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ORIGINAL RESEARCH

A One Health Perspective on the Plasmid Backbone Preference and Evolutionary Adaptation of *tmexCD-toprJ* in *Klebsiella spp*

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Background: Antimicrobial resistance (AMR) poses a critical One Health challenge, linking human, animal, and environmental health through the movement of multidrug-resistant (MDR) bacteria and resistance determinants. The *tmexCD-toprJ* gene cluster, an efflux pump conferring high-level resistance to tigecycline and eravacycline. However, its plasmid backbone preferences and evolutionary trajectories in *Klebsiella spp*. remain insufficiently characterized.

Methods: This study investigated the plasmid backbone preference and evolutionary characteristics of *tmexCD-toprJ*-harboring plasmids in *Klebsiella spp*. using whole-genome sequencing of three clinical strains carrying *tmexCD-toprJ* collected from 2018 to 2023. Conjugation assays, comparative genomics, and global epidemiological analysis were performed to assess plasmid mobility, genetic context, and evolutionary direction under the One Health framework.

Results: All three isolates (K7, K36, and K307) exhibited MDR and harbored major resistance genes, including bla_{IMP-4}, *mcr-1.1*, and bla_{NDM-1}, respectively. The plasmid from K36 was transferable to EC600 (frequency, 10^{-7}), confirming cross-species mobility. Global database analysis revealed that *tmexCD-toprJ*-positive *Klebsiella spp*. isolates (n=92) originated mainly from humans (59.8%), followed by animals (37.0%) and environments (3.3%). Phylogenetic and plasmid analyses the *tmexCD1-toprJ1* variant was mainly associated with these hybrid plasmids, frequently co-localizing with *sul1, qnrB*, and *strA/B* to form stable "tigecycline–aminoglycoside–sulfonamide" co-resistance modules. In contrast, *tmexCD2-toprJ2* was more often inserted into classical resistant plasmids.

Conclusion: These findings demonstrate that *tmexCD-toprJ* has evolved as a highly mobile resistance determinant within *Klebsiella spp*. disseminating across the human–animal–environment interface via hybrid plasmids and horizontal gene transfer. This underscores the urgent need for integrated One Health surveillance and containment strategies to mitigate plasmid-mediated multidrug resistance and its global public health impact.

Keywords: tmexCD-toprJ, Klebsiella spp, one health, horizontal gene transfer, hybrid plasmids

Introduction

Antimicrobial resistance (AMR) has become one of the most pressing global health threats of the 21st century, undermining the efficacy of antibiotics and jeopardizing medical and veterinary treatment outcomes.[1–5] The One Health approach, recognizing the interconnectedness of human, animal, and environmental health, has become an essential framework for addressing the multifactorial nature of AMR.[6–9] According to the One Health High-Level Expert Panel (OHHLEP), this approach seeks to sustainably balance and optimize health across species and ecosystems, emphasizing the need for collaborative surveillance and intervention strategies.[10]

Among the numerous resistance threats, carbapenem-resistant *Klebsiella spp*. (CRK) have emerged as critical priority pathogens due to their association with severe infections and high mortality rates.[11] Tigecycline and eravacycline, regarded as last-resort options against CRK, are increasingly compromised by emerging resistance

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mechanisms.[12–15] Recently, the *tmexCD-toprJ* gene cluster, encoding a resistance–nodulation–division (RND) efflux pump, has been identified as a novel mobile determinant conferring high-level resistance to tigecycline, eravacycline, and multiple other antibiotics. Since its first report in *Klebsiella pneumoniae* in 2020,[16] *tmexCD-toprJ* has been detected across multiple Enterobacteriaceae species (eg, *E. coli, Salmonella*)[17–19] and ecological niches[20–23] (eg, food-producing animals, wastewater, and soils). Such findings illustrate a complete One Health transmission chain linking clinical, agricultural, and environmental ecosystems. In this cycle, antimicrobial use in animal husbandry promotes the colonization and evolution of this gene within the animal microbiome. Resistant bacteria or genes can then be transmitted to humans through the food chain, environmental contamination, or direct contact. Additionally, the misuse of antibiotics in human healthcare creates further selective pressure, contributing to the spread of resistance through the environment.[24–26]

Although the *tmexCD-toprJ* gene has been increasingly identified in *Enterobacteriaceae* from animal and environmental reservoirs, existing research has largely concentrated on the phenotypic and strain-level characterization of these isolates,[20–23] while the genetic vehicles driving its dissemination, particularly plasmid backbones, remain insufficiently explored within *Klebsiella* species. In particular, insufficient analysis has been conducted on the selection preferences and evolutionary paths of different *tmexCD-toprJ* variants across diverse plasmid types, which limits our ability to anticipate their spread. Plasmid evolutionary analyses are therefore essential to understand how horizontal gene transfer (HGT) connects resistance dissemination across the One Health interface. Moreover, the genetic diversity of its plasmid backbones, its association with other resistance determinants such as *mcr* and bla_{NDM}, and its potential for cross-species horizontal transfer have not been systematically elucidated. Understanding the plasmid preference of *tmexCD-toprJ* dissemination is critical for anticipating the emergence of multidrug-resistant strains.

In this study, we investigated the molecular and evolutionary characteristics of three *tmexCD-toprJ*-positive *Klebsiella spp*. isolates collected from Zhongnan Hospital of Wuhan University, Hubei Province, China from January 2018 to December 2023. Combining whole-genome sequencing (WGS), conjugation assays, and global genomic epidemiology, our objectives were to systematically analyze the characteristics of hybrid *tmexCD-toprJ*-harboring plasmids, subtype-specific plasmid backbone preferences and evolutionary trajectories, and the formation of stable co-resistance modules in *Klebsiella spp*. This integrative analysis provides novel insights into the plasmid-mediated evolution of *tmexCD-toprJ* and offers a scientific basis for designing One Health-based strategies to curb multidrug resistance.

Materials and Methods

Bacterial Isolates and Identification

This study was conducted at Zhongnan Hospital of Wuhan University (Hubei, China).[27–29] As part of routine clinical carbapenemase phenotyping surveillance, 345 CRK isolates were collected from January 2018 to December 2023. Duplicate isolates from the same patient were excluded based on patient identification and initial isolation date. This ensured the inclusion of only unique isolates in the analysis. All strains were preliminarily screened for carbapenem resistance (meropenem MIC ≥4 mg/L) and tigecycline non-susceptibility (MIC ≥4 mg/L) using the VITEK 2 Compact system (bioMérieux, France). Among 26 tigecycline non-susceptible isolates, three strains (K7, K36, K307) were confirmed to carry *tmexCD-toprJ* by whole-genome sequencing. Strains were subcultured on Luria-Bertani (LB) agar (Oxoid, UK) at 37°C for 24 hours and identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; Microflex LT, Bruker Daltonics, Germany) with standard library matching for species identification, and verified by ribosomal multilocus sequence typing (rMLST, https://pubmlst.org/species-id).

For isolates recovered from non-sterile sites, infection or colonization was adjudicated by review of medical records, integrating compatible clinical informations and the treating physician's intention to treat. For respiratory specimens, isolates were considered colonization when culture positivity occurred in the absence of pneumonia-consistent symptoms/imaging and without antimicrobial escalation, whereas cases meeting these criteria were classified as infection.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was initially performed using the VITEK II system (bioMérieux, France) for broad-spectrum antibiotic testing. The results were further confirmed through broth microdilution for tigecycline and eravacycline and agar dilution for other antibiotics, following Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. The antimicrobial susceptibility panel included carbapenems, tetracycline derivatives (tigecycline and eravacycline), polymyxins, and other commonly used antibiotics. Polymyxin susceptibility was interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023) (http://www.eucast.org/) breakpoints (susceptible ≤ 2 mg/L, resistant > 2 mg/L), while eravacycline was evaluated according to the China Antimicrobial Surveillance Network (CHINET, 2023) (www.chinets.com) criteria (susceptible ≤ 1 mg/L). Quality control strains were included in each batch to ensure the accuracy of results. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains. If control MICs deviated from expected ranges, the experiments were repeated to confirm the reliability of the data.

Conjugation Transfer Assay

In the conjugative transfer experiment, donor strains were clinical CRK isolates (K7, K36, K307) harboring *tmexCD-toprJ*, and rifampicin-resistant *E. coli* EC600 as the recipient strain. Donor and recipient strains were cultured on LB agar at 37°C for 18 hours, then transferred to LB broth and incubated at 37°C with shaking until they reached the logarithmic growth phase. The donor and recipient strains were then mixed at a 1:1 ratio and incubated statically at 37°C for an additional 18 hours to promote conjugation. Transconjugants were selected on LB agar containing rifampicin (1024 mg/L) and tigecycline (2 mg/L).

Putative transconjugants were confirmed via polymerase chain reaction (PCR) targeting *tmexCD-toprJ*, followed by Sanger sequencing (Wuhan Tianyi Huayu Gene Technology Co., Ltd, Wuhan, China) to validate sequence identity (≥99% coverage, ≥98% similarity). The positive transconjugant is *Escherichia coli*, and it is positive for *tmexCD-toprJ*. The conjugation frequency was calculated as the number of transconjugants per donor, with results expressed as the mean value of triplicate experiments.[30] This method was used to quantify the efficiency of plasmid transfer.

Whole-Genome Sequencing and Genomic Analysis

Genomic DNA was extracted from clinical Klebsiella isolates using a bacterial genomic DNA extraction kit (Aidlab Biotechnologies Co., Ltd, Beijing, China), following the manufacturer's protocol. The general genomic characteristics were comprehensively assessed using Illumina sequencing for clinical strains. Sequencing services were provided by Personal Biotechnology Co., Ltd. (Shanghai, China). Genome assembly was performed using SPAdes v3.15.5 and Canu v2.2.[31] Antimicrobial resistance genes, virulence genes, and plasmid replicon types were identified by aligning the assembled genomes against the ResFinder, VFDB, and PlasmidFinder databases via the Center for Genomic Epidemiology (https://www.genomicepidemiology.org/). The presence of *tmexCD-toprJ* variants and mobile genetic elements was confirmed using Kleborate v2.3.2.[32] Plasmid mobility and structural modules were analyzed using VRprofile2 (https://tool2-mml.sjtu.edu.cn/VRprofile/).[33] Moreover, plasmids were compared with the homologous plasmid sequences in the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and visualized using BLAST Ring Image Generator (BRIG) v0.95[34] and EasyFig v2.2.3.[35] Phylogenetic relationships among *tmexCD-toprJ*-carrying plasmids were reconstructed using concatenated conserved genes (MCP, portal, terL) aligned by MAFFT (v7.526),[36] trimmed with trimAl (v1.5.0),[37] and inferred using FastTree (v2.1.10)[38] for tree construction. Phylogenetic trees were visualized via Itol (https://itol.embl.de/).[39]

Public Database Retrieval

To characterize the global distribution and evolutionary features of *tmexCD-toprJ*-harboring plasmids in *Klebsiella spp*., we screened publicly available genomes from the NCBI Genome database (last accessed: December, 2024). Genomes were included if they belonged to the genus *Klebsiella*, contained *tmexCD-toprJ* (any subtype) identified by sequence-based screening, and provided sufficient contig or plasmid sequence context for plasmid replicon typing and genetic context analysis. Genomes were excluded if they were duplicates from the same isolate submission, lacked adequate

assembly quality for plasmid context inference, or contained incomplete *tmexCD-toprJ* regions. For isolates with multiple submissions, only one representative genome was retained. The resulting dataset was used for phylogenetic reconstruction, plasmid backbone classification, and co-resistance module analysis.

Results

Clinical Characteristics of Patients with Klebsiella Infections

Three *Klebsiella* strains (K7, K36, and K307) were isolated from patients with severe underlying conditions, including liver cirrhosis, multiple trauma, and intracranial aneurysm. Notably, the patient from whom isolate K7 was recovered was receiving immunosuppressant therapy at the time of sampling. The infections presented as urinary tract infections (K7) and respiratory infections (K36 and K307). According to the predefined clinical criteria, all three isolates were adjudicated as infection strains. The isolates were recovered in June 2019 (K36), November 2019 (K7), and October 2022 (K307), indicating sporadic detection during the 2018–2023 surveillance period. The hospital stays were 60, 17, and 58 days, with a mean of 46.3 days. Two patients recovered after prolonged antibiotic therapy, whereas one patient (K307) died due to septic complications.

All patients received broad-spectrum empirical therapy including β-lactam/β-lactamase inhibitor combinations, carbapenems, and tigecycline. Treatment responses varied, likely due to multidrug resistance and host comorbidities. Detailed clinical features are summarized in Table 1.

Genomic Characteristics of the Three tmexCD-toprJ-Positive Isolates

WGS and comparative genomic analyses revealed distinct genetic architectures among the three *Klebsiella* isolates. Two strains (K7 and K36) were identified as *Klebsiella pneumoniae*, and one (K307) as *Klebsiella variicola*, based on rMLST results. Multilocus sequence typing (MLST) classified the isolates as ST485 (K7), ST63-2LV (K36), and ST264-1LV (K307).

All three strains possessed large circular chromosomes (5.1–5.4 Mb) and multiple plasmids, ranging from 30 to 300 kb in size. Plasmid profiling demonstrated notable diversity and complexity: K7 harbored a large hybrid plasmid pK7-1-tmexCD, carrying *tmexCD2-toprJ2* and bla_{IMP-4}. K36 contained two major plasmids: pK36-1-tmexCD (carrying *tmexCD1-toprJ1* and *mcr-1.1*) and pK36-2-mcr8.2. K307 possessed plasmid pK307-3-tmexCD (*tmexCD1-toprJ1*) along with two bla_{NDM-1}-bearing plasmids. These plasmids belonged predominantly to the IncFIB, IncHI1B, and IncR incompatibility groups, with most displaying hybrid plasmid backbones—indicating extensive recombination.

The distribution of resistant genes showed that all three strains carried *tet (A), aac (6')-Ib-cr* and other drug-resistant genes besides *tmexCD-toprJ* (Table 2). Moreover, Strain K36 harbored a significant mutation in the *ompK36* gene (see supplementary figure S1), which has been implicated in decreased porin expression and increased resistance to carbapenems. The combined effect of the *ompK36* mutation and bla_{DHA} gene was likely the primary mechanism underlying carbapenem resistance in K36. In contrast, no significant mutations in outer membrane proteins were detected in strains K7 and K307; however, these strains harbored bla_{IMP-4} and bla_{NDM}, respectively, which were the main determinants of their carbapenem resistance. No acquired virulence genes were detected in any strain; however, multiple insertion sequences (*IS26, IS903, Tn3*) were widely distributed throughout their genomes, indicating high genomic plasticity. Collectively, these findings suggest that the *tmexCD-toprJ*-positive Klebsiella isolates maintain complex plasmid structures that facilitate horizontal gene transfer and resistance gene co-localization. Detailed results are presented in Table 2.

Antimicrobial Susceptibility Profiles of tmexCD-toprJ-Positive Isolates

All three isolates exhibited high-level multidrug resistance (MDR), with elevated minimum inhibitory concentrations (MICs) to β-lactams, carbapenems, aminoglycosides, fluoroquinolones, and tetracyclines. Specifically, all isolates demonstrated resistance to meropenem (MIC ≥ 8 μg/mL), imipenem, ceftazidime, cefepime, aztreonam, ciprofloxacin, gentamicin, and amikacin.

The three isolates displayed variable susceptibility to last-resort antibiotics: K36 was resistant to colistin but remained susceptible to ceftazidime/avibactam, indicating the presence of mcr genes but absence of carbapenemase activity neutralized

Table 1 Clinical Characteristics of Patients with *Klebsiella* Infection

Patients	Isolates	Sequence Types	Age/ Gender	Wards	Underlying Diseases	Specimen	Isolated Time	Days in Hospital	Empirical Treatment	Discharge Status
1	K7	ST485	67/ Female	Hepatobiliary surgery	Decompensated cirrhosis after autoimmune hepatitis	Urine	November, 2019	60	MFX, MEM, TGC, AMK, TZP, IPM	Improved
2	K36	ST63-2LV	54/ Male	ICU	Multiple trauma	Sputum	June, 2019	17	IPM, AMK, PB, TGC, MEM	Improved
3	K307	ST264-1LV	69/ Female	Neurosurgery	Basilar artery aneurysm	Sputum	October, 2022	58	IPM, BPM, CSL, CAZ	Died

Abbreviations: ICU, intensive care unit; MXF, moxifloxacin; MEM, meropenem; TGC, tigecycline; AMK, amikacin; TZP, piperacillin-tazobactam; IPM, imipenem; PB, polymyxin B; BPM, biapenem; CSL, cefoperazone/sulbactam; CAZ, ceftazidime.

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Table 2 Basic Information of the Three Isolates Investigated in This Study

Isolate	Species	MSLT	Chromosome or Plasmid/Size	Plasmid Type	Resistance Genes
K7	*Klebsiella pneumoniae*	ST485	Chromosome (5309442bp)	–	$bla_{SHV-191}$, $bla_{SHV-110}$, bla_{SHV-27}, *fosA, OqxA, OqxB*
			pK7-1-tmexCD (319684bp)	IncHI1B,	*tmexCD2-toprJ2, aac (3)-IId, aac (6')-Ib-cr,* bla_{SFO-1}, bla_{TEM-1B}, bla_{IMP-4}, *qnrS1, catB3*
			pK7-2 (56118bp)	IncR	*aadA1, aph (6)-Id, aph (3")-Ib,* bla_{LAP-2}, bla_{OXA-10}, *cmlA1, floR, qnrS1, arr-2, sul2, tet (A), dfrA14, strA, strB*
			pK7-3 (4091bp)	Col440I	-
K36	*Klebsiella pneumoniae*	ST63-2LV	Chromosome (5178805bp)	-	$bla_{SHV-110}$, bla_{SHV-81}, *fosA6, OqxA, OqxB*
			pK36-1-tmexCD (315778bp)	IncHI1B, IncFIB	*tmexCD1-toprJ1, mcr-1.1, aadA1, aadA3, aph (6)-Id, aph (3')-Ib, aph (4)-Ia, aac (3")-IV, armA,* bla_{DHA-1}, *msr (E), mph (E), cmlA1, qnrB4, sul1, sul3*
			pK36-2-mcr8.2 (134839bp)	IncFIA, IncFII, IncQ1	*mcr-8.2, aac (3)-IId, aadA16, aph (6)-Id, aph (3')-Ia, aph (3")-Ib, aac (6')-Ib-cr, mph (A), floR, arr-3, sul1, sul2, tet (A), dfrA27, strA, strB*
K307	*Klebsiella variicola*	ST264-1LV	Chromosome (5440965bp)	-	bla_{LEN19}, bla_{LEN16}, *fosA, OqxA, OqxB*
			pK307-1-NDM (388308bp)	IncHI1B, IncU	*aac (3)-IId, aadA16, aadA2,* $bla_{CTX-M-14}$, bla_{TEM-1C}, bla_{NDM-1}, *mph (A), catA2, qnrS1, arr-3, sul1, dfrA12, dfrA27*
			pK307-2 (250454bp)	IncFIB, IncFII	*aac (3)-IIa, aph (6)-Id, aph (3')-Ib, aac (6')-Ib-cr,* $bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}, *catB3, qnrB1, sul2, tet (A), dfrA14, strA, strB*
			pK307-3-tmexCD (150817bp)	IncFII, IncR	*tmexCD1-toprJ1, aph (6)-Id, aph (3")-Ib, strA, strB*
			pK307-4-NDM (44962bp)	IncX3	bla_{NDM-1}
			pK307-5 (3951bp)	Col (pHAD28)	-
			pK307-6(3335bp)	ColRNAI	-

by avibactam. K7 and K307 were susceptible to colistin but resistant to ceftazidime/avibactam, consistent with the presence of metallo-β-lactamases (bla_{IMP-4}, bla_{NDM-1}). Tigecycline and eravacycline MICs exceeded the non-susceptible thresholds (tigecycline MIC≥4 μg/mL, eravacycline MIC≥1 μg/mL), confirming that the *tmexCD-toprJ*-encoded RND efflux pumps confer cross-resistance to novel tetracycline antibiotics. Detailed results are presented in Table 3.

Overall, these data indicate that the coexistence of *tmexCD-toprJ* with *mcr,* bla_{NDM}, and bla_{IMP} in diverse plasmid backgrounds creates a formidable multidrug-resistant phenotype, severely limiting therapeutic options and underscoring the clinical relevance of plasmid-mediated resistance evolution.

Table 3 Antimicrobial Susceptibility Testing Results of Clinical *Klebsiella* Isolates and Transconjugants/Transformants

Strain	MIC (μg/mL)												
	MEM	IMP	CAZ	CAZ/AVI	TGC	COL	FEP	ATM	CIP	CHL	AMK	GEN	ERV
Clinical isolates													
K7	8	4	>128	>128	32	2	16	>128	>128	>128	4	>128	4
K36	8	16	>128	4	32	>128	8	>128	>128	>128	>128	>128	4
K307	8	4	>128	>128	16	2	128	>128	16	>128	8	>128	4

(*Continued*)

Table 3 (Continued).

Strain	MIC (μg/mL)												
	MEM	IMP	CAZ	CAZ/AVI	TGC	COL	FEP	ATM	CIP	CHL	AMK	GEN	ERV
Recipients													
E. coli EC600	<0.125	<0.125	0.25	<0.125	<0.125	<0.125	<0.125	0.5	0.125	4	2	1	0.125
Transconjugants													
EC600 :: pK36-1-tmexCD	<0.125	0.25	0.125	<0.125	4	32	<0.125	<0.125	2	32	>128	>128	2
EC600 :: pK36-2-mcr8.2	<0.125	0.25	0.25	0.25	0.5	16	<0.125	<0.125	1	>128	2	8	0.25
Control strain													
ATCC 25922	<0.125	<0.125	0.5	0.25	0.25	0.5	<0.125	<0.125	<0.125	8	2	0.5	0.064
ATCC 700603	<0.125	<0.125	4	0.5	0.5	1	8	8	4	32	1	1	0.125

Abbreviations: MEM, meropenem; IPM, imipenem; CAZ, ceftazidime; CAZ/AVI, ceftazidime/avibactam; TGC, tigecycline; COL, colistin; FEP, cefepime; ATM, aztreonam; CIP, ciprofloxacin; CHL, chloramphenicol; AMK, amikacin; GEN, gentamicin; ERV, eravacycline.

Conjugative Transfer and Plasmid Mobility

Both donor and recipient strains were cultured under controlled laboratory conditions, and the experiments were repeated three times to ensure the reliability of the results. Multiple trials demonstrated that plasmids from K36 were transferable to *E. coli* EC600, whereas those from K7 and K307 were not. The transfer frequencies of pK36-1-tmexCD and pK36-2-mcr8.2 were 8.07×10^{-7} and 9.68×10^{-6} per donor strain, respectively. The transconjugants were confirmed by PCR and sequencing of *tmexCD-toprJ*.

After conjugation, antimicrobial susceptibility testing was performed on the *E. coli* EC600 transconjugants harboring pK36-1-tmexCD and pK36-2-mcr8.2. The results showed phenotypic profiles consistent with the resistance genes carried on the transferred plasmids. Specifically, the EC600 :: pK36-1-tmexCD transconjugant exhibited elevated MIC to tigecycline (≥4 μg/mL), eravacycline, and other tetracyclines, confirming the functional expression of the *tmexCD-toprJ*-encoded RND efflux pump. The EC600 :: pK36-2-mcr8.2 transconjugant demonstrated resistance to colistin, consistent with the presence of the mcr-8.2 gene. No significant changes were observed for β-lactams or quinolones, indicating that the resistance phenotypes were specifically conferred by the respective plasmids. These findings confirm that both plasmids retained their expected resistance functions following successful conjugative transfer. Detailed results are presented in Table 3.

The successful transfer of plasmids from K36 underscores the potential for horizontal gene transfer to spread multidrug resistance among different bacterial species, potentially complicating the treatment of *Klebsiella* infections in clinical settings.

Comparative and Genetic Analysis of the Three tmexCD-toprJ Plasmids

pK7-1-tmexCD and pK36-1-tmexCD were hybrid plasmids, which carried not only *tmexCD-toprJ* but also bla_{IMP-4} and *mcr1.1*, respectively. Interestingly, the pK7-1-tmexCD carried an IncHI1B-like replicon type, while pK36-1-tmexCD contained both IncHI1B and IncFIB replicon types, and pK307-3-tmexCD carried IncFII(K), IncFII(pAR0022), and IncR replicon types.

Although the three plasmids (pK7-1-tmexCD, pK36-1-tmexCD, and pK307-3-tmexCD) showed low similarity, pK7-1-tmexCD shared a highly similar plasmid frames with pA324-IMP, p2019SCSN059_tmexCD_333k, and pFK8966-1 (Figure 1a); pK36-1-tmexCD was similar to pKP1961-1, pSYCC1_tmex_287 and pSBH193-1 (Figure 1b); and pK307-3-tmexCD was similar to pKP9, p1220-CTXM and pKP398-1 (Figure 1c). These findings suggested that these are well-evolved plasmids adapted to *Klebsiella spp*., and when bacteria acquired these plasmids, they gained the resistance phenotypes encoded by these plasmids.

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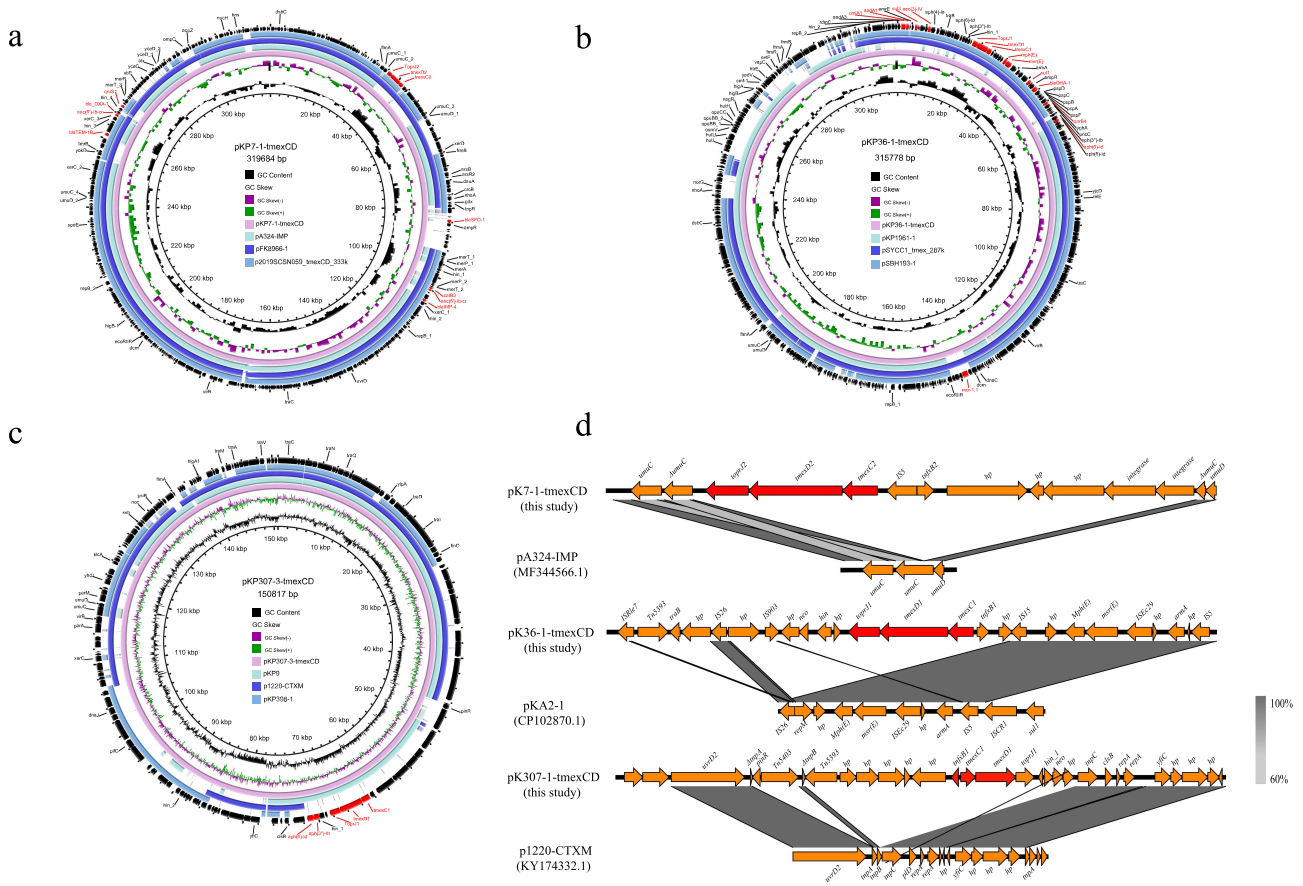

Figure 1 Comparative genomic analysis of *tmexCD-toprJ* plasmids in strains K7, K36, K307 and publicly homologous plasmids. (**a**) Comparative analysis of pK7-1-tmexCD with reference plasmids. From inner to outer rings: sequences of pA324-IMP, pFK8966-1, and p2019SCSN059_tmexCD_333k. Antibiotic resistance genes are highlighted in red. (**b**) Comparative analysis of pK36-1-tmexCD with reference plasmids. From inner to outer rings: sequences of pKP1961-1, pSYCC1_tmex_287, and pSBH193-1. Antibiotic resistance genes are highlighted in red. (**c**) Comparative analysis of pK307-3-tmexCD with reference plasmids. From inner to outer rings: sequences of pKP9, p1220-CTXM, and pKP398-1. Antibiotic resistance genes are highlighted in red. (**d**) Linear comparison of *tmexCD-toprJ* carrying regions in K7, K36, K307 plasmids with public plasmids from the NCBI Nucleotide database, *tmexCD-toprJ* is marked in red.

Therefore, monitoring the horizontal spread of these plasmids in *Klebsiella spp*. and other species remains essential. To further analyze the genetic background of the *tmexCD-toprJ* plasmids, the structures of the three plasmids were investigated in detail. Easyfig analysis showed that there were significant differences in the insertion sites of *tmexCD-toprJ* gene cluster among the three strains: *tmexCD2-toprJ2* was inserted into the *umuC* gene in K7 and produced a direct repeat sequence, *tmexCD1-toprJ1* was inserted into *IS26* in K36, and K307 in the *Tn3* family of transposase genes (Figure 1d). This diversity of insertion patterns suggested that *tmexCD-toprJ* may spread in different hosts through multiple transposition mechanisms. In addition, the insertion of int genes and hypothetical protein-coding genes on both sides of the region further supported the high mobility of the region. These findings further demonstrated that the propagation of *tmexCD-toprJ* was not only dependent on plasmid conjugation but may also spread across genomes through transposition and site-specific recombination.

Prevalence and Host Distribution of tmexCD-toprJ Plasmids

To further investigate the evolution of *tmexCD-toprJ* plasmids in *Klebsiella spp*., genomes were retrieved from the NCBI genome database (as of December 22, 2024). A total of 92 *tmexCD-toprJ*-positive *Klebsiella spp*. strains, comprising 94 *tmexCD-toprJ* plasmids, met the screening criteria. The majority of these strains were *K. pneumoniae* (75/94, 81.5%), followed by *K. quasipneumoniae* (10/94, 10.9%) (Figure 2a). In terms of host origin, human strains accounted for the highest proportion (55/94, 59.8%), primarily from respiratory tract, blood, or urine samples, while strains of animal origin (34/94, 37.0%) and environmental strains (3/94, 3.3%) were less frequent but still significant (Figure 2b). This

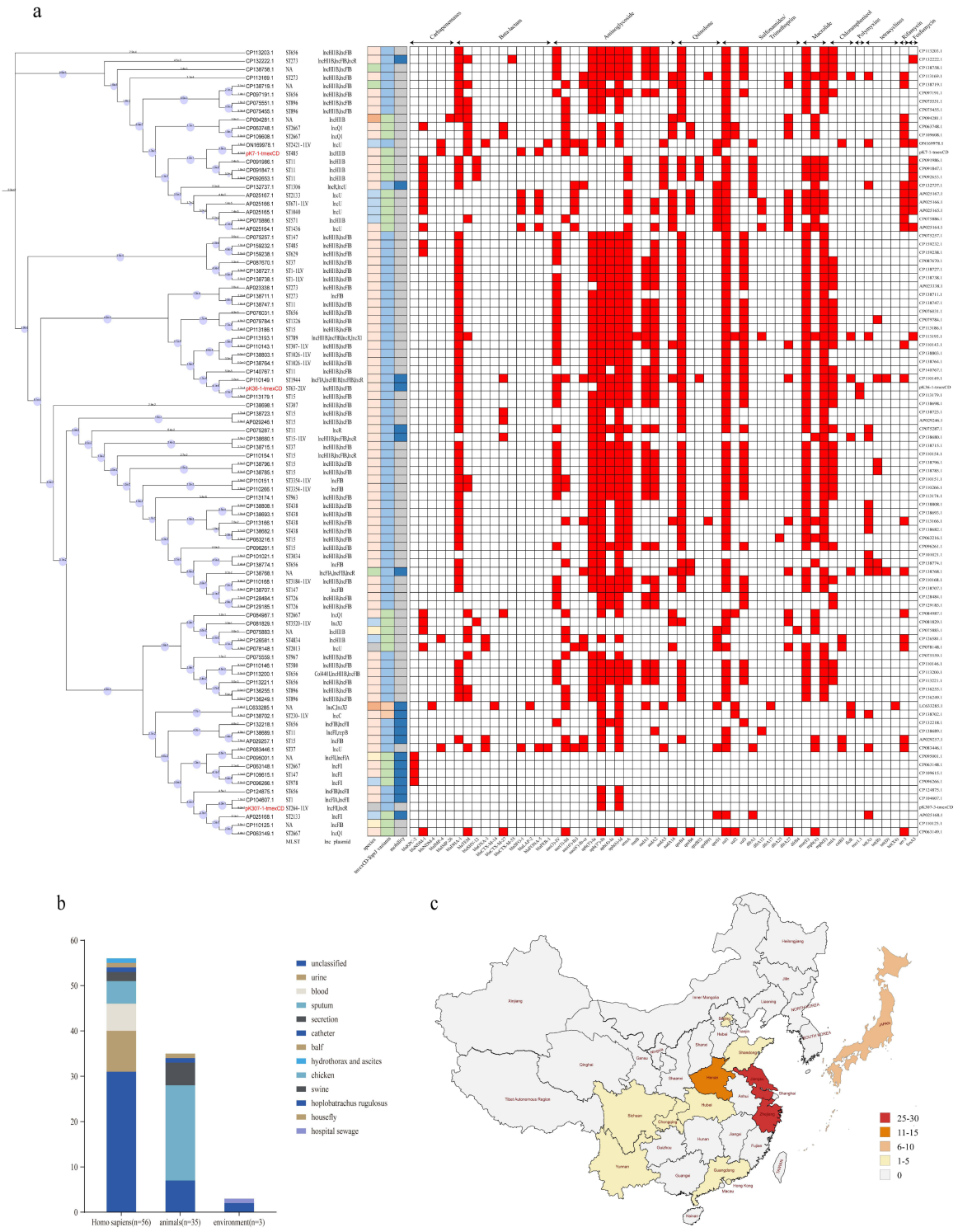

Figure 2 Evolutionary analysis of *tmexCD-toprJ*-positive plasmids in *Klebsiella spp.* and distribution of host origins. (**a**) Phylogenetic tree analysis of plasmid genomes with MLST, plasmid replicons, strain types, conjugative potential, and distribution of antibiotic resistance. (**b**) Stacked bar chart of host origin distribution of *tmexCD-toprJ* positive strains. (**c**) Heatmap of geographical distribution of *tmexCD-toprJ* positive strains.

highlights the role of antibiotic use in animal husbandry and environmental reservoirs in the spread of drug-resistant genes. Geographical distribution analysis revealed that the majority of the *tmexCD-toprJ* positive strains were found in China (84/94, 91.3%) and Japan (8/94, 8.7%), with the strains distributed across 11 provinces in China (Figure 2c).

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MLST analysis (Figure 2a) revealed that these 92 strains covered 41 different ST types, with ST15 (11/94, 12.0%), ST656 (8/94, 8.7%), and ST11 (7/94, 7.6%) forming a mainly clonal group, representing 28.3% of the total strains. Based on the *tmexCD-toprJ* gene sequence analysis (Figure 2a), three variants were identified: *tmexCD1-toprJ1* (68/94, 72.3%), *tmexCD2-toprJ2* (24/94, 25.5%), and *tmexCD3-toprJ3* (2/94, 2.1%). Phylogenetic analysis suggested that these three variants likely originated from a common ancestor but have since diverged into different branches.

These findings indicated that the plasmid diversity of *tmexCD-toprJ* in *K. pneumoniae* was found to be significantly higher than in other species, suggesting that *K. pneumoniae* may serve as an "evolutionary hub" for the horizontal gene transfer of this gene to other species. Furthermore, *tmexCD-toprJ* plasmids showed widespread across different ST types in *Klebsiella*, indicating high host adaptability and transmissibility, mainly through horizontal gene transfer mechanisms such as conjugation and transformation, rather than clonal expansion of specific sequence types (STs).

Plasmid Characterization and Co-Localization of Resistance Genes

Plasmid incompatibility group analysis (Figure 2a) revealed that *tmexCD-toprJ* was predominantly distributed in IncFIB (62/94, 66.0%) and IncHI1B (60/94, 63.8%) plasmids. A significant proportion of these plasmids were hybrid plasmids (61/94, 64.9%), with the majority being IncFIB/IncHI1B hybrid plasmids (46/61, 75.4%). Further analysis indicated that the *tmexCD1-toprJ1* variant was mainly located on hybrid plasmids (59/68, 86.8%), predominantly IncFIB/IncHI1B hybrids (46/59, 78.0%). In contrast, the *tmexCD2-toprJ2* variant was primarily distributed on non-hybrid plasmids (23/24, 95.8%), which were dispersed among IncHI1B (8/24, 33.3%), IncU (5/24, 20.8%), IncF (5/24, 20.8%), and IncQ (4/24, 16.7%). The *tmexCD3-toprJ3* variant was represented by only two isolates and was not subjected to further analysis.

In addition to plasmid-level comparisons, a phylogenetic analysis based solely on the *tmexCD-toprJ* gene cluster sequences was performed (see supplementary figure S2), the phylogeny revealed two major evolutionary clades corresponding to *tmexCD1-toprJ1* and *tmexCD2-toprJ2*, each showing distinct evolutional preferences. Combined with the above results, specifically, *tmexCD1-toprJ1* was enriched in hybrid IncFIB/IncHI1B plasmids (86.8%), supporting its role in driving the formation of multidrug co-resistance modules in highly recombinogenic plasmid environments. In contrast, *tmexCD2-toprJ2* was more frequently integrated into non-hybrid plasmids (95.8%) with carbapenemase genes (such as bla_{KPC}, bla_{NDM}) (20/24, 83.3%), suggesting that this variant has adapted to classical carbapenemase plasmids through transposon-mediated insertion. These results indicate distinct evolutionary trajectories: tmexCD1-toprJ1 tends to undergo structural recombination to form hybrid plasmids, whereas tmexCD2-toprJ2 integrates into classical resistance plasmids, facilitating parallel dissemination under varying antimicrobial pressures.

Resistance gene co-localization analysis (Figure 2a) showed that *tmexCD-toprJ* plasmids commonly carried multiple resistance genes, with *sul1* (76/94, 80.9%), *strA/B* (74/94, 78.7%), and *qnrB* (66/94, 70.2%) being the most frequently co-localized genes. In both IncFIB and IncHI1B plasmids, *tmexCD-toprJ* exhibited high co-occurrence rates with *strA/B, sul1, qnrB*, and bla_{DHA-1} (>85%). These findings suggested that *tmexCD-toprJ* co-evolves with multiple common resistance genes, forming a "tigecycline-aminoglycoside-sulfonamide" co-resistance module and contributing to cross-resistance to tigecycline and other antibiotics. These results demonstrated that the evolution and dissemination of *tmexCD-toprJ* are driven by its subtypes. By adapting to distinct plasmid backbones and integrating diverse resistance genes, they ultimately propagate through ecosystems as multidrug- or pan-drug-resistant, thereby shaping their current complex profile of resistance transmission.

Discussion

The rising prevalence of antimicrobial resistance represents one of the most pressing challenges across human medicine, veterinary science, and environmental health.[1–5] In this context, the "One Health" concept has been increasingly recognized as an essential framework for understanding and containing the spread of resistant pathogens and resistance genes.[6–9] It emphasizes that the health of people, animals, and the environment is closely interconnected, and that effective resistance control requires integrated surveillance and intervention across all these domains.[36,37] This study provides a genomic and epidemiological characterization of *tmexCD-toprJ*-carrying *Klebsiella spp*. within the One Health framework, revealing its evolutionary plasmid selection preferences, co-evolution with multiple resistance determinants, and high potential for interspecies transmission. Three clinical isolates carrying *tmexCD-toprJ* were

identified from carbapenem-resistant, tigecycline-non-susceptible strains collected between 2018 and 2023. Genomic analysis demonstrated that these strains co-harbored bla_{NDM-1}, bla_{IMP-4}, and *mcr* genes, forming MDR genetic backgrounds that significantly limit treatment options in clinical settings. Importantly, conjugation experiments confirmed that *tmexCD-toprJ*-bearing plasmids can be horizontally transferred with measurable efficiency, underscoring their mobility and public health relevance.

As an RND-type efflux pump, *tmexCD-toprJ* confers resistance to tigecycline, eravacycline, and other antimicrobials. Since its initial identification, this determinant has been reported in various Enterobacteriaceae from clinical, animal, and environmental settings. Our source-tracking analysis showed that *tmexCD-toprJ*-positive *Klebsiella* strains are not confined to human infections—they are also present in animals (37.0%) and the environment (3.3%). This distribution highlights the role of non-human reservoirs in the persistence and amplification of this resistance determinant. Antimicrobial use in livestock production may select for *tmexCD-toprJ*-carrying strains in animals, from which they can enter the food chain or environment.[20–23,40,41] Conversely, human-derived strains can reach environmental compartments through wastewater, completing a transmission cycle that is characteristic of One Health challenges.

This study found an absence of clonal specificity for *tmexCD-toprJ* among *Klebsiella* strains. The gene cluster was identified in multiple STs and even different species (*K. pneumoniae* and *K. variicola*), indicating that its dissemination is not driven by clonal expansion of a particular strain. Instead, phylogenetic and genomic analyses strongly support that horizontal gene transfer plays the dominant role in its spread. Notably, the *tmexCD1-toprJ1* and *tmexCD2-toprJ2* exhibit distinct evolutionary preferences, *tmexCD1-toprJ1* is enriched in hybrid plasmids (IncFIB/IncHI1B) with high recombination potential, while *tmexCD2-toprJ2* tends to integrate into non-hybrid traditional resistant plasmids (eg, bla_{NDM}, bla_{KPC}), suggesting parallel evolutionary routes shaped by different selective pressures.

According to previous reports, the spread of *tmexCD-toprJ* is still primarily dominated by *tmexCD1-toprJ1*.[42,43] Hence, our global dataset revealed that *tmexCD-toprJ* is predominantly carried by hybrid plasmid systems, especially the IncFIB/IncHI1B type. More than 64% of *tmexCD-toprJ*-positive plasmids identified in *Klebsiella* were such hybrid structures, which have been associated with broad host range and increased stability across bacterial species. These plasmids did not only harbor *tmexCD-toprJ*; they also frequently co-carried multiple additional resistance genes, including *sul1, strA/B, qnrB*, and in some cases carbapenemase (bla_{IMP-4}) or polymyxin resistance (*mcr*) genes. This co-localization created a "tigecycline–aminoglycoside–sulfonamide" co-resistance module, significantly limiting therapeutic options.

At the structural level, mobile genetic elements (MGEs) likely played a key role in *tmexCD-toprJ* mobilization and in the formation of hybrid IncFIB/IncHI1B backbones. In our isolates, *tmexCD-toprJ* showed variable insertion sites (eg, within *umuC* or adjacent to *IS* and transposase regions), supporting multiple transposition or recombination routes that could shuffle resistance modules across plasmid scaffolds. Such MGE-mediated plasticity provided a mechanistic basis for the evolutionary success of *tmexCD-toprJ* in *Klebsiella spp.*

Despite its strengths, this study has certain limitations that should be addressed in future research. Firstly, although our experimental and comparative genomic analyses demonstrate interspecies transfer potential and provide evidence of past recombination events that have generated hybrid plasmid backbones, this study offers limited direct evidence of real-time transmission chains within specific clinical, animal, or environmental settings; therefore, any discussion of dissemination dynamics across the One Health interface should be interpreted as inference rather than direct observation. Nevertheless, In this context, we propose several plausible drivers that are consistent with our findings and prior reports, including selective pressure from antimicrobial use in food-producing animals, co-selection mediated by non-tigecycline resistance determinants co-carried on the same plasmids (eg, sulfonamides, aminoglycosides, quinolones, and beta-lactams), and the presence of mixing reservoirs such as wastewater and other environmental compartments that facilitate contact between human- and animal-associated microbiota. Finally, the three clinical isolates were collected in 2019 (two isolates) and 2022 (one isolate), indicating sporadic detection over the 6-year study period; while one patient infected with the *tmexCD1-toprJ1*-positive isolate (K307) died, clinical outcomes are strongly influenced by host factors and infection severity, and subtype-specific associations with mortality cannot be established from this limited sample size. Larger, prospective datasets integrating human, animal, and environmental sampling will be required to validate subtype-specific risks and to more definitively resolve evolutionary trajectories and transmission patterns.

Conclusion

Based on three clinical isolates and comparative analyses of publicly available genomes, our data suggest that *tmexCD-toprJ* exhibits subtype-associated plasmid backbone preferences in *Klebsiella spp*. The frequent co-occurrence of this gene cluster with many resistant genes on hybrid IncFIB/IncHI1B plasmids reflects an alarming trend toward multi-resistance convergence. Nevertheless, given the limited number of clinical isolates and potential sampling biases in public databases, the inferred evolutionary directions should be interpreted cautiously and verified in larger, independent collections. However, it is also clear that conventional infection control measures based solely on clonal typing (eg, MLST) are insufficient to track and contain *tmexCD-toprJ* dissemination. Since the gene spreads primarily via plasmids, surveillance strategies should incorporate plasmid typing and focus on monitoring mobile genetic elements across human, animal, and environmental isolates. These findings emphasize that controlling *tmexCD-toprJ* dissemination requires an integrated One Health strategy, encompassing coordinated antimicrobial stewardship, environmental monitoring, and genomic surveillance. Strengthening molecular surveillance at the human–animal–environment interface will be crucial to interrupt the transmission cycle of this "super-resistance determinant" and to mitigate its impact on global health security.

Abbreviations

AMR, antimicrobial resistance; MDR, multidrug-resistant; OHHLEP, the One Health High-Level Expert Panel; CRK, carbapenem-resistant *Klebsiella spp*.; RND, resistance–nodulation–division; WGS, whole-genome sequencing; STs, sequence types; LB, Luria-Bertani; AST, antimicrobial susceptibility testing; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; CHINET, the China Antimicrobial Surveillance Network; PCR, polymerase chain reaction; MLST, multilocus sequence typing.

Data Sharing Statement

All data used in this study are presented in this published article and supplementary files. Genome sequencing data is publicly available in the NCBI GenBank database under BioProject accession number PRJNA1358567.

Ethics Approval

This study was conducted in accordance with the Declaration of Helsinki. Samples were coded and analysis was performed using an anonymized database. All information involving human participants in this study was approved by the medical ethics committee of Zhongnan Hospital of Wuhan University (2021128K). Informed consent was waived by the ethics committee due to the retrospective nature of the study and the use of anonymized clinical samples. All data were analyzed anonymously.

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.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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