to control the spread of high-risk resistance plasmids.

The worldwide prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) presents a significant public health challenge. The major carbapenemases include KPC (class A), IMP, VIM, and NDM (class B), and OXA-48 (class D), all of which contribute to nosocomial outbreaks. Recent findings have

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highlighted the emergence of clinical isolates co-producing multiple carbapenemases, further complicating the already limited therapeutic options (*1*). This phenomenon is associated with various plasmid-borne carbapenemase genes. Among them, IncP plasmids are notable for their broad host range, high conjugation efficiency, and frequent carriage of resistance genes, facilitating widespread dissemination and microbial adaptation through mobile genetic elements and recombination hotspots (*2*). In this study, a novel IncP6 plasmid that concurrently harbors the $bla_{\text{KPC-2}}$ and $bla_{\text{IMP-4}}$ genes in a *K. pneumoniae* clinical isolate was identified and designated as KpBSI024.

The strain KpBSI024 was isolated in 2023 from a patient with a bloodstream infection admitted to an intensive care unit (ICU) at a tertiary hospital in Shanghai, China, as part of a surveillance study. Antimicrobial susceptibility testing revealed resistance to β-lactam/β-lactam inhibitors and carbapenems, with MICs of 16 μg/mL for both meropenem and imipenem, and reduced susceptibility to ceftazidime-avibactam (Table 1). Whole-genome sequencing using Illumina and Nanopore platforms (Supplementary Methods) revealed that KpBSI024 belongs to ST1514 and KL109, a rare lineage previously associated only with carbapenem-susceptible *K. pneumoniae* (*3*). The genome comprised a chromosome and five plasmids (IncFIB, IncFII, IncQ1, IncP6, and Col440I), with $bla_{\text{KPC-2}}$ and $bla_{\text{IMP-4}}$ co-located on the IncP6 plasmid pKpBSI024-3 (Figure 1A). In addition, KpBSI024 harbors the aminoglycoside resistance genes *aac(3)-IId, aac(6')-Ib-cr*, *aadA16, aph(3')-Ia*, *aph(3'')-Ib*, and *aph(6)-Id*; β-lactam resistance genes $bla_{\text{CTX-M-3}}$, $bla_{\text{SHV-61}}$, $bla_{\text{TEM-1A}}$, and $bla_{\text{TEM-1B}}$; rifamycin resistance gene *ARR-3*; amphenicol resistance gene

TABLE 1. Antimicrobial susceptibility profiles of CRKP KpBSI024 and other strains used in the transfer assay of the resistant plasmid pKpBSI024-3 (p3)*.

Antimicrobial agent†	Antimicrobial susceptibility results (MIC, μg/mL)§				
	***K. pneumoniae* KpBSI024**	***K. pneumoniae* KpBSI024-p0**	***E. coli* C600-pA**	***E. coli* C600-pA-p0-p3**	***E. coli* ATCC 25922¶**
Aztreonam	≥64 (R)	32 (R)	≤1 (S)	≥64 (R)	≤1 (S)
Imipenem	≥16 (R)	≥16 (R)	≤0.25 (S)	≥16 (R)	≤0.25 (S)
Meropenem	≥16 (R)	8 (R)	≤0.25 (S)	≥16 (R)	≤0.25 (S)
Cefepime	≥32 (R)	≥32 (R)	≤0.12 (S)	≥32 (R)	≤0.12 (S)
Ceftazidime	≥64 (R)	≥64 (R)	0.5 (S)	≥64 (R)	≤0.12 (S)
Ceftazidime-avibactam	64/4 (R)	32/4 (R)	0.25/4 (S)	128/4 (R)	≤0.12 (S)
Cefoperazone-sulbactam	≥64 (R)	≥64 (R)	≤8 (S)	≥64 (R)	≤8 (S)
Ticarcillin-clavulanate	≥128 (R)	≥128 (R)	≤8 (S)	≥128 (R)	≤8 (S)
Piperacillin-tazobactam	≥128 (R)	≥128 (R)	8 (S)	≥128 (R)	≤4 (S)
Trimethoprim-sulfamethoxazole	≥320 (R)	≥320 (R)	≤20 (S)	≤20 (S)	≤20 (S)
Amikacin	≤2 (S)	≤2 (S)	≤2 (S)	≤2 (S)	4 (S)
Tobramycin	8 (I)	8 (I)	≥16 (R)	≥16 (R)	≤1 (S)
Ciprofloxacin	≥4 (R)	≥4 (R)	≤0.25 (S)	≥4 (R)	≤0.25 (S)
Levofloxacin	≥8 (R)	≥8 (R)	0.5 (S)	4 (R)	≤0.12 (S)
Doxycycline	≥16 (R)	≥16 (R)	≥16 (R)	≥16 (R)	≤0.5 (S)
Minocycline	4 (S)	8 (I)	8 (I)	4 (S)	≤1 (S)
Tigecycline	≤0.5 (S)	1 (S)	1 (S)	≤0.5 (S)	≤0.5 (S)
Polymyxin	1 (S)	≤0.5 (S)	2 (S)	≤0.5 (S)	≤0.5 (S)

Abbreviation: MIC=minimum inhibitory concentration; R=resistant; S=susceptible; I=intermediate; CRKP=carbapenem-resistant *Klebsiella pneumoniae*; *E. coli*=*Escherichia coli*; CLSI=Clinical and Laboratory Standards Institute.

* The clinical isolate of CRKP KpBSI024 contains the mobilizable plasmid pKpBSI024-3 (p3) co-harboring $bla_{\text{KPC-2}}$ and $bla_{\text{IMP-4}}$. The strain information is provided in Supplementary Figure S1 and Supplementary Table S1 (available at https://weekly.chinacdc.cn/).

† The MIC was determined by the VITEK2 Compact system except for ceftazidime-avibactam. The MIC of ceftazidime-avibactam was determined using the microbroth dilution method.

§ Bacterial antimicrobial susceptibility was interpreted based on the CLSI guidelines 2025 (M100).

¶ *E. coli* ATCC 25922 was used as the quality control strain.

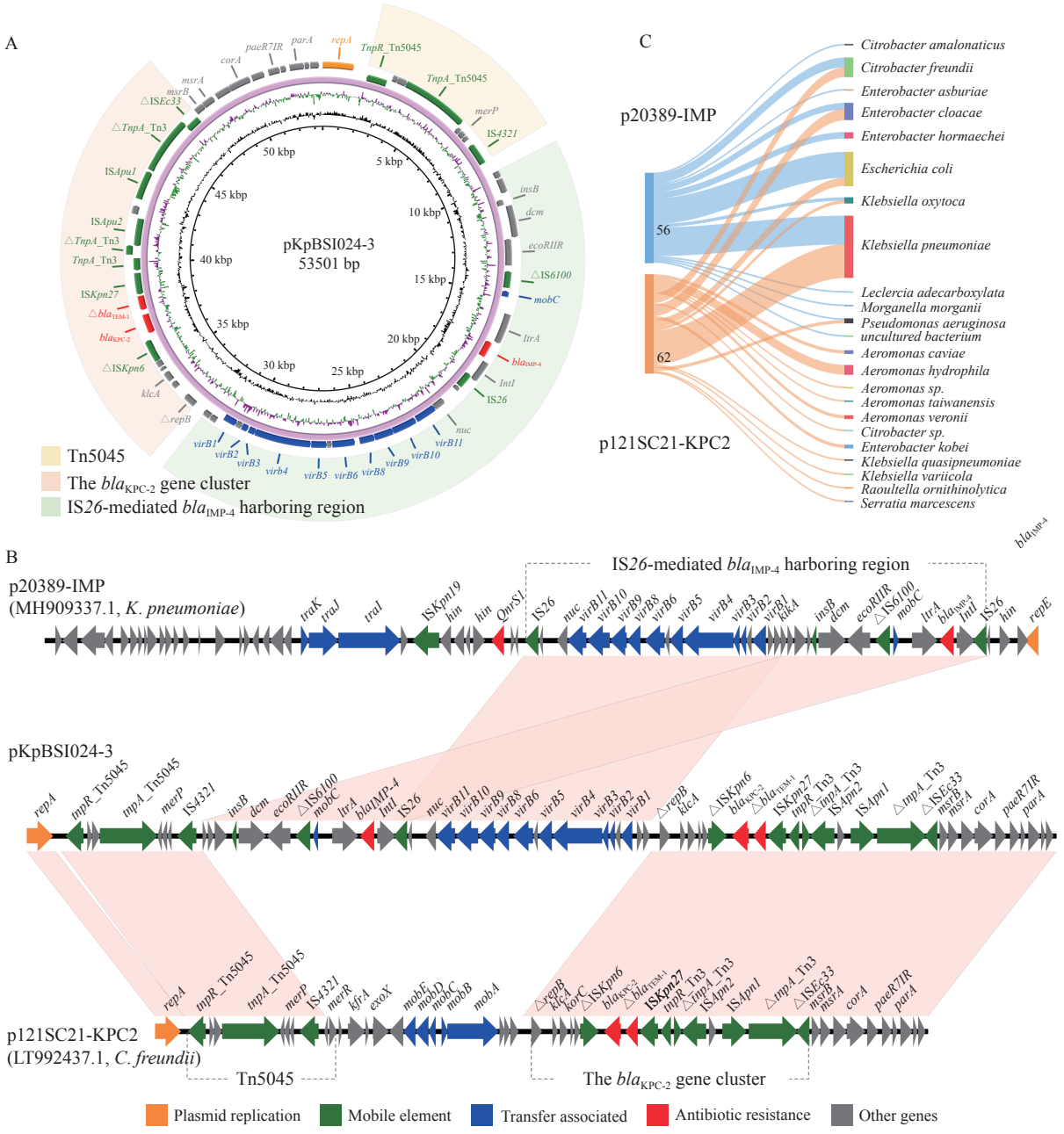

FIGURE 1. Organizational schematic of the bla_{KPC-2} and bla_{IMP-4}-carrying plasmid pKpBSI024-3 from the *K. pneumoniae* clinical isolate KpBSI024. (A) Plasmid map annotated by gene function; (B) Linear comparison of pKpBSI024-3 with reference plasmids p121SC21-KPC2 and p20389-IMP; (C) Bacterial hosts carrying similar plasmids with >90% coverage and >99% identity.
Note: For (A), orange: replication; green: mobile elements; blue: transfer; red: resistance; For (C), based on NCBI *nr* database analysis.

floR; quinolone resistance genes *qnrS1, qnrB91, OqxA, OqxB*, and *mdf(A)*; folate pathway antagonist resistance genes *sul1, sul2*, and *dfrA27*; tetracycline resistance gene *tet(A)*; macrolide resistance gene *mph(A)*; and fosfomycin resistance gene *fosA*.

VRprofile2 analysis indicated that pKpBSI024-3 encodes a type IV secretion system but lacks the relaxase gene and chaperone protein, which are essential for conjugation (Figure 1B). Attempts to transfer pKpBSI024-3 from *K. pneumoniae* KpBSI024 into *E. coli* (C600-pA) via conjugation were unsuccessful. However, the mobilization of pKpBSI024-3 was achieved at a frequency of $(2.51\pm2.00)\times10^{-7}$ using the helper conjugative plasmid

pKP2648-34 (Supplementary Material, Supplementary Tables S1–S2, and Supplementary Figure S1, available at https://weekly.chinacdc.cn/). The self-transmissible pKP2648-34, which is 34 kb in size and lacks any antibiotic resistance genes (*4*), was first introduced into KpBSI024. Following acquisition of the pKpBSI024-3 plasmid, *E. coli* (C600-pA) exhibited resistance to carbapenems and ceftazidime-avibactam similar to the donor strain KpBSI024 (Table 1). Growth curve analysis demonstrated that transfer of the 53-kb plasmid pKpBSI024-3 did not impose a substantial metabolic burden on the recipient strain (Supplementary Figure S2, available at https://weekly.chinacdc.cn/).

Comparative genomic analysis revealed that pKpBSI024-3 originated through recombination between two plasmids: IncP6 plasmid p121SC21-KPC2 (55% coverage, 100% identity) (*5*) and IncN plasmid p20389-IMP (43% coverage, 99.99% identity) (*6*) (Figure 1B). Sequence analysis indicated a complex mosaic arrangement, wherein the $bla_{\text{KPC-2}}$ gene resides within a Tn3-based transposon exhibiting a disrupted structure (Δ*ISEc33*-Tn*3*-IS*Apu1*-orf-IS*Apu2*-IS*Kpn27*-Δ$bla_{\text{TEM-1}}$-$bla_{\text{KPC-2}}$-Δ*ISKpn6-korC-klcA-*Δ*repB*). This structure resembles the Tn*1722* transposon unit, a major vehicle for $bla_{\text{KPC-2}}$ dissemination frequently identified in China, characterized by the conserved *ISKpn27-*bla_{KPC-2}*-*Δ*ISKpn6* core. However, unlike typical Tn*1722*, this variant lacks the *tnpR* and *tnpA* transposition genes, and includes multiple additional insertion sequences (e.g., IS*Apu1*, IS*Apu2*, and IS*Ec33*). These findings suggest that sequential insertion events and extensive structural rearrangements contributed to the current mosaic architecture of pKpBSI024-3. Meanwhile, the $bla_{\text{IMP-4}}$ gene is embedded within a class 1 integron associated with IS*26*. Additional mobile genetic elements, including Tn*5045* and IS*4321*, further emphasize the complexity of the plasmid's recombination processes. Based on these findings, it was hypothesized that pKpBSI024-3 was formed through a multi-step recombination process. Initially, the IS*26*-flanked region containing $bla_{\text{IMP-4}}$ was excised from p20389-IMP via IS*26*-mediated homologous recombination, forming a circular intermediate. This structure subsequently integrated into the backbone of p121SC21-KPC2. Notably, this insertion event appeared to be accompanied by deletion of a contiguous region associated with conjugative transfer, resulting in a rearranged backbone.

Furthermore, it is notable that the two plasmids involved in the recombination event leading to pKpBSI024-3 exhibit differing transfer capabilities. The plasmid p20389-IMP is self-transmissible, whereas p121SC21-KPC2 contains a mobilization module (*mobA-mobE*) and may transfer with the assistance of a conjugative plasmid (Figure 1B). Additionally, genomic comparisons with p121SC21-KPC2 and p20389-IMP from the NCBI *nr* database revealed highly similar plasmids across multiple bacterial hosts and datasets. p20389-IMP has 56 similar plasmids, while p121SC21-KPC2 has 62. These exhibited >90% query coverage and >99% nucleotide identity (Figure 1C). The bacterial hosts involved primarily include *K. pneumoniae*, *E. coli*, and *C. freundii*, spanning more than 20 species. Their distribution is geographically diverse, encompassing more than nine countries, with a significant prevalence in China and Spain (Supplementary Table S3, available at https://weekly.chinacdc.cn/). The widespread presence of these highly similar plasmids highlights the potential for recombination and the associated risk of broad dissemination.

DISCUSSION

The identification of *K. pneumoniae* KpBSI024 harboring an IncP6 plasmid containing both $bla_{\text{KPC-2}}$ and $bla_{\text{IMP-4}}$ represents a notable advancement in our understanding of multidrug resistance evolution. Although KPC and IMP carbapenemase genes have been individually associated with various plasmid replicon types, including IncP-type plasmids, this is the first report of their co-localization within a single IncP plasmid. In contrast to prior studies documenting IncP plasmids carrying bla_{IMP} in *Pseudomonas* or bla_{KPC} variants in *Klebsiella* (*7*–*8*), this study highlights a novel evolutionary convergence, underscoring the increased recombination capacity and heightened risk of multidrug resistance. Furthermore, this finding differs from the recently characterized IncN-IncR plasmid co-harboring bla_{KPC} and bla_{IMP} in *K. pneumoniae* ST1393, where recombination was facilitated by IS*Kpn19* and IS*Kpn27* (*9*). These observations emphasize the evolutionary pressures driving plasmid recombination and the emergence of complex resistance mechanisms. Despite the requirement for a helper conjugative plasmid to facilitate mobilization of pKpBSI024-3, its genomic resemblance to plasmids identified across diverse bacterial hosts suggests the potential for widespread

dissemination.

Plasmid pKpBSI024-3 exemplifies the importance of recombination in promoting genetic diversity among resistance determinants. The interplay of Tn*3*-based transposons, IS*26*-associated integrons, and multiple IS elements likely contributed to the integration of resistance genes from various plasmid sources. Such mosaic plasmids pose a considerable threat to antimicrobial treatment, as the co-expression of serine- and metallo-carbapenemases can synergistically compromise the efficacy of available therapies, including next-generation inhibitors such as ceftazidime-avibactam.

From an epidemiological standpoint, the ST1514 lineage remains relatively rare, with prior reports predominantly involving carbapenem-susceptible isolates. The acquisition of a high-risk plasmid such as pKpBSI024-3 may signify a potential shift toward multidrug resistance within this lineage. The limited conjugation observed under laboratory conditions contrasts with the widespread detection of similar plasmids in diverse bacterial hosts, implying possible alternative mechanisms of horizontal gene transfer or environmental adaptation.

The extensive repertoire of resistance genes present on pKpBSI024-3, including those mediating resistance to β-lactams, aminoglycosides, quinolones, and fosfomycin, illustrates the therapeutic difficulties associated with such plasmids. The observed resistance to ceftazidime-avibactam is particularly concerning, as this agent constitutes one of the few remaining treatment options for CRKP infections. The potential for interspecies plasmid transfer further exacerbates these concerns.

The public health implications of such plasmids are profound, as they undermine the efficacy of critical antimicrobial agents and complicate infection control strategies. Continuous surveillance of high-risk plasmids and their derivatives should be prioritized. Moreover, there is an urgent need for novel therapeutic strategies to combat plasmid-mediated multidrug resistance. One promising approach involves the development of CRISPR-Cas-based systems engineered to selectively eliminate resistant plasmids (*10*).

In conclusion, the identification of the IncP6 plasmid co-harboring $bla_{\text{KPC-2}}$ and $bla_{\text{IMP-4}}$ exemplifies the dynamic nature of resistance gene dissemination in *K. pneumoniae*. The public health implications of such plasmids are substantial. Genomic surveillance efforts should prioritize the detection of plasmid-mediated resistance genes, particularly those capable of co-harboring multiple carbapenemases. Rigorous monitoring of plasmid dynamics and resistance gene transmission is essential to prevent the emergence of untreatable infections. Additionally, the development of novel therapeutic strategies targeting plasmid stability and mobility may offer promising approaches to mitigate the spread of multidrug resistance.

Conflicts of interest: No conflicts of interest.

Ethical statement: Approved by the Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (No. RJ2019NO1-3).

Funding: Supported by the National Key Research and Development Program of China (2024YFE0199000) , the National Natural Science Foundation of China (82000011), Hainan Provincial Natural Science Foundation of China (825RC764) and the Decision-Making Consultation Project of Shanghai Jiao Tong University (JCZXSJB2023-13).

doi: 10.46234/ccdcw2025.178

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Submitted: May 14, 2025
Accepted: August 01, 2025
Issued: August 08, 2025

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SUPPLEMENTARY MATERIAL

Whole-genome Sequencing, Assembly, and Annotation

Genomic DNA from the carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolate KpBSI024 was extracted and subjected to both short-read and long-read sequencing using the Illumina HiSeq and Oxford Nanopore MinION platforms, respectively. For Illumina data, read quality was assessed using FastQC, and low-quality bases and adapter sequences were trimmed with fastp (v0.24.0) (*1*), achieving a Q30 > 99%. For Nanopore data, reads shorter than 1 kb were excluded. Hybrid genome assembly was conducted with Unicycler (v0.5.0) (*2*) using default parameters, in which high-quality Illumina reads were used to polish Nanopore reads to enhance assembly accuracy. All chromosomes and plasmids were circularized, confirming complete assembly. Genome annotation was performed using Prokka (v1.12) (*3*), while functional profiling, including antimicrobial resistance gene (ARG) identification, plasmid replicon typing, and detection of mobile genetic elements, was carried out using VRprofile2 (*4*). This tool internally runs Abricate (https://github.com/tseemann/abricate) with the ResFinder (v4.6.0) (*5*) and PlasmidFinder (v2.0.1) (*6*) databases, applying ≥80% coverage and ≥80% identity thresholds. All identified ARGs exhibited >90% coverage and identity. Sequence typing was performed using MLST (v2.0.9) (*7*). Additional analyses utilized BRIG (v0.95) (*8*) and EasyFig (v3.0.0) (*9*).

Bacterial Strains and Conjugation Assays

Antimicrobial susceptibility testing was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (*10*). *K. pneumoniae* KpBSI024 and *E. coli* C600 were used for conjugation assays (Supplementary Figure S1). The *E. coli* C600-p0 and C600-pA strains were obtained from previous studies (*11*–*12*). Detailed information on bacterial strains and plasmids is provided in Supplementary Table S1; primer sequences are listed in Table S2. All strains were cultured in lysogeny broth (LB) at 37 °C with appropriate antibiotics. Conjugation assays were conducted as previously described (*11*). Briefly, overnight cultures of donor and recipient strains were diluted 1:100 in fresh LB medium and incubated at 220 rpm and 37 °C. When cultures reached an OD_{600} of 0.6, 1 mL of each donor and recipient culture was harvested, washed with PBS, resuspended in 100 μL of 10 mmol/L $MgSO_4$ mixed at a 1:1 ratio, and spotted 20 μL mixed cells onto pre-warmed LB agar plates. After overnight incubation at 37 °C, cells were recovered, resuspended in LB medium, serially diluted, and plated on LB agar containing selective antibiotics. When KpBSI024 was the recipient for selection, plates contained 50 μg/mL ceftazidime-avibactam. To select for transconjugants acquiring pKpBSI024-3 in *E. coli* C600-pA, plates contained 50 μg/mL ceftazidime-avibactam, 50 μg/mL apramycin, and 100 μg/mL rifamycin. For transconjugants carrying pKP2648-34, plates contained 200 μg/mL hygromycin, 50 μg/mL apramycin, and 100 μg/mL rifamycin. Following overnight incubation, colony-forming units (CFUs) were enumerated. Transconjugants were confirmed by PCR, and conjugation frequency was calculated as the number of confirmed transconjugants divided by the total

SUPPLEMENTARY TABLE S1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Note	Source
Strain		
KpBSI024	*K. pneumoniae*, ST1514, *bla*$_{KPC-2}$+, *bla*$_{IMP-4}$+, Mem^R, CAZ^R	This study
KpBSI024-p0	*K. pneumoniae*, Transconjugant carrying p0 from C600-p0, Mem^R, CAZ^R	This study
C600-p0	*E. coli*, Transconjugant carrying p0 from KP2648, Rif^R	Wang, et al. (*12*)
C600-pA	*E. coli*, Recipient in the conjugation assay, Apr^R, Rif^R	Zhang, et al. (*11*)
C600-pA-p0-p3	*E. coli*, Transconjugant carrying p0, p3 and pACYC184-Apr; Mem^R, CAZ^R, Apr^R, Rif^R	This study
Plasmid		
p3 (pKpBSI024-3)	Mobilizable, natural plasmid in CRKP KpBSI024, *bla*$_{KPC-2}$+, *bla*$_{IMP-4}$+	This study
p0 (pKP2648-34)	Conjugative, helper plasmid in KP2648 with *hph* insertion, Hm^R	Wang, et al. (*12*)
pA (pACYC184-Apr)	Non-mobilizable, p15A origin of replication, Apr^R	Bartolomé, et al. (*13*)

Abbreviation: CRKP=carbapenem-resistant *Klebsiella pneumoniae*; Apr^R=apramycin resistance; Hm^R=hygromycin resistance; CAZ^R=ceftazidime-avibactam resistance; Rif^R=rifampicin resistance; Mem^R=meropenem resistance.

SUPPLEMENTARY TABLE S2. Oligonucleotide primers used in this study.

Name	Sequence (5'–3')	Description
C600-F	GGGCAAACTCACTCAATTTCTGG	Validation *E. coli* C600
C600-R	CATATCCATCGCCCGGAATATGAAT	
KpBSI024-F	ATGGCTGGTGGTACAGGTAG	Validation *K. pneumoniae* KpBSI024
KpBSI024-R	CGCGTTGGATATAACCATAGCC	
pKpBSI024-3-F	CGTCTAGTTCTGCTGTCTTG	Validation plasmid pKpBSI024_3
pKpBSI024-3-R	CTTGTCATCCTTGTTAGGCG	
pKP2648-34-F	GATACCCTGGCCTTTTAGCC	Validation plasmid pKP2648_34
pKP2648-34-R	TTGACGAAGCAGGGGTAATC	

SUPPLEMENTARY TABLE S3. Distribution of plasmids similar to p20389-IMP and p121SC21-KPC2 identified in the NCBI *nr* database.

Query	Target	Target species	Target length	Query cover (%)	Identities (%)	E value	Collection date	Geographic location
LT992437.1	OW849094.1	*Enterobacter cloacae*	40,714	100	100.00	0	2016	Spain
LT992437.1	CP110880.1	*Enterobacter kobei*	42,506	100	100.00	0	2019/08	China
LT992437.1	OW969881.1	*Citrobacter freundii*	41,032	100	99.99	0	2016	Spain
LT992437.1	OW849032.1	*Raoultella ornithinolytica*	40,356	100	99.99	0	2016	Spain
LT992437.1	OW970224.1	*Klebsiella pneumoniae*	40,441	100	99.98	0	2016	Spain
LT992437.1	OW969860.1	*Klebsiella pneumoniae*	40,441	100	99.98	0	2015	Spain
LT992437.1	OW968457.1	*Klebsiella pneumoniae*	40,730	100	99.98	0	2015	Spain
LT992437.1	OW968248.1	*Klebsiella pneumoniae*	40,424	100	99.98	0	2016	Spain
LT992437.1	OW968211.1	*Klebsiella pneumoniae*	40,509	100	99.98	0	2016	Spain
LT992437.1	OW968195.1	*Klebsiella pneumoniae*	40,815	100	99.98	0	2015	Spain
LT992437.1	OW849136.1	*Klebsiella oxytoca*	40,441	100	99.98	0	2018	Spain
LT992437.1	OW848999.1	*Klebsiella pneumoniae*	40,577	100	99.98	0	2016	Spain
LT992437.1	KY913901.1	*Klebsiella oxytoca*	40,275	100	99.98	0	–	China
LT992437.1	CP032895.1	*Enterobacter kobei*	43,125	100	99.97	0	2017	China
LT992437.1	OW849539.1	*Klebsiella pneumoniae*	40,359	100	99.95	0	2015	Spain
LT992437.1	CP080103.1	*Klebsiella quasipneumoniae*	40,407	100	99.93	0	2018	Argentina
LT992437.1	CP093216.1	*Escherichia coli*	43,534	100	99.75	0	2020/06/25	Croatia
LT992437.1	OW849084.1	*Citrobacter freundii*	40,203	100	99.74	0	2018	Spain
LT992437.1	OW969726.1	*Enterobacter cloacae*	40,408	99	99.97	0	2014	Spain
LT992437.1	OW848977.1	*Enterobacter cloacae*	40,289	99	99.97	0	2016	Spain
LT992437.1	OW849381.1	*Escherichia coli*	40,408	99	99.97	0	2016	Spain
LT992437.1	OW849079.1	*Enterobacter cloacae*	40,646	99	99.97	0	2016	Spain
LT992437.1	CP182930.1	*Citrobacter freundii*	46,509	99	99.62	0	2022	China
LT992437.1	MN539620.1	*Citrobacter sp.*	40,013	98	99.99	0	–	China
LT992437.1	OW967267.1	*Citrobacter freundii*	40,836	98	99.99	0	2018	Spain
LT992437.1	OW969792.1	*Klebsiella pneumoniae*	39,797	98	99.98	0	2014	Spain
LT992437.1	OW848983.1	*Escherichia coli*	39,780	98	99.98	0	2016	Spain
LT992437.1	CP040685.1	*Pseudomonas aeruginosa*	40,180	98	99.50	0	2017/09/22	China
LT992437.1	MH909348.1	*Klebsiella pneumoniae*	42,055	96	100.00	0	2013	China
LT992437.1	OW969822.1	*Klebsiella pneumoniae*	43,808	96	100.00	0	2014	Spain
LT992437.1	OW969816.1	*Klebsiella pneumoniae*	39,199	96	100.00	0	2012	Spain

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Query	Target	Target species	Target length	Query cover (%)	Identities (%)	E value	Collection date	Geographic location
LT992437.1	OW969809.1	*Klebsiella pneumoniae*	39,216	96	100.00	0	2012	Spain
LT992437.1	OW969787.1	*Enterobacter cloacae*	39,386	96	100.00	0	2012	Spain
LT992437.1	CP028566.1	*Aeromonas hydrophila*	38,976	96	100.00	0	2015	China
LT992437.1	CP026224.1	*Aeromonas sp. ASNIH3*	39,148	96	100.00	0	2014	USA
LT992437.1	KR014106.1	*Aeromonas hydrophila*	44,451	96	100.00	0	–	China
LT992437.1	AP019194.1	*Aeromonas hydrophila*	39,071	96	100.00	0	2018/08	Japan
LT992437.1	OW969906.1	*Citrobacter freundii*	43,157	96	100.00	0	2015	Spain
LT992437.1	CP079843.1	*Escherichia coli*	41,744	96	100.00	0	2020/09	China
LT992437.1	AP022283.1	*Aeromonas veronii*	47,395	96	100.00	0	2019/02/05	Japan
LT992437.1	AP022253.1	*Aeromonas hydrophila*	39,071	96	100.00	0	2018/08/06	Japan
LT992437.1	MN477223.1	*Enterobacter cloacae*	39,013	96	99.99	0	–	China
LT992437.1	OW970313.1	*Klebsiella pneumoniae*	39,114	96	99.99	0	2013	Spain
LT992437.1	OW969914.1	*Klebsiella variicola*	39,199	96	99.99	0	2016	Spain
LT992437.1	OW969903.1	*Klebsiella pneumoniae*	39,388	96	99.99	0	2014	Spain
LT992437.1	OW969864.1	*Klebsiella pneumoniae*	39,148	96	99.99	0	2013	Spain
LT992437.1	OW969856.1	*Klebsiella pneumoniae*	39,199	96	99.99	0	2014	Spain
LT992437.1	OW969852.1	*Klebsiella pneumoniae*	39,216	96	99.99	0	2013	Spain
LT992437.1	OW969826.1	*Klebsiella pneumoniae*	39,182	96	99.99	0	2013	Spain
LT992437.1	OW969796.1	*Klebsiella pneumoniae*	39,182	96	99.99	0	2013	Spain
LT992437.1	MH624130.1	*Aeromonas taiwanensis*	53,205	96	99.99	0	–	China
LT992437.1	CP079826.1	*Aeromonas veronii*	51,662	96	99.99	0	2019/06	China
LT992437.1	MH909350.1	*Klebsiella pneumoniae*	39,014	96	99.98	0	2013	China
LT992437.1	CP018968.1	*Escherichia coli*	44,320	96	99.94	0	2011	Vietnam
LT992437.1	KU578314.1	*Pseudomonas aeruginosa*	38,939	96	99.91	0	–	China
LT992437.1	CP109826.1	*Serratia marcescens*	39,600	95	100.00	0	2021/10	China
LT992437.1	AP022277.1	*Enterobacter cloacae*	46,974	95	99.99	0	2019/02/05	Japan
LT992437.1	OW969689.1	*Citrobacter freundii*	38,347	94	100.00	0	2016	Spain
LT992437.1	OW848790.1	*Citrobacter freundii*	38,483	94	100.00	0	2018	Spain
LT992437.1	CP163081.1	*Citrobacter freundii*	45,124	94	99.99	0	2016/05	China
LT992437.1	AP022243.1	*Aeromonas caviae*	53,629	94	99.97	0	2018/08/06	Japan
LT992437.1	AP019197.1	*Aeromonas caviae*	53,629	94	99.97	0	2018/08	Japan
MH909337.1	CP064181.1	*Citrobacter amalonaticus*	52,790	100	100.00	0	2013/01/18	China
MH909337.1	MF344557.1	*Klebsiella pneumoniae*	56,893	100	100.00	0	–	China
MH909337.1	CP028486.1	*Escherichia coli*	52,864	100	100.00	0	2017/02/07	China
MH909337.1	KT989599.1	*Citrobacter freundii*	53,653	100	100.00	0	–	China
MH909337.1	CP098781.1	*Enterobacter hormaechei*	52,492	100	99.99	0	2017/03	China
MH909337.1	MH909334.1	*Klebsiella pneumoniae*	53,619	100	99.99	0	2011	China
MH909337.1	KU051709.1	*Escherichia coli*	51,600	100	99.98	0	–	China
MH909337.1	CP040895.1	*Leclercia adecarboxylata*	94,635	100	99.98	0	2018/11	China
MH909337.1	CP073923.1	*Klebsiella pneumoniae*	54,314	100	99.98	0	2015/12/23	China
MH909337.1	CP059714.1	*Enterobacter hormaechei*	52,787	100	99.98	0	2019/08/08	China
MH909337.1	KU862632.1	*Klebsiella pneumoniae*	51,591	100	99.97	0	–	–

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Query	Target	Target species	Target length	Query cover (%)	Identities (%)	E value	Collection date	Geographic location
MH909337.1	KT982616.1	*Escherichia coli*	54,449	100	99.96	0	–	China
MH909337.1	KT982615.1	*Escherichia coli*	54,449	100	99.96	0	–	China
MH909337.1	KT989376.1	*Escherichia coli*	54,449	100	99.96	0	–	China
MH909337.1	KT982618.1	*Escherichia coli*	51,589	100	99.95	0	–	China
MH909337.1	KU886034.1	*Klebsiella pneumoniae*	51,599	100	99.93	0	–	China
MH909337.1	MK036890.1	*Klebsiella pneumoniae*	56,047	99	100.00	0	2009	China
MH909337.1	MH909339.1	*Klebsiella pneumoniae*	51,393	99	100.00	0	–	China
MH909337.1	CP102437.1	*Klebsiella pneumoniae*	163,393	99	100.00	0	2021/09/05	China
MH909337.1	KX711879.1	*Pseudomonas aeruginosa*	51,207	99	99.99	0	–	China
MH909337.1	CP052763.1	*Klebsiella pneumoniae*	52,578	99	99.99	0	2013/12/15	China
MH909337.1	MF072962.1	*Citrobacter freundii*	51,104	99	99.99	0	–	China
MH909337.1	KM977631.1	*Klebsiella pneumoniae*	50,742	99	99.99	0	–	China
MH909337.1	CP090265.1	*Escherichia coli*	52,202	99	99.99	0	2020	China
MH909337.1	CP050160.1	*Escherichia coli*	60,074	99	99.98	0	2016/09/28	China
MH909337.1	KU051708.1	*Klebsiella pneumoniae*	51,469	99	99.97	0	–	China
MH909337.1	MH909328.1	*Klebsiella pneumoniae*	50,480	99	99.97	0	–	China
MH909337.1	KU051707.1	*Escherichia coli*	51,362	99	99.96	0	–	China
MH909337.1	MH909336.1	*Klebsiella pneumoniae*	50,717	99	99.91	0	–	China
MH909337.1	ON882014.1	*Klebsiella pneumoniae*	52,398	99	99.79	0	–	China
MH909337.1	CP096924.1	*Enterobacter hormaechei*	55,933	98	100.00	0	2012	China
MH909337.1	KT989598.1	*Enterobacter cloacae*	54,669	98	99.96	0	–	–
MH909337.1	MH727565.1	*Citrobacter freundii*	51,795	98	99.92	0	2014/09/17	China
MH909337.1	KT982613.1	*Klebsiella pneumoniae*	50,979	98	99.91	0	–	China
MH909337.1	KU051710.1	*Citrobacter freundii*	50,546	97	100.00	0	–	China
MH909337.1	KY913900.1	*Klebsiella oxytoca*	61,680	97	99.93	0	–	China
MH909337.1	MW590809.1	*Klebsiella oxytoca*	62,892	96	100.00	0	–	–
MH909337.1	CP050159.1	*Enterobacter cloacae*	56,780	96	99.99	0	2014/06/03	China
MH909337.1	CP090255.1	*Escherichia coli*	49,457	96	99.98	0	2018	China
MH909337.1	CP092465.1	*Citrobacter freundii*	59,165	94	100.00	0	2021/09/27	China
MH909337.1	CP093156.1	*Enterobacter asburiae*	63,489	94	100.00	0	2019/06/19	China
MH909337.1	CP077825.1	*Klebsiella pneumoniae*	49,579	94	100.00	0	2018	Australia
MH909337.1	CP066846.1	*Escherichia coli*	60,935	94	100.00	0	2020/06	China
MH909337.1	CP098488.1	*Enterobacter hormaechei*	57,389	94	100.00	0	2019	China
MH909337.1	KM660724.1	*Morganella morganii*	57,797	94	100.00	0	–	USAn
MH909337.1	CP046118.1	*Enterobacter cloacae*	62,663	94	100.00	0	2017/03/29	China
MH909337.1	CP025965.2	*Klebsiella pneumoniae*	59,730	94	99.98	0	2017	China
MH909337.1	CP050859.2	*Klebsiella pneumoniae*	59,764	94	99.97	0	2016	China
MH909337.1	CP091489.1	*Enterobacter cloacae*	114,676	94	99.95	0	2020/12/13	China
MH909337.1	MW574949.1	*Escherichia coli*	67,069	93	99.99	0	–	Sweden
MH909337.1	MW574945.1	*Escherichia coli*	59,328	93	99.99	0	–	Sweden
MH909337.1	MW574936.1	*Escherichia coli*	67,066	93	99.99	0	–	Sweden
MH909337.1	CP096922.1	*Citrobacter freundii*	62,214	92	99.98	0	2014	China

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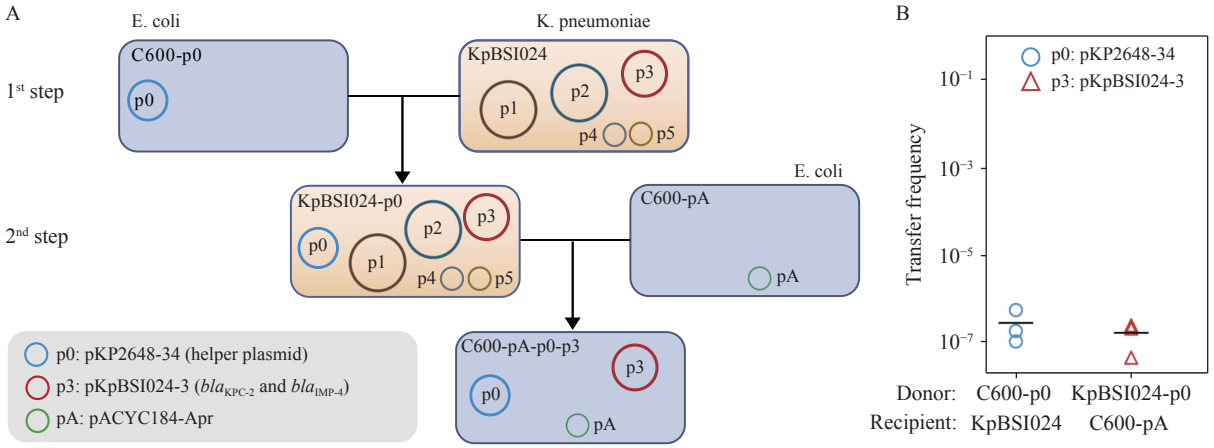
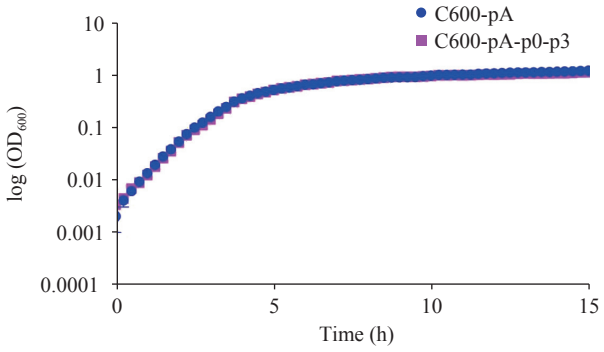
Query	Target	Target species	Target length	Query cover (%)	Identities (%)	E value	Collection date	Geographic location
MH909337.1	LC663729.1	*uncultured bacterium*	67,562	91	99.99	0	–	–
MH909337.1	KJ933392.1	*Escherichia coli*	72,800	91	99.99	0	–	USA
MH909337.1	OP378618.1	*Escherichia coli*	205,325	91	99.98	0	–	–

Note: "–" means data not available.
Abbreviation: NCBI=National Center for Biotechnology Information.



SUPPLEMENTARY FIGURE S1. Transfer of the mobilizable plasmid pKpBSI024-3 (p3), co-harboring *bla*KPC-2 and *bla*IMP-4, facilitated by the conjugative plasmid pKP2648-34 (p0). (A) Schematic representation of the two-step conjugation assay. In the first step, the helper plasmid pKP2648-34 (p0) was transferred from donor *E. coli* C600-p0 to the recipient CRKP *K. pneumoniae* KpBSI024. In the second step, pKpBSI024-3 (p3) was transferred into *E. coli* C600-pA with the assistance of pKP2648-34 (p0); (B) Transfer frequencies of the mobilizable plasmid pKpBSI024-3 (p3) and the conjugative plasmid pKP2648-34 (p0). Donor strains included *E. coli* C600-p0 and *K. pneumoniae* KpBSI024-p0, while recipient strains were *K. pneumoniae* KpBSI024 and *E. coli* C600-pA.
Abbreviation: CRKP=carbapenem-resistant *Klebsiella pneumoniae*; *E.coli=Escherichia coli*.



SUPPLEMENTARY FIGURE S2. Growth curves of *E. coli* C600-pA and its transconjugant *E. coli* C600-pA-p0-p3.

number of recipient cells. All conjugation assays were performed in triplicate.

Bacterial Growth Curve Analysis

To assess growth, overnight cultures of recipient strains and corresponding transconjugants were diluted 1:100 into fresh LB medium, incubated at 37 °C, and shaken at 220 rpm. To generate growth curves, OD_{600} readings were taken every 15 minutes using a spectrophotometer, with three technical replicates for each strain.

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