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ORIGINAL RESEARCH

Co-Production of KPC-2 and NDM-5 in a Carbapenem-Resistant *Klebsiella Pneumoniae* Clinical Isolate: Genetic Insights and Risks

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Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), particularly strains co-producing KPC and NDM carbapenemases, poses a severe global health threat due to limited treatment options. Understanding the genetic drivers of resistance and transmission is critical.

Methods: A multidrug-resistant CRKP strain, KP3T58, co-harboring bla_{KPC-2} and bla_{NDM-5}, was isolated from an ICU patient. Whole-genome sequencing and comparative genomic analyses were performed. Plasmid transferability was assessed via conjugation assays using *E. coli* EC600 as the recipient. Virulence phenotypes were evaluated through siderophore production (CAS assay), capsule quantification (uronic acid), serum resistance, and Galleria mellonella infection models. Antimicrobial susceptibility was determined (Vitek-2; CLSI/EUCAST standards).

Results: KP3T58 exhibited resistance to nearly all antibiotics except polymyxin. The strain's multidrug-resistant (MDR) phenotype resulted from a combination of chromosomal mutations and the multiple plasmid-borne resistance genes. Whole-genome analysis identified three key plasmids: a conjugative plasmid pKP3T58_1 (IncFIB/FII/R type, carrying a large resistance gene cluster); a conjugative plasmid pKP3T58_2 (IncI1-I type, high-frequency transfer, carrying bla_{NDM-5}); and a non-conjugative plasmid pKP3T58_3 (IncFII type, carrying bla_{KPC-2}). Conjugation assays confirmed that pKP3T58_1 and pKP3T58_2 could transfer individually or jointly to *E. coli* EC600, while pKP3T58_3 (retaining only a partial T4SS) could co-transfer to recipient cells with the assistance of pKP3T58_2. MLST confirmed clonal persistence of the high-risk ST11 lineage within the patient. Phylogenetic analysis revealed KP3T58's clustering within a dominant epidemic ST11-KL64 subclade prevalent in China. Multiple virulence assays (including siderophore production, capsule quantification, serum resistance, and *Galleria mellonella* infection models) demonstrated that KP3T58 lacks a typical hypervirulent phenotype but retains a certain level of pathogenicity.

Conclusion: This study demonstrates clonal evolution of ST11 CRKP to co-produce KPC-2 and NDM-5 within a host, clustering within a prevalent epidemic lineage. Critically, we provide experimental evidence that mobilizable plasmids retaining only a partial T4SS can undergo horizontal transfer when assisted by conjugative plasmids, fundamentally expanding our understanding of resistance dissemination. The convergence of high-risk epidemiology, MDR, and novel plasmid transfer mechanisms in strains like KP3T58 necessitates enhanced surveillance and urgent molecular investigation into the transmission dynamics of these threats.

Keywords: *Klebsiella pneumoniae*, KPC-2, NDM-5, carbapenemases, ST11

Introduction

The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) challenges clinical management and global public health. The main issue is the extremely limited antibiotic treatment options, which makes CRKP infections difficult to treat and threatens patient outcomes and the healthcare system.[1] Carbapenem resistance in *K. pneumoniae* mainly results from

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carbapenemases (β-lactamase enzymes), particularly *Klebsiella pneumoniae* carbapenemase (KPC; class A serine enzymes) and New Delhi metallo-β-lactamase (NDM; metallo-β-lactamases requiring zinc).[2] What is even more alarming is that *K. pneumoniae* strains co-existing with *bla*KPC and *bla*NDM can obtain or spread extra antimicrobial resistance genes, such as extended-spectrum β-lactamase (ESBL) genes, fluoroquinolone resistance genes, tetracycline resistance genes and aminoglycoside resistance genes. This results in a high level of resistance to most of the routinely employed antibiotics, creating serious obstacles for therapeutic treatment.[3]

Healthcare-associated infections remain a significant challenge, particularly due to the increasing prevalence of multidrug-resistant (MDR) organisms. KPC-producing and NDM-producing *K. pneumoniae* (KPC-Kp and NDM-Kp) are undoubtedly concerning pathogens, characterized by limited treatment options, high mortality rates, and the capacity to trigger outbreaks in healthcare settings.[4] A previous investigation of an NDM outbreak indicated that the losses caused by ward closures, temporary admission restrictions, or delayed discharges due to such outbreaks were enormous.[5]

In this study, we identified a multidrug-resistant *K. pneumoniae* strain (KP3T58) isolated from a clinical patient, exhibiting resistance to nearly all antibiotics except polymyxin. Whole-genome sequencing (WGS) revealed the coexistence of bla_{KPC-2} and bla_{NDM-5} alongside three critical plasmids. Through conjugation assays, we confirmed the transferability of these high-risk genetic determinants and further investigated the virulence phenotype of KP3T58. This work comprehensively characterizes a clinical CRKP co-producing KPC and NDM, highlighting the urgent threat posed by such dual-carbapenemase strains.

Materials and Methods

Bacterial Isolates and Case Report

A 70-year-old male was admitted to the intensive care unit (ICU) of The Second Affiliated Hospital of Xiamen Medical College due to brainstem hemorrhage. Upon admission, chest CT findings indicated that the patient concurrently suffered from chronic bronchitis and emphysema. During hospitalization at this tertiary care center, *K. pneumoniae* was detected in the patient's sputum. To control the infection, piperacillin – tazobactam was initiated at a dosage of 4.5 grams every 12 hours. However, after three weeks of treatment, sputum culture following bronchoscopy still yielded *K. pneumoniae*, with the isolate showing intermediate susceptibility to piperacillin-tazobactam (MIC = 16 µg/mL). Therapy was subsequently changed to ceftazidime (1 gram every 8 hours) and linezolid (0.6 gram every 8 hours) for one week. Due to deteriorating pulmonary status, meropenem (2 grams every 8 hours) was administered. During meropenem treatment, bronchoalveolar lavage fluid culture was positive for a CRKP isolate (KP3T58). This isolate exhibited resistance to ceftazidime-avibactam, tigecycline, and carbapenems. The patient died of respiratory failure caused by severe pulmonary infection three weeks after KP3T58 detection. Figure 1 summarizes the microbiological details, timeline, and antibiotic regimens.

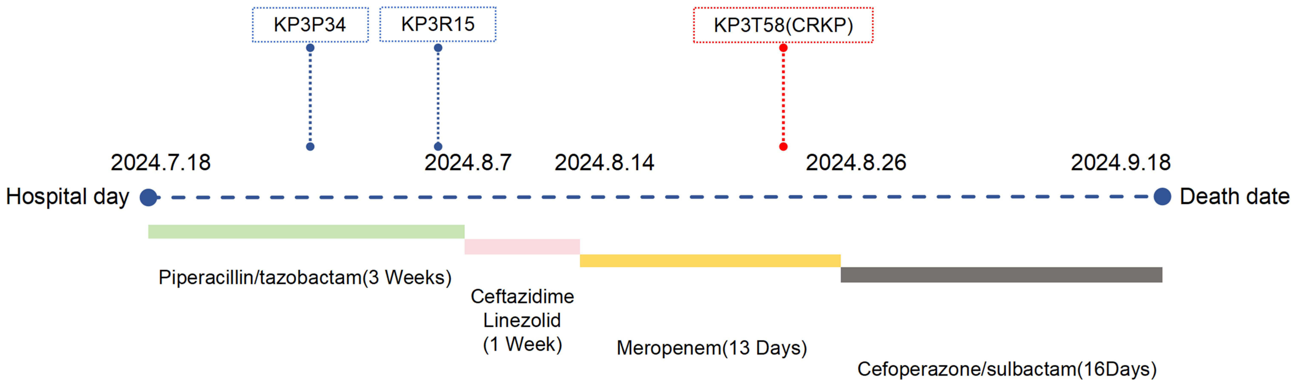

Figure 1 The patient's treatment and infection timeline.

Antimicrobial Susceptibility Testing

Isolates were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS, BioMérieux, France). Antimicrobial susceptibility testing was performed using the Vitek-2 system (Vitek-AST-N334/N335 cards), with ceftazidime-avibactam (CZA) susceptibility determined by broth microdilution. Tigecycline breakpoints followed FDA criteria (susceptible ≤2 mg/L, intermediate 4 mg/L, resistant ≥8 mg/L); polymyxin breakpoints followed EUCAST 2023 standards (https://www.eucast.org); all other breakpoints adhered to CLSI M100 guidelines. *Escherichia coli* ATCC 25922 served as the quality control strain.

Whole-Genome Sequencing and Bioinformatics Analysis

Genomic DNA from KP3T58 was extracted using the Qiagen DNA extraction Kit (Qiagen, Germany). Genome sequencing was performed on the PacBio HiFi and Illumina NovaSeq 6000 platforms. Long-read data (third-generation sequencing) was assembled using hifiasm (v0.19.5). The assembly was error-corrected using pilon (v1.24) and the clean short-read (second-generation) sequencing data. We used Kleborate (https://github.com/katholt/Kleborate/) for multilocus sequence typing (MLST) and serotype analysis.[6] ResFinder 4.6.0 was used to identify chromosomal mutations and acquired resistance genes.[7] PlasmidFinder (v2.1) identified plasmid replication origins, resistance genes, and virulence factors.[8] OriTfinder analyzed plasmid conjugative and mobilizable capabilities.[9] Additionally, VRprofile was used for analysis and annotation of insertion sequences (ISs) and transposons (Tns).[10]

Multilocus Sequence Typing (MLST)

MLST was performed on the first two *K. pneumoniae* isolates (KP3P34 and KP3R15) recovered from patient sputum samples. PCR amplification and sequencing of seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB, and tonB*) were conducted as previously described.[11] Allele numbers and sequence types (STs) were assigned using the Pasteur Institute's Klebsiella pneumoniae MLST database (http://bigsdb.pasteur.fr/klebsiella/).

Phylogenetic Analysis

Genomic sequences and metadata of 35 *K. pneumoniae* strains (34 public isolates from China co-harboring *bla*KPC and *bla*NDM, plus clinical strain KP3T58) were obtained from NCBI. Core genome SNP (cgSNP) analysis was performed using Parsnp v1.2 with KP3T58 as the reference genome, and the resulting phylogenetic tree was visualized and annotated via iTOL (https://itol.embl.de/).

Comparative Genomic Analyses

Sequence alignment was performed using BLASTn. For plasmid comparison, Proksee (https://proksee.ca/) was employed used to generate circular maps comparing KP3T58 plasmids with other representative plasmids. The genetic environments surrounding antibiotic resistance genes were investigated using Easyfig (version 2.25). Nucleotide sequences were aligned using ClustalW in Jalview 2.11.4.0. Amino acid sequence alignment of Tet(A) was performed using ESPript 3.0.[12]

Conjugation Assay

Conjugation assays assessed transfer of resistance plasmids from *K. pneumoniae* KP3T58 (donor) to *E. coli* EC600 (recipient). Donor and recipient strains, grown to logarithmic phase, were mixed (1:1 ratio), centrifuged (8,000g, 1 min), and resuspended in 20 µL of 10 mM $MgSO_4$. The mixture was spotted onto Luria-Bertani (LB) agar and incubated overnight at 37°C. Serial dilutions were plated on LB agar supplemented with selective antibiotics: tetracycline (10 mg/L; *tet(A)*), bleomycin (10 mg/L; *ble*), gentamicin (15 mg/L; *rmtB*), and for the recipient, rifampicin (600 mg/L).

Transconjugants were identified by MALDI-TOF MS. The presence of *tet(A), rmtB,* $bla_{NDM\text{-}5}$, and $bla_{KPC\text{-}2}$ in transconjugants was confirmed by PCR using primers listed in Table 1. Conjugation frequency (CF) was calculated as: CF = [Number of transconjugants (CFU/mL)] / [Number of donor cells (CFU/mL)].

Table 1 Oligonucleotides for PCR

Name	Sequence (5′-3′)
bla$_{KPC-2}$ F	ATGTCACTGTATCGCCGTCT
bla$_{KPC-2}$ R	TTACTGCCCGTTGACGCC
bla$_{NDM-5}$ F	ATGGAATTGCCCAATATTATGCACCCG
bla$_{NDM-5}$ R	TCAGCGCAGCTTGTCGGC
qnrS1 F	GCGACTTTCGACGTGCTAAC
qnrS1 R	CGTGGCATAAATTGGCACCC
tet(A) F	GTGAAACCCAACAGACCCCT
tet(A) R	TCAGCGATCGGCTCGTTG
sul1 F	AAATTTCGCGAGGGTTTCCG
sul1 R	CGATCGAAATGCTGCGAGTC
rmtB F	ATATGTCACCCCGGAATCGC
rmtB R	CATCCTGCAGGGCAAAGGTA

Plasmid Stability Testing and Growth Assays

Plasmid stability in transconjugants was evaluated as described previously.[13] Fitness was assessed by growth curve analysis. Transconjugants and the recipient strain were cultured overnight in LB, diluted to an OD600 of 0.01, and incubated at 37°C for 24h. OD600 was measured every 30 minutes.[14]

Serum Killing Assay

To evaluate the capacity of strains to withstand serum-mediated killing, a serum resistance assay was conducted following previously published procedures.[15] Briefly, mid-log phase bacterial cells at a concentration of colony-forming units (CFU) per milliliter were combined with normal human serum, sourced from healthy human volunteers, in a 1:3 ratio. The mixture was then incubated at 37°C for 2 hours. Subsequently, after serial dilution, the bacteria were plated onto LB agar and incubated overnight at 37°C to enumerate the viable bacteria. Informed consent was obtained from the donors prior to using their serum.

Quantitative Siderophore Production Assay

To assess the capacity of bacterial supernatants to chelate iron, the researchers employed the chrome azurol S (CAS) assay in accordance with the standardized procedures.[16] Briefly, 1 µL of stationary-phase, iron-chelated cultures was placed onto CAS plates. After incubation at 37°C for 48 hours, the formation of orange halos was used as an indicator to detect siderophore production.

Capsule Quantification

To evaluate the mucoviscosity of *K. pneumoniae* KP3T58, uronic acid extraction and quantification were carried out following a previously reported protocol.[15] Specifically, an overnight culture in LB medium underwent dilution at a ratio of 1:100 into fresh medium and was incubated at 37°C for 6 h. Then, 500 µL of the culture was combined with 100 µL of 1% Zwittergent 3–12 detergent. The mixture was heated at 50°C for 20 min and subsequently centrifuged at 13,000×g for 5 min. Next, 300 µL of the supernatant was mixed with 1.2 mL of absolute ethanol and centrifuged again at 13,000×g for 5 min. The obtained pellet was dried and resuspended in 200 µL of sterile water. Subsequently, 1.2 mL of tetraborate solution (12.5 mM sodium tetraborate in sulfuric acid) was added. The solution was incubated at 100°C for 5 min, followed by rapid cooling on ice for a minimum of 10 min. Finally, 20 µL of hydroxyphenyl reagent was added. After a 5 - minute incubation at room temperature, the optical density (OD) was measured at 520 nm.

G. Mellonella in vivo Infection Model

To assess the pathogenicity of *K. pneumoniae* strains KP3T58, *Galleria mellonella* infection assays were conducted following established protocols.[17] First, the caterpillars were stored at 4°C; those weighing between 150 and 200mg were then carefully selected. Two groups were established: a treatment group and a control group. The treatment group was inoculated with 10 μL of a bacterial suspension at a concentration of 1×10^6 colony-forming units (CFU)/mL, while the control group received 10 μL of normal saline.

Each treatment group consisted of at least 30 caterpillars, which were evenly divided into three Petri dishes. All setups were kept at 37°C. Caterpillar survival rates were documented through daily observations over a three-day period.

Statistical Analysis

Data analyses were conducted using GraphPad Prism 8.0.2 software. The results were presented with a two-tailed non-parametric Student's *t*-test. For the survival data obtained from in vivo and in vitro experiments, the Log Rank test (Mantel-Cox) was employed for analysis. P-values < 0.05 were considered significant.

Nucleotide Accession Number

The complete genome sequence of *K. pneumoniae* KP3T58 has been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI), with the accession number PRJNA1206428.

Result

K. Pneumoniae KP3T58 Was a MDR Strain

K. pneumoniae KP3T58 exhibited high-level resistance to ceftazidime/avibactam and tigecycline, carbapenems (ertapenem, meropenem, and imipenem), β-lactam inhibitors (amoxicillin/clavulanic acid,piperacillin/tazobactam, ticarcillin/clavulanic acid, cefoperazone/sulbactam), β-lactam antimicrobials (cefuroxime, ceftazidime,ceftriaxone, cefepime, aztreonam), aminoglycosides (amikacin, tobramycin), quinolones (ciprofloxacin, levofloxacin), tetracyclines (doxycycline, minocycline), it exhibited susceptibility solely to colistin (Table 2).

Table 2 Antimicrobial Drug Susceptibility Profiles

Antibiotics	**MIC (mg/L)/Antimicrobial Susceptibility**							
	Isolates			**Transconjugant**				**Strains**
	KP3P34	**KP3R15**	**KP3T58**	***E. coli* EC600-p1**	***E. coli* EC600-p2**	***E. coli* EC600-p1-p2**	***E. coli* EC600-p2-p3**	***E. coli* EC600**
Ampicillin/sulbactam	≤2/S	16/I	≥32/R	4/S	≥32/R	≥32/R	≥32/R	≤2/S
Piperacillin/tazobactam	≤4/S	8/S	≥128/R	≤4/S	≥128/R	≥128/R	≥128/R	≤4/S
Cefuroxime	2/S	2/S	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	2/S
Cefuroxime axetil	2/S	2/S	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	2/S
Cefoxitin	≤4/S	≤4/S	≥64/R	≤4/S	≥64/R	≥64/R	≥64/R	≤4/S
Ceftazidime	≤1/S	≤1/S	≥64/R	0.5/S	≥64/R	≥64/R	≥64/R	≤1/S
Ceftriaxone	≤0.25/S	≤0.25/S	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤0.25/S
Cefoperazone/sulbactam	≤8/S	≤8/S	≥64/R	≤8/S	≥64/R	≥64/R	≥64/R	≤8/S
Cefepime	≤1/S	≤1/S	16/R	2/S	8 SDD	16/R	16/R	≤1/S
Ertapenem	≤0.5/S	≤0.5/S	≥8/R	≤0.12/S	≥8/R	≥8/R	≥8/R	≤0.5/S
Imipenem	≤1/S	≤1/S	≥16/R	≤0.25/S	≥16/R	8/R	≥16/R	≤1/S
Amikacin	≤2/S	≤2/S	≥64/R	4/S	4/S	4/S	≥64/R	≤2/S
Levofloxacin	≤0.25/S	0.5/S	≥8/R	≤0.12/S	≤0.12/S	0.25/S	0.5/S	≤0.25/S
Tigecycline	≤0.5/S	≤0.5/S	≥8/R	2/S	≤0.5/S	2/S	≤0.5/S	≤0.5/S
Trimethoprim/sulfamethoxazole	≤20/S	≤20/S	≥320/R	≥320/R	≤20/S	≥320/R	≤20/S	≤20/S
Ceftazidime/avibactam	0.25/4/S	0.25/4/S	64/4/R	0.25/4/S	64/4/R	64/4/R	64/4/R	0.25/4/S
Colistin	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S

Abbreviations: MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant; SDD, susceptible dose-dependent; p1,pKP3T58_ 1;p2,pKP3T58_ 2;p3, pKP3T58_ 3.

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Genomic Characteristics of K. Pneumoniae KP3T58

WGS analysis using Kleborate typed strain KP3T58 as sequence type ST11 and capsule type KL64. Hybrid assembly with Circos revealed a circular chromosome of 5,534,638 bp (accession no. CP177330) with a GC content of 57.0%. Strain KP3T58 carried 23 resistance determinants associated with its MDR phenotype (Table 3). Chromosomal resistance genes included *bla*$_{\text{SHV-11}}$, *aadA2, and qacE*. Notably, three point mutations were identified in the OmpK37 porin (I70M, I128M, N230G), and seven mutations were detected in the transcriptional repressor AcrR (P161R, G164A, F172S, R173G, L195V, F197I, K201M), which regulates the OqxAB efflux pump. Virulome analysis showed that KP3T58 carried multiple virulence-associated factors, such as iron uptake systems (yersiniabactin and Ent siderophore), type 1 and type 3 fimbriae, capsule, and type 6 secretion systems (T6SS-I). However, the isolate lacked multiple virulence genes involved in siderophore biosynthesis, including *iucABCD, iroBCD*, and *rmpA*.

Plasmid Characteristics of K. Pneumoniae KP3T58

Genomic analysis indicated that plasmid pKP3T58_1 (323,738 bp, CP177331), classified as an IncFIB(K)/IncFII (pKP91)/IncR plasmid with a GC content of 52%. It carried multiple resistance genes, including *tet(A),qnrS1,bla*$_{\text{CTX-M-14}}$,*bla*$_{\text{TEM-1}}$,*bla*$_{\text{LAP-2}}$,*aph(3")-Ib* and *aph(6)-Id* (Table 3). OriTfinder analysis indicated that pKP3T58_1 was conjugative, as it contained a complete conjugative apparatus (*oriT* site, relaxase, type-4 secretion system (T4SS), and type-4 coupling protein (T4CP)).

The resistance gene *bla*$_{NDM-5}$ was located on plasmid pKP3T58_2 (108,400 bp, CP177332), an IncI1-I type plasmid. This plasmid also contained the *sul1* and *ble* drug-resistance genes. Bioinformatics analysis further confirmed pKP3T58_2 as aconjugative plasmid.

Plasmid pKP3T58_3 (56,083 bp, CP177333), assigned to the IncFII type, exhibited a 52% GC content and contained four resistance genes: *bla*$_{\text{KPC - 2}}$, *rmtB, bla*$_{\text{TEM - 1B}}$, and *bla*$_{\text{CTX - M - 65}}$. In contrast to pKP3T58_1 and pKP3T58_2,

Table 3 Genomic Information of the K. Pneumoniae KP3T58

Characteristics	**KP3T58**				
	Chromosome	**pKP3T58_1**	**pKP3T58_2**	**pKP3T58_3**	**pKP3T58_4**
Incapability group		IncFIB/IncFII/IncR	IncI1-I	IncFII	ColRNAI
Accession no.	CP177330	CP177331	CP177332	CP177333	CP177334
GC content (%)	57	52	50	52	56
Mobile ability		T4SS T4CP oriT Relaxase	T4SS T4CP oriT Relaxase	T4SS	NO
Resistant genes	*bla*$_{\text{SHV-11}}$ *aadA2* *qacE*	*bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{TEM-1B}}$ *bla*$_{\text{LAP-2}}$ *tet(A)* *qnrS1* *aph(3")-Ib* *aph(6)-Id* *sul1* *sul2* *qacE / dfrA1 / catA2*	*bla*$_{\text{NDM-5}}$ *sul1* *ble* *qacE*	*bla*$_{\text{KPC-2}}$ *rmtB* *bla*$_{\text{TEM-1B}}$ *bla*$_{\text{CTX-M-65}}$	NO
Virulence factors	yersiniabactin	NO	NO	NO	NO
Chromosomal mutations					
acrR	P161R,G164A,F172S,R173G,L195V,F197I,K201M				
ompk37	I70M,I128M,N230G				

pKP3T58_3 lacked autonomous conjugative ability, due to an incomplete conjugation system, specifically the absence of *oriT*, a relaxase, and T4CP.

Plasmid pKP3T58_4 (11,970 bp, CP177334), with a GC content of 56%, was devoid of resistance or virulence genes and belonged to the ColRNAI type.

MLST Confirms ST11 Clonal Persistence

MLST analysis assigned both carbapenem-susceptible isolates (KP3P34, KP3R15) and the carbapenem-resistant isolate KP3T58 to ST11 (Pasteur scheme), confirming clonal persistence within the patient.

KP3T58 Clusters Within Dominant Epidemic ST11 Clade

Phylogenetic analysis of 34 *K. pneumoniae* strains co-harboring *bla*KPC and *bla*NDM carbapenemase genes from nationwide surveillance and the clinical isolate KP3T58 revealed that KP3T58 clustered within the dominant epidemic ST11-KL64 clade, indicating it is a prevalent ST11-KL64 clone circulating in China (Figure 2).

Comparative Genomic and Linear Comparison

Comparative analysis showed that plasmid pKP3T58_ 1 had 80–84% coverage and 99.9–100% identity with punnamed1 (CP040176.1) of *K. pneumoniae* strain 2e from Chongqing, p82_1 (CP101547.1) of KP82 from Yunnan, and pKP309 (CP089881.1) of KP309 from Shanghai, all isolated from within China (Figure 3A).

Further analysis revealed pKP3T58_1 contained two large antibiotic resistance gene clusters.The *bla*$_{\text{TEM-1B}}$ gene, together with other resistance genes (*sul2,aph(3")-Ib,aph(6)-Id,bla*$_{\text{LAP-2}}$,*qnrS1*), formed a 17,803-bp antimicrobial resistance (AMR) region. The genomic context upstream of *bla*$_{\text{TEM-1B}}$ was homologous to plasmid pF16KP0064-1 (CP052173.1) from Seoul, South Korea. A similar genetic environment for *qnrS1* was observed when comparing pKP3T58_1 with the JNK002 plasmid unnamed2 (NZ_CP061963.1) (Figure 3B). The presence of multiple insertion sequences (ISs) and recombinase genes suggested this AMR region likely arose from successive insertion and recombination events. Furthermore, comparative analysis identified a point mutation (Leu294Val) in the *tet(A)* gene of pKP3T58_1 (Figure 3C).

Comparative analysis of plasmid pKP3T58_2 revealed potential evolutionary pathways for antibiotic resistance gene acquisition.pZYST1C2 (NZ_CP031615.1) from Heilongjiang, China, pKP11-2 (OW848878.1) from Catalan, Spain, and pKP-NDM-5 (NZ_CP084746.1) from Zhejiang, China, respectively, exhibited 80–98% coverage and 99.9–100% identity with pKP3T58 _ 2 (Figure 3D).

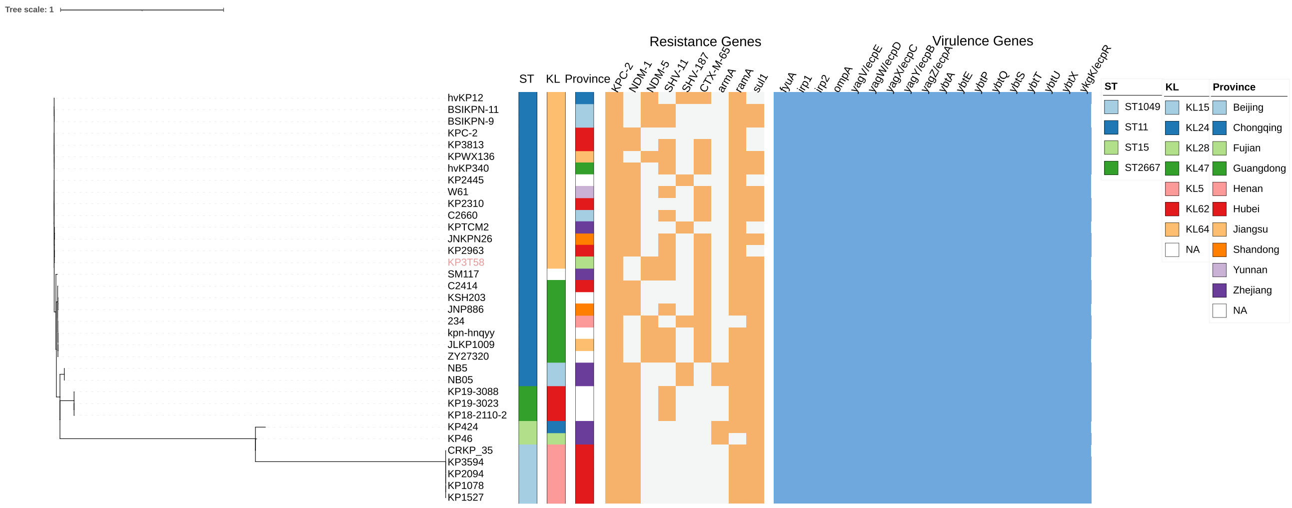

Figure 2 Phylogeny of 35 *K. pneumoniae* isolates co-harboring *bla*KPC and *bla*NDM based on core genome SNP analysis. The tree includes 34 public isolates from China and the clinical strain KP3T58. Annotations indicate multilocus sequence typing (MLST; ST), capsular serotype (KL), Chinese provinces of origin, and distributions of antimicrobial resistance genes and virulence genes.

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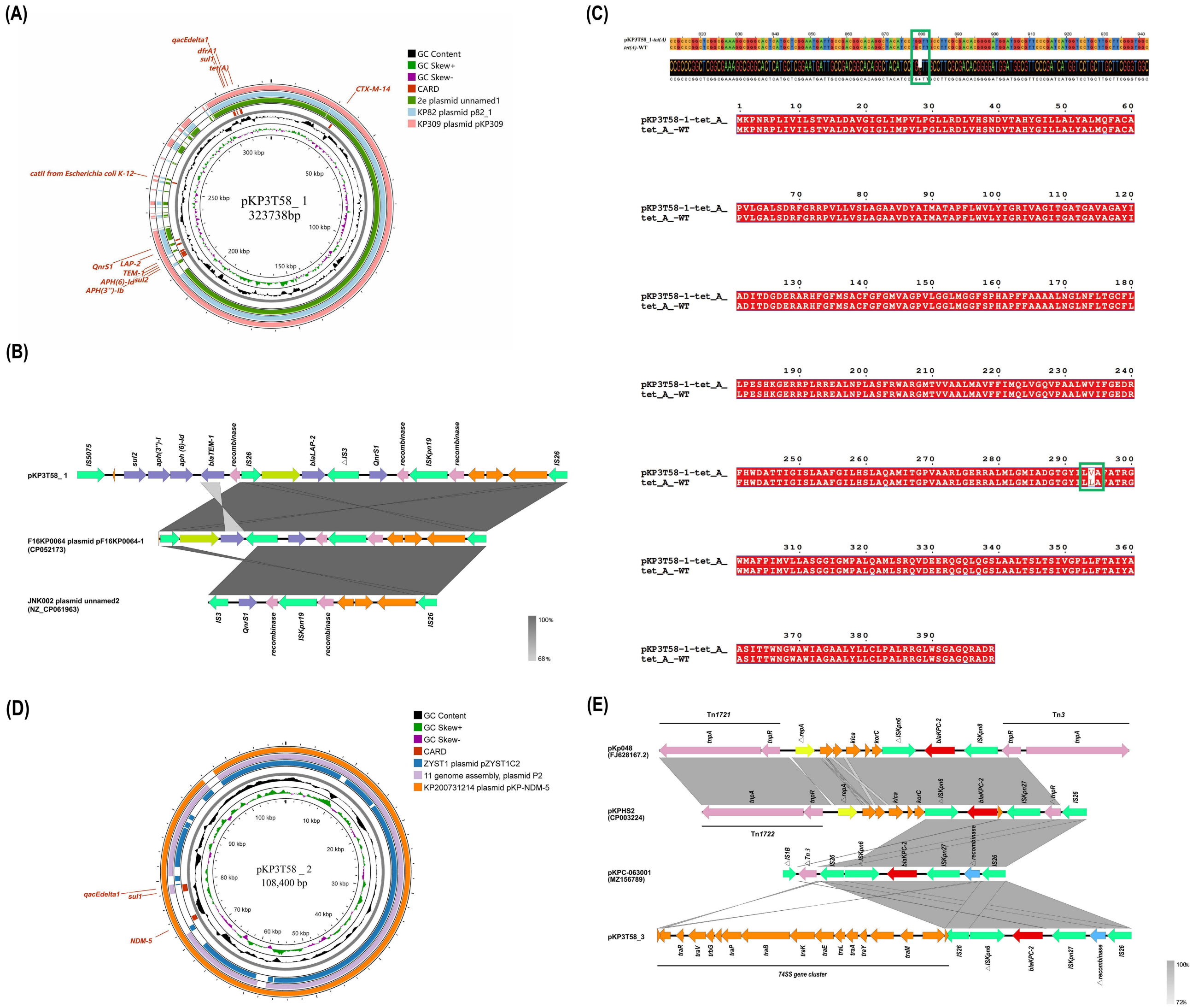

Figure 3 Comparative analysis of pKP3T58_ 1, pKP3T58_ 2 and pKP3T58_ 3 with other reference plasmids. (**A**) Genome alignment was performed with pKP3T58_ 1 (CP177331), plasmid unnamed1 (CP040176.1), p82_1 (CP101547.1) and pKP309 (CP089881.1). (**B**) Alignment of the genetic environment surrounding *bla*$_{\text{TEM-1B}}$ with pF16KP0064-1(CP052173.1) and plasmid unnamed2(NZ_CP061963.1). (**C**) Nucleotide and amino acid sequence alignments between the *tet(A)* of pKP3T58_1 and the wild - type. (**D**) Genome alignment was performed with pKP3T58_ 2(CP177332), pZYST1C2 (NZ_CP031615.1), pKP11 - 2 (OW848878.1), and pKP - NDM - 5 (NZ_CP084746.1). (**E**) Comparison of the genetic environment surrounding *bla*$_{KPC-2}$.

Structural analysis of the *bla*$_{KPC-2}$ locus identified IS*kpn27* and IS*kpn6* flanking the gene upstream and downstream, respectively. Notably, this resistance cassette was entirely embedded within an IS*26*-bounded region. A complete T4SS was detected downstream of *bla*$_{KPC-2}$, containing essential conjugation genes such as *traA, traB, traE, traK*, and *traM* (Figure 3E).

The Non-Conjugative Plasmid pKP3T58_3 Was Mobilized with the Assistance of Conjugative Plasmid pKP3T58_2

Given the three key resistance plasmids and their potential for interbacterial transfer, we assessed the dissemination risk of antimicrobial resistance associated with strain KP3T58. Conjugation assays demonstrated that conjugative plasmids pKP3T58_1 and pKP3T58_2 could transfer individually or together to *E. coli* EC600. Notably, pKP3T58_2 exhibited an exceptionally high conjugation frequency (1.1×10^{-2} - 1.8×10^{-2}). Contrary to expectations, pKP3T58_3 (lacking complete conjugative elements) was mobilized from KP3T58 to *E. coli* EC600 at a lower frequency (4.2×10^{-6} - 5.8×10^{-5}) with the help of the conjugative helper plasmid pKP3T58_2. Antimicrobial susceptibility profiles of recipient cells and transconjugants are summarized in Table 1. PCR and agarose gel electrophoresis confirmed the presence of resistance genes in all transconjugants.

Plasmid stability assays showed that all KP3T58-derived plasmids were stably maintained in transconjugants during serial passages. Furthermore, *E. coli* EC600 harboring these drug-resistant plasmids showed no significant growth defect, ensuring their persistent maintenance in bacterial populations.

In vitro and in vivo Virulence of KP3T58

Subsequently, we aimed to investigate whether KP3T58 possesses hypervirulent traits. For this purpose, *K. pneumoniae* strain HS11286 (classical strain, ST11)[18] served as the virulence-negative control, while *K. pneumoniae* NTUH-K2044 (ST23, KL1)[19] was used as the virulence-positive control.

Quantitative siderophore production assays revealed that KP3T58 produced significantly fewer siderophores (13 mm halo diameter) than the positive control NTUH-K2044 (22 mm), comparable to the negative control HS11286 (12 mm) (Figure 4A).

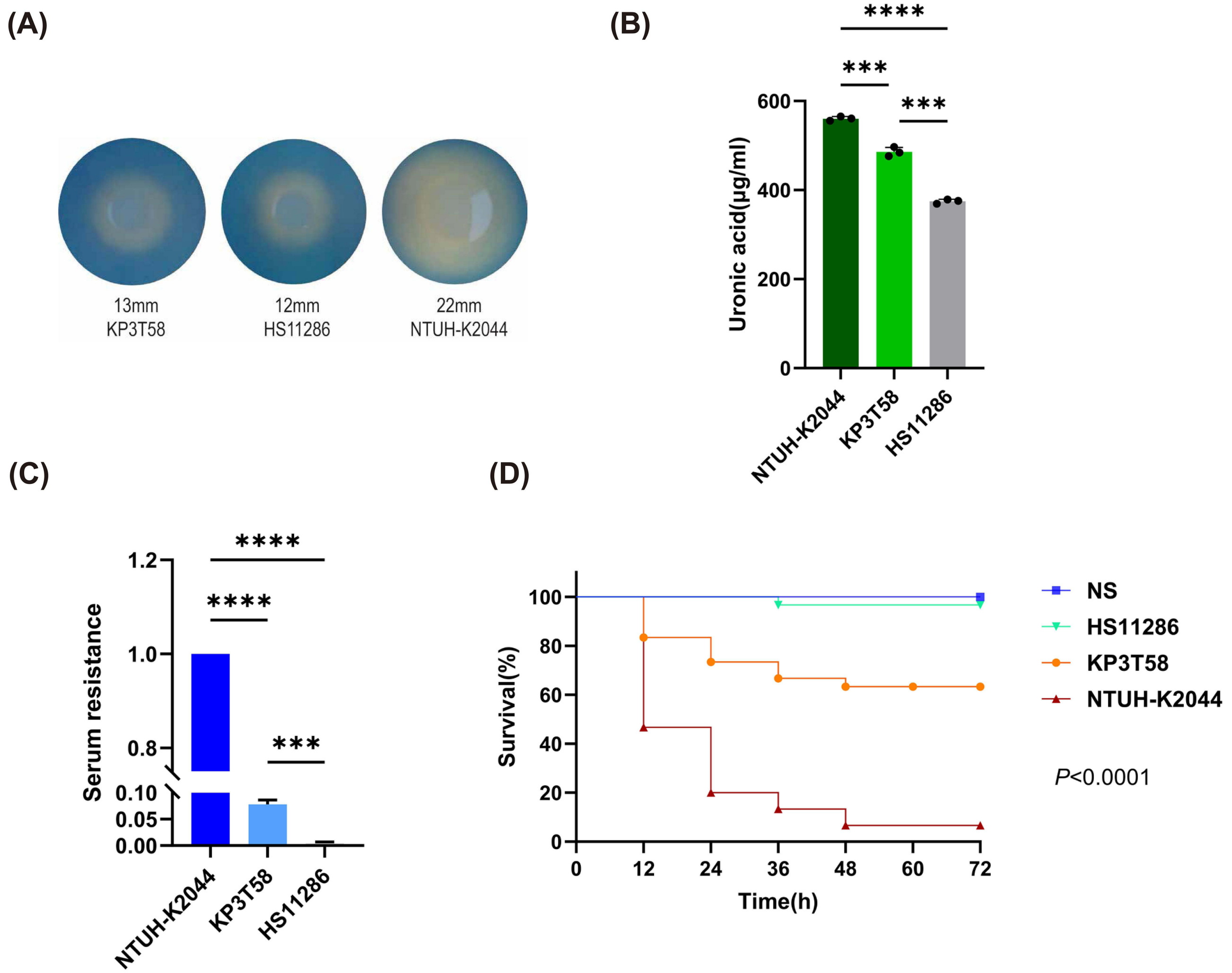

Figure 4 The virulence phenotypes and levels of KP3T58. (**A**) Siderophores production determined by CAS agar plate. (**B**) The production of capsule measured based on uronic acid levels. (**C**) The survival rate (%) evaluated by serum resistance assay. (**D**) The survival curves of *G. mellonella* infected by KP3T58, NTUH-K2044 and HS11286. Note:NS (normal saline). Unpaired two-sided Student's t-tests were performed for uronic acid production and the survival rate in the serum resistance assay. ****P* < 0.001; *****P* < 0.0001. A log - rank (Mantel–Cox) test was employed for the assessment of the survival curves. A significant difference (*P*<0.0001) was observed between these groups.

Capsule quantification via uronic acid measurement demonstrated that KP3T58 produced less uronic acid than NTUH-K2044, but slightly more than HS11286 (Figure 4B). Similar trends were observed in serum resistance assays (Figure 4C).

Pathogenicity assessment using the *Galleria mellonella* infection model showed that KP3T58-infected larvae exhibited a 63% survival rate at 72 hours post-infection, significantly lower than the negative control HS11286 (96%), but substantially higher than the positive control NTUH-K2044 (6%) (Figure 4D).

Discussion

Over the past decade, the global prevalence of MDR K. pneumoniae, particularly carbapenem-resistant variants (CRKP), has escalated significantly, posing a critical public health challenge.[20,21] To elucidate the origin of such high-risk strains, we investigated the clonal and epidemiological context of KP3T58. MLST analysis confirmed that both carbapenem-susceptible isolates (KP3P34, KP3R15) and the carbapenem-resistant isolate KP3T58 from the same patient belong to ST11. This suggests that resistance in KP3T58 likely evolved through acquired resistance mechanisms—such as plasmid acquisition or horizontal gene transfer—rather than new clonal invasion. The ST11 clone demonstrated prolonged persistence within the host, developing resistance under antibiotic pressure. Phylogenetic analysis of 35 *K. pneumoniae* strains co-harboring KPC and NDM carbapenemases from nationwide surveillance further revealed that KP3T58 clustered within a predominant epidemic subclade characterized by co-production of KPC and NDM carbapenemases in ST11-KL64 clones circulating in China. This clustering pattern, combined with the prevalence of ST11-KL64 among dual-carbapenemase producers in the national dataset, suggests that the ST11-KL64 lineage may serve as a key epidemiological vehicle for the acquisition and dissemination of the KPC-NDM co-production phenotype in China. Its dominance positions KP3T58 within a high-risk transmission network potentially driven by this successful clone.

KP3T58 exhibited near-pan-resistance to clinically used antibiotics, including ceftazidime-avibactam (CZA) and tigecycline. CZA, a β-lactam/β-lactamase inhibitor combination effective against KPC-producing CRKP, is compromised by NDM enzymes due to their zinc-dependent hydrolysis mechanism, which avibactam cannot inhibit.[22–24] Critically, unlike KPC-variant-mediated CZA resistance, NDM production does not restore carbapenem susceptibility. Moreover, the stable CZA resistance phenotype in such strains may facilitate horizontal dissemination of resistance genes within bacterial populations.

Plasmid pKP3T58_1, harboring three replicons (IncFIB, IncFII, and IncR), circumvented incompatibility through its multi-replicon configuration, enabling stable coexistence with other plasmids. This large conjugative plasmid carried multiple resistance genes, including *qnrS1, tet(A)*, and diverse β-lactamases. The *qnrS1* gene was embedded within a complex AMR region (ΔIS*3*–IS*Kpn19*) alongside other determinants (IS*5075-aph(3")-Ib-aph(6)-Id-* bla_{TEM-1}-IS*26*-bla_{LAP-2}). Comparative analysis with plasmids pF16KP0064-1 and pJNK002 indicated that this region arose from successive insertion and recombination events. Functionally, *qnrS1* acquisition elevated levofloxacin MICs in transconjugants, while chromosomal *acrR* mutations (mediating fluoroquinolone resistance via RND efflux pump dysregulation) synergistically enhanced resistance.[25] Although *qnrS1* alone confers low-level resistance, it expands the mutant selection window, promoting high-level resistance emergence.[26,27]

Tigecycline resistance in KP3T58 likely stems from efflux pump upregulation. While *rpsJ* mutations and *tet(A)* variants are established tigecycline resistance mechanisms,[28–31] comparative analysis excluded *rpsJ* alterations. However, we identified a Leu294Val substitution in *tet(A)*, which—combined with RND efflux activity—may explain the reduced tigecycline susceptibility in transconjugants. Prior studies corroborate that such mutations diminish tigecycline sensitivity and potentiate MDR phenotypes.[32]

Conjugation assays revealed exceptionally high transfer efficiency of pKP3T58_2 (IncI1-I), consistent with its phylogenetic clustering within an epidemic subclade and suggesting conserved dissemination mechanisms. Comparative genomics indicated high sequence similarity with plasmid pKP-NDM-5 from Zhejiang Province, implying regional spread of this plasmid lineage. In East Asia, bla_{KPC-2} frequently localizes to IncFII-type plasmids,[33] creating an ecological niche for co-dissemination with pKP3T58_2. Critically, KPC-NDM co-production confers elevated resistance to carbapenems and CZA, demanding urgent surveillance of pKP3T58_2-like plasmids.

The *bla*KPC gene resided on an IncFII-type plasmid, the primary vector for bla_{KPC-2} spread in ST11.[34] Structural analysis revealed a unique genetic environment flanked by IS*Kpn27* and IS*26*, with a truncated IS*Kpn6* and an incomplete recombinase gene upstream. Notably, no ΔTn3 homolog was detected downstream; instead, a complete T4SS was present. This architecture —distinct from predominant Tn*1721* transposons in Chinese isolates—highlights how IS accumulation enhances plasticity around bla_{KPC-2}, facilitating carbapenem resistance dissemination.[35,36]

Previous studies define mobilizable plasmids as those incapable of autonomous transfer due to defective conjugative machinery, yet capable of horizontal transfer via "hitchhiking" with helper conjugative plasmids. Conventional knowledge dictates that such plasmids retain, at minimum, an *oriT* site to enable co-transfer. In our conjugation assays, plasmid pKP3T58_3—containing only a partial T4SS—was successfully co-transferred with the conjugative plasmid pKP3T58_2. This unconventional finding expands the mechanistic paradigm of plasmid dissemination. Notably, mobilizable plasmids may possess greater dissemination potential and broader host ranges than conjugative plasmids, potentially attributable to fewer protospacer sequences vulnerable to CRISPR-Cas targeting.[37,38] Consequently, the observed co-transfer of pKP3T58_3 raises significant concerns regarding the accelerated dissemination of antimicrobial resistance genes.

Although KP3T58 lacked hallmark hypervirulence genes (*iucABCD, iroBCD, rmpA*) and assays confirmed atypical hypervirulence, its yersiniabactin system poses substantial risks. This siderophore enhances respiratory colonization and pneumonia development, potentially enabling acquisition of additional virulence determinants.[39,40] While reports of carbapenem-resistant hypervirulent *K. pneumoniae* (hv-CRKP) are increasing,[17] most global CRKP infections remain opportunistic healthcare-associated infections (HAIs).[41] High-risk groups (neonates, elderly, immunocompromised) in ICUs—where CRKP mortality reaches 48.9%[42]—are particularly vulnerable. Critically, the within-host evolution of resistance in ST11 underscores the need for decolonization protocols targeting susceptible carriers pre-resistance emergence. Antibiotic pressure concurrently drives convergence of MDR and hypervirulence, exacerbating therapeutic challenges.[43]

Conclusion

In summary, this study systematically characterized the resistome of the clinical isolate and conducted in-depth genomic analysis of key resistance gene contexts. MLST analysis confirmed clonal persistence of the ST11 lineage within the host, while phylogenetic positioning revealed KP3T58's clustering within a dominant epidemic subclade circulating in China. Critically, we experimentally demonstrated that mobilizable plasmids retaining only a T4SS —despite lacking core conjugative machinery—can achieve horizontal transfer when assisted by conjugative plasmids. This discovery fundamentally reshapes our understanding of plasmid dissemination mechanisms. Given these novel insights into plasmid transmission, coupled with the high-risk epidemiological context of the strain, and considering the potential role of the ST11-KL64 clone as a major disseminator of the KPC-NDM co-production phenotype in China, we conclude that the spread of CRKP co-producing KPC and NDM carbapenemases demands enhanced surveillance. Targeted monitoring of this high-prevalence, genetically adaptable lineage may be critical for understanding and interrupting the transmission dynamics of dual-carbapenemase resistance.

Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital of Xiamen Medical College (2025010). Written informed consent was obtained from the deceased patient's next-of-kin for the publication of this case report and associated data.

Author Contributions

All authors made a significant contribution to the work reported, whether 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 report no conflicts of interest in this work.

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