Infection and Drug Resistance

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ORIGINAL RESEARCH

Genomic and Phenotypic Analysis of *bla*$_{KPC-2}$ Associated Carbapenem Resistance in *Klebsiella aerogenes*: Insights into Clonal Spread and Resistance Mechanisms Across Hospital Departments in Beijing

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Purpose: This study conducted an phenotypic and whole-genome sequencing analysis with *Klebsiella aerogenes* to elucidate its clinical epidemiological characteristics, antimicrobial resistance (AMR) phenotype, biofilm formation ability and hemolytic activity testing, AMR genes and phylogenetic relationships, so as to provide a further understanding of the intra-hospital strain transmission.

Methods: Samples were collected from a hospital in Beijing between 2020 and 2022. All strains underwent bacterial identification, antimicrobial susceptibility testing (AST) using the VITEK-2 compact system. Biofilm formation ability and hemolytic activity were tested. Second-generation sequencing was applied to all strains, with those carrying the *bla*$_{KPC}$ gene were selected for third-generation sequencing. Whole-genome analysis identified resistance genes, plasmid types, MLST typing, and phylogenetic relationships. Plasmids were assembled to detect plasmid structures and AMR gene location.

Results: Among the 42 *K. aerogenes* isolates, 21 were carbapenem-resistant *K. aerogenes* (CRKA). All strains exhibited strong biofilm formation and no hemolytic activity. Most were sourced from sputum (83.3%). CRKA demonstrated extensive resistance to antibiotics, particularly β-lactamase inhibitors and Cefotetan. This resistance pattern was closely associated with the presence of an IncFII(pHN7A8) plasmid, which carried multiple resistance genes, including *bla*$_{KPC-2}$, *bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$, *rmtB* and a large number of mobile elements. The majority of CRKA strains clustered within the same branch of the phylogenetic tree, exhibiting minimal single nucleotide polymorphism (0–13 SNPs) differences, and they shared the same sequence type (ST292), resistance genes, and plasmids, originating from different departments, suggesting clonal transmission among the hospital.

Conclusion: Our research reveals that the clonal transmission of CRKA occurs across various departments within the hospital. The widespread resistance observed in CRKA, attributed to the presence of *bla*$_{KPC}$ and ESBLs genes, underscores the need for heightened vigilance to prevent the further dissemination of CRKA within the hospital and, potentially, throughout the wider community.

Keywords: *Klebsiella aerogenes*, carbapenem resistance, *bla*$_{KPC-2}$, clonal transmission

Received: 22 January 2024
Accepted: 29 April 2024
Published: 3 July 2024

Introduction

Klebsiella aerogenes, formerly known as *Enterobacter aerogenes*, belongs to the *Enterobacteriaceae* family and is classified as a Gram-negative facultative anaerobic bacterium. It is a prevalent opportunistic pathogen associated with various infections, including urinary tract infections, pneumonia, and bacteremia.[1,2] *K. aerogenes* are often associated with nosocomial infections, and prolonged use of broad-spectrum antibiotics, especially in immunocompromised patients, represents a critical risk factor for acquiring these infections.[3] Carbapenem antibiotics are considered one of the most effective broad-spectrum β-lactam antibiotics for the treatment of multidrug-resistant *Enterobacteriaceae* infections.[4] However, the widespread clinical use of carbapenems has led to the global dissemination of carbapenemase-producing *Enterobacteriaceae* (CRE) strains, resulting in a serious clinical challenge, treatment failure, and high mortality rates, and thus posing a significant public health threat.[5,6] The primary mechanism of resistance to carbapenem antibiotics is the production of carbapenemase enzymes. Carbapenemases are a type of β-lactamase enzyme capable of hydrolyzing penicillins, cephalosporins, monobactams, and carbapenems.[7] The genes encoding carbapenemases in *Enterobacteriaceae* mainly include Class A carbapenemases (bla_{KPC}, bla_{SME}, bla_{IMI}) and Class B carbapenemases (bla_{NDM}, bla_{IMP}, bla_{VIM}).[8] In *K. aerogenes* and various other *Enterobacteriaceae* bacteria, carbapenem resistance is still primarily driven by bla_{KPC} gene.[9] The prevalence of carbapenem-resistant *Klebsiella* strains, particularly those expressing bla_{KPC-2} has been increasing in clinical specimens submitted from various departments.[10] Due to the presence of CRE isolates, these bacterial strains typically display extensive resistance. This trend poses new challenges for clinical antimicrobial therapy in the management of infections caused by these multidrug-resistant strains.[11]

The aim of this study was to investigate the localized transmission of *K. aerogenes* within the hospital setting. The main objectives of the research included conducting antimicrobial susceptibility testing, biofilm formation ability and hemolytic activity testing, whole-genome sequencing (WGS), and bioinformatics analysis. By exploring the epidemiological characteristics, antibiotic resistance profiles, and genomic features, we aimed to delineate the transmission-evolutionary relationships and population structure among the strains. Additionally, we sought to identify the presence and location of carbapenemase genes within the strains.

Methods

Bacterial Strain Collection

This research involves a retrospective analysis conducted on *K. aerogenes* strains obtained from a Beijing Hospital between 2020 and 2022. We collected a total of 42 distinct strains of hospital-acquired *K. aerogenes* from a diverse range of patients across various departments and specimen sources, as detailed in Supplementary Table S1.

Antimicrobial Susceptibility Testing (AST) and Species Identification

In this study, the VITEK-2 compact system, manufactured by bioMérieux in Marcy l'Étoile, France, was employed for identifying bacterial strains and assessing their susceptibility to antimicrobial agents. The interpretation of these results followed the guidelines outlined in document M100-S32 by the Clinical and Laboratory Standards Institute (CLSI).[12] To determine susceptibility to tigecycline, we used the E-test from bioMérieux. It's important to note that CLSI does not establish specific breakpoints for tigecycline. Therefore, we adopted the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), as provided on their website (http://www.eucast.org/).

Biofilm Detection

The 42 strains of *K. aerogenes* were inoculated onto LB agar plates and incubated for 16–18 hours at 37°C in an inverted incubator. Then, 1 μL of the inoculation loop was used to pick individual colonies into 2 mL of API 0.85% NaCl medium and the turbidity was adjusted to 0.5 MacFarland. 980 μL of clean liquid LB medium and 20 μL of 0.5 MacFarland bacterial suspension were added to the CORNING 24-well plates and each strain was subjected to three parallel treatments simultaneously. A negative control group was established and each group was also subjected to three parallel treatments. The well plates were incubated in a constant temperature incubator at 37°C for 24 h. Then the well plates were removed, the bacterial liquid was aspirated, and each well was washed three times with sterile water. 1 mL of

crystal violet solution at a concentration of 0.1% was added to each well and then placed in a room temperature shaker at low speed and slow agitation for 20 minutes. The staining solution was gently pipetted out along the wall of the wells, and each well was washed three more times with sterile water, dried at room temperature, and then decolorized by adding 1.5 mL of 95% ethanol to each well. The 24-well plate was placed on a shaker and shaken slowly for 20 minutes. The absorbance value at the wavelength of 600 nm (OD_{600}), was read on the Microplate reader, manufactured by Molecular Devices in California, the United States, and the measurement was made three times in each well, and the measurement results were recorded. Clean LB liquid medium was used as the negative control, and two times of the OD_{600} value of the negative control group was used as the cut-off value (Dc), and the experimental data were taken as the average of three times (D value). If the D value is less than Dc, the strain does not form a biofilm. If the D value is greater than Dc but less than twice Dc, it is considered a weak biofilm indicator. If the D value exceeds twice Dc, it is considered a strong biofilm indicator.

Hemolytic Activity Assay

Hemolytic activity of *K. aerogenes* strains was evaluated. All strains were cultured in an LB liquid medium overnight. Then, they were subjected to the streak plate method on Blood Agar Plate and cultured at 37°C for 24 h to observe the hemolytic cycle of colonies on plates. Hemolytic activity can be divided into α-hemolysis, β-hemolysis, and γ-hemolysis.[13]

Whole Genome Sequencing

In this study, we sequenced the entire genomes of 42 *K. aerogenes* strains. Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), and sequencing was conducted on Illumina MiSeq platforms following the manufacturer's guidelines (Illumina, San Diego, CA, USA). Raw genome sequences were processed for quality by trimming and filtering to eliminate adapter sequences and low-quality paired-end reads using Trimmomatic (v0.3.13).[14] Subsequently, we employed SPAdes (v3.15.2)[15] for de novo assembly, reconstructing sequence reads into draft continuous sequences contigs. Additionally, we selectively chose two strains carrying bla_{KPC-2} and bla_{KPC-3} for long-read genome sequencing using an Oxford Nanopore MinION sequencer. Third-generation sequencing data underwent filtration using Filtlong software to exclude reads shorter than 500 base pairs and eliminate low-quality reads and adapter sequences, yielding a high-quality dataset. A hybrid assembly of both second-generation and third-generation sequencing data was performed using Unicycler v0.4.8,[16] enabling us to obtain complete chromosomal and plasmid sequences of the bacterial strains.

Phylogenetic and Bioinformatics Analysis

To explore the evolutionary propagation relationships among the *K. aerogenes* strains, we constructed a phylogenetic tree comprising 42 strains obtained in our study. Moreover, to determine the global evolutionary position of *K. aerogenes* strains from China, we expanded our analysis by including 679 sequences previously assembled and downloaded from NCBI (Supplementary Table S1). By integrating these additional sequences, we gained a broader perspective on the phylogenetic placement of Chinese *K. aerogenes* strains within a global context. The identification of single-nucleotide polymorphisms (SNPs) within the core genome was carried out using the snippy v4.6.0 pipeline (https://github.com/tseemann/snippy), with the *K. aerogenes* strain Ka37751 (NCBI accession number: NZ_CP041925.1) serving as the reference genome. Alignment of the genomes to the reference genome was accomplished using BWA-mem v1.2.0. SNPs were detected using SAMtools v1.12[17] and FreeBayes v1.3.5.[18] The identification and removal of homologous recombination events were performed using Gubbins v2.4.1.[19] Core SNPs were extracted using SNP-sites v2.5.1.[20] For the construction of the phylogenetic tree, IQ-TREE v1.6.10[21] was employed. The general time-reversible nucleotide substitution model and the gamma rate estimation model (GAMMA) were selected, with 1000 bootstrap samplings conducted for robustness estimation. The resulting phylogenetic tree was visualized and customized using the online ITOL platform (https://itol.embl.de/).

To ensure that all 42 strains belong to the *K. aerogenes* species, we performed species homogeneity identification using the Average Nucleotide Identity (ANI).[22] According to the PubMLST typing scheme,[23] the assembled genomes

were subjected to multilocus sequence typing (MLST) analysis using MLST tool v2.19.0.[24] The presence of antimicrobial resistance (AMR) genes was predicted using the ABRicate in conjunction with the Comprehensive Antibiotic Resistance Database (CARD),[25] while the assembled sequences were compared against the PlasmidFinder database[26] to enable the identification of plasmid replicon types. The pairwise SNP distance matrix for each pair of strains was computed using the SNP-dists software, based on the FASTA sequence alignment generated by Snippy. The Plasflow v1.1[27] was employed to predict plasmid sequences within the assembled genome sequences using the Third Generation sequencing. The predicted plasmid sequences were further analyzed by conducting online Basic Local Alignment Search Tool (BLAST) searches on the National Center for Biotechnology Information (NCBI) website to identify plasmids that exhibited similarity to the plasmids in our study. The locations of plasmid replicons, IS sequences and AMR genes were determined using PlasmidFinder v2.0.1,[26] ISfinder,[28] and ResFinder v4.0.15,[29] respectively. The complete plasmid sequences were annotated using Prokka v1.14.6.[30] The comparative analysis of plasmids was visualized using the BRIG,[31] which generated plasmid comparison circular diagrams. Genomic assemblies were mapped against the selected plasmids using BLAST[32] to determine whether the strains carried the corresponding plasmids.

Data and Statistical Analysis

Statistical descriptions of the epidemiological characteristics were conducted separately for CRKA and Non-CRKA. The differences in antibiotic resistance between the two groups were assessed using the *chi-square test*. For cases where the *chi-square test* assumptions were not met, the *Fisher's exact test* was employed. All the aforementioned analyses were performed using the R software. A *P*-value less than 0.05 was considered statistically significant.

Results

The Epidemiological Characteristics of the K. Aerogenes Strains

In this study, 42 *K. aerogenes* strains were collected, comprising 21 CRKA strains and 21 Non-CRKA strains (Table 1). The collections were exclusively located in Beijing, and the sampling period spanned from 2020 to 2022. In terms of department distribution, the majority of cases originated from the Cardiovascular Internal Medicine Department (n=14)

Table 1 The Epidemiological Characteristics of 42 K. Aerogenes Strains in This Study

Distribution	No. Isolates (%)		
	CRKA (n=21)	Non-CRKA (n=21)	Total (n=42)
Year			
2020	2(9.5)	5(23.8)	7(16.7)
2021	2(9.5)	9(42.9)	11(26.2)
2022	17(81.0)	7(33.3)	24(57.1)
Source			
Urine	1(4.8)	5(23.8)	6(14.3)
Sputum	19(90.5)	16(76.2)	35(83.3)
Bronchoalveolar Lavage Fluid	1(4.8)	0(0.0)	1(2.4)
Department			
Department of Respiratory Medicine	6(28.6)	8(38.1)	14(33.3)
Department of Cardiovascular Medicine	6(28.6)	8(38.1)	14(33.3)
Department of Gastroenterology	1(4.8)	2(9.5)	3(7.1)
Department of General Surgery	0(0.0)	1(4.8)	1(2.4)
Department of Surgical Intensive Care	0(0.0)	1(4.8)	1(2.4)
Department of Oncology	3(14.3)	1(4.8)	4(9.5)
Department of Neurology	4(19.0)	0(0.0)	4(9.5)
Department of Nephrology	1(4.8)	0(0.0)	1(2.4)

and the Respiratory Internal Medicine Department (n=14). The isolated strains were mainly sourced from sputum (83.3%), followed by urine (14.3%), and bronchoalveolar lavage fluid (2.4%).

Antimicrobial Susceptibility Testing

Antimicrobial sensitivity results are shown in Table 2. Among the 42 strains, the resistance rate to ticarcillin/clavulanic acid was the highest (83.3%), followed by cefotetan (81.0%), piperacillin/tazobactam (76.2%) and amoxicillin (69.0%), and only one isolate was resistant to trimethoprim/sulfamethoxazole. According to the antibiotic resistance profile, 30 strains (71.4%) were classified as multidrug-resistant (MDR), and 21 strains (50%) were resistant to at least one of the carbapenem antibiotics (imipenem or meropenem), which were defined as CRKA. Excluding trimethoprim/sulfamethoxazole, ciprofloxacin and levofloxacin, CRKA exhibited significantly higher resistance rates to various antibiotics compared to Non-CRKA. Notably, CRKA displayed complete resistance to ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime/avibactam, and cefotetan, along with high-level resistance to cefepime (95.2%) and amoxicillin (95.2%).

Biofilm Detection

The biofilm detection results (Supplementary Table S2) show that the D values for all strains exceed twice the Dc value, thereby classifying them as strong biofilm formers. Further comparison between CRKA and Non-CRKA strains revealed no significant difference (P = 0.29) in the Dc values between the two groups (Supplementary Figure S1).

Hemolytic Activity

The results, as shown in Supplementary Figure S2, indicate that all the *K. aerogenes* strains exhibited γ-hemolysis, meaning that they do not exhibit hemolytic activity.

Table 2 Comparing Differences in Antibiotic Resistance Rates Among 42 K. Aerogenes Strains

Antibiotic	Resistance, n (%)				
	CRKA (n=21)	Non-CRKA (n=21)	Total (n=42)	χ2	*P*
Amikacin	16(76.2)	1(4.8)	17 (40.5)	22.235	<0.05
Tobramycin	16(76.2)	1(4.8)	17(40.5)	22.235	<0.05
Amoxicillin	20(95.2)	9(42.9)	29(69.0)	13.480	<0.05
Tigecycline	14(66.7)	3(14.3)	17(40.5)	11.958	<0.05
Trimethoprim/ sulfamethoxazole	0(0.0)	1(4.8)	1(2.4)	-	>0.05
Ciprofloxacin	8(38.1)	6(28.6)	14(33.3)	0.429	>0.05
Levofloxacin	7(33.3)	3(14.3)	10(23.8)	2.100	>0.05
Ticarcillin/ clavulanic acid	21(100.0)	14(66.7)	35(83.3)	6.171	<0.05
Piperacillin/tazobactam	21(100.0)	11(52.4)	32(76.2)	13.125	<0.05
Ceftazidime/avibactam	21(100.0)	5(23.8)	26(61.9)	25.846	<0.05
Imipenem	21(100.0)	0(0.0)	21(50.0)	42.000	<0.05
Meropenem	21(100.0)	0(0.0)	21(50.0)	42.000	<0.05
Doxycycline	17(81.0)	8(38.1)	25(59.5)	8.005	<0.05
Minocycline	17(81.0)	10(47.6)	27(64.3)	5.081	<0.05
Cefotetan	21(100.0)	13(61.9)	34(81.0)	7.566	<0.05
Cefepime	20(95.2)	3(14.3)	23(54.8)	27.776	<0.05
MDR	21(100.0)	9(42.9)	30(71.4)	16.800	<0.05

Note: The choice between Pearson's chi-squared test, Yates' corrected chi-squared test, or Fisher's exact probability method depends on total and expected frequencies. If chi-squared test conditions are not met ("-"), Fisher's exact probability method is used.

Detection of AMR Gene Determinants

CRKA strains exhibited a higher average carriage rate of AMR genes compared to Non-CRKA strains (Table 3). The β-lactamase resistance genes detected include $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$, $bla_{\text{DHA-1}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{LAP-2}}$. Notably, $bla_{\text{KPC-2}}$ was widely found in 76.2% of CRKA strains, and the carriage rates of $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-65}}$ in CRKA were significantly greater than in Non-CRKA. The study identified several aminoglycoside resistance genes, including *aac(3)-IId, aadA16, aph(3')-Ia*, and *rmtB*. Notably, *rmtB* had a high detection rate of 76.2% in CRKA, while only one Non-CRKA strain was found to harbor it. *aac(3)-IId*, and *aadA16* were detected in a small number of Non-CRKA strains. Additionally, tetracycline resistance, represented by the *tet(A)* gene, was found in four Non-CRKA strains. Nine strains were found to have the fluoroquinolone resistance genes q*nrS1*, with one strain possessing *aac(6')-Ib-cr* and another strain possessing *qnrB4* among the Non-CRKA strains. Additionally, a few sulfonamide resistance genes, including *sul1, sul2, dfrA14*, and *dfrA27*, were detected. Only one CRKA strain was found to carry *mcr-1*.

Phylogenetic Analysis of K. Aerogenes Strains

We drew a maximum-likelihood phylogenetic tree based on 721 genomes (42 genomes in this study and 679 globally distributed strains, as detailed in Supplementary Table S1) to investigate the population structure and evolutionary position of the *K. aerogenes* strains in this study (Figure 1). The strains were primarily sourced from six countries, including Brazil, Canada, China, Germany, Singapore, and the United States. The phylogenetic tree was divided into six

Table 3 Antimicrobial Resistance Genes of CRKA and Non-CRKA Isolates

Resistance genes	The number of resistance genes (N, %)				
	CRKA (n=21)	Non-CRKA (n=21)	Total (n=42)	χ2	*P*
aac(3)-IId	0(0.0)	2(9.5)	2(4.8)	0.525	>0.05
aac(6')-Ib-cr	0(0.0)	1(4.8)	1(2.4)	-	>0.05
aadA16	0(0.0)	1(4.8)	1(2.4)	-	>0.05
aph(3')-Ia	2(9.5)	4(19.0)	6(14.3)	0.194	>0.05
arr-3	0(0.0)	1(4.8)	1(2.4)	-	>0.05
bacA	3(14.3)	10(47.6)	13(31.0)	5.459	<0.05
catII	7(33.3)	1(4.8)	8(19.0)	3.860	<0.05
$bla_{\text{CTX-M-3}}$	2(9.5)	3(14.3)	5(11.9)	0.000	>0.05
$bla_{\text{CTX-M-65}}$	16(76.2)	1(4.8)	17(40.5)	22.235	<0.05
dfrA14	1(4.8)	0(0.0)	1(2.4)	-	>0.05
dfrA27	0(0.0)	1(4.8)	1(2.4)	-	>0.05
$bla_{\text{DHA-1}}$	0(0.0)	1(4.8)	1(2.4)	-	>0.05
$bla_{\text{KPC-2}}$	16(76.2)	0(0.0)	16(38.1)	25.846	<0.05
$bla_{\text{KPC-3}}$	1(4.8)	1(4.8)	2(4.8)	0.000	>0.05
$bla_{\text{LAP-2}}$	0(0.0)	3(14.3)	3(7.1)	1.436	>0.05
mcr-1	1(4.8)	0(0.0)	1(2.4)	-	>0.05
mdtB	2(9.5)	6(28.6)	8(19.0)	1.390	>0.05
$bla_{\text{NDM-5}}$	1(4.8)	0(0.0)	1(2.4)	-	>0.05
qnrB4	0(0.0)	1(4.8)	1(2.4)	-	>0.05
qnrS1	4(19.0)	5(23.8)	9(21.4)	0.000	>0.05
rmtB	16(76.2)	1(4.8)	17(40.5)	22.235	<0.05
sul1	0(0.0)	2(9.5)	2(4.8)	0.525	>0.05
sul2	0(0.0)	1(4.8)	1(2.4)	-	>0.05
$bla_{\text{TEM-1}}$	18(85.7)	5(23.8)	23(54.8)	16.243	<0.05
tet(A)	0(0.0)	4(19.0)	4(9.5)	2.487	>0.05

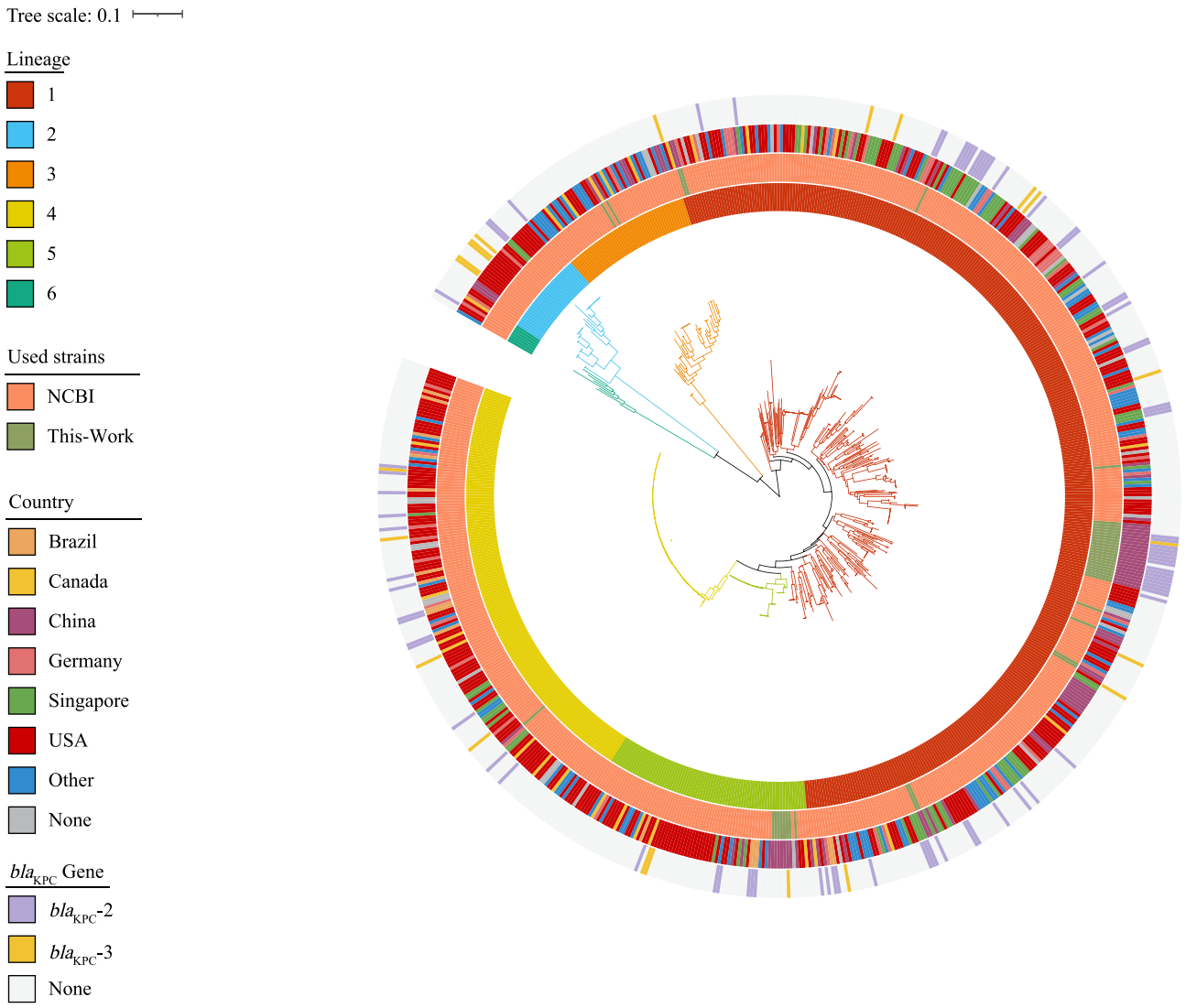

Figure 1 Phylogenetic tree of the global *K. aerogenes* strains displaying the position of Chinese KA strains. The rings, from inner to outer, labeled with different colors indicate lineage, used strains, country and bla_{KPC} gene, respectively.

lineages, with Lineage 1 being the predominant branch. Among the 42 strains in this study, all lineages were represented except Lineages 2 and Lineages 6, but they were mainly distributed in Lineage 1 (n=31) and Lineage 5 (n=8).

K. aerogenes carrying the bla_{KPC} gene exhibit two predominant types: bla_{KPC-2} and bla_{KPC-3}. The distribution of these types spans diverse lineages and countries. Specifically, there are 82 strains (11.4%) characterized by the presence of bla_{KPC-2}, and 22 strains (3.1%) identified with bla_{KPC-3}.

The Average Nucleotide Identity among the isolates was greater than 95%, indicating that they belonged to *K. aerogenes* species (Supplementary Figure S3). A phylogenetic tree was constructed based on 122,864 core genome SNPs, incorporating the 42 self-tested strains and the reference strain, Ka37751. The tree revealed four distinct branches (C1-C4) within the population (Figure 2). Branch C1 comprised 18 strains, C2 had 8 strains, C3 contained 14 strains, and C4 was consisted of 2 strains. Notably, branch C1 emerged as the dominant branch, representing 42.8% of the total strains. The MLST analysis revealed five major sequence types (STs): ST292, ST14, ST12, ST311 and ST435. A new ST type was identified, which was a variant of ST192. Compared to ST192, there is a change in the *pryG* housekeeping gene, with the base at position 231 transitioning from T to C. The branches showed a close correlation with specific ST types, with all ST292 strains belonging to C1 and all ST311 strains belonging to C2. The phylogenetic tree reveals that most of the CRKA strains (highlighted in red) clustered in the main branch C1. Compared to other lineages, this lineage

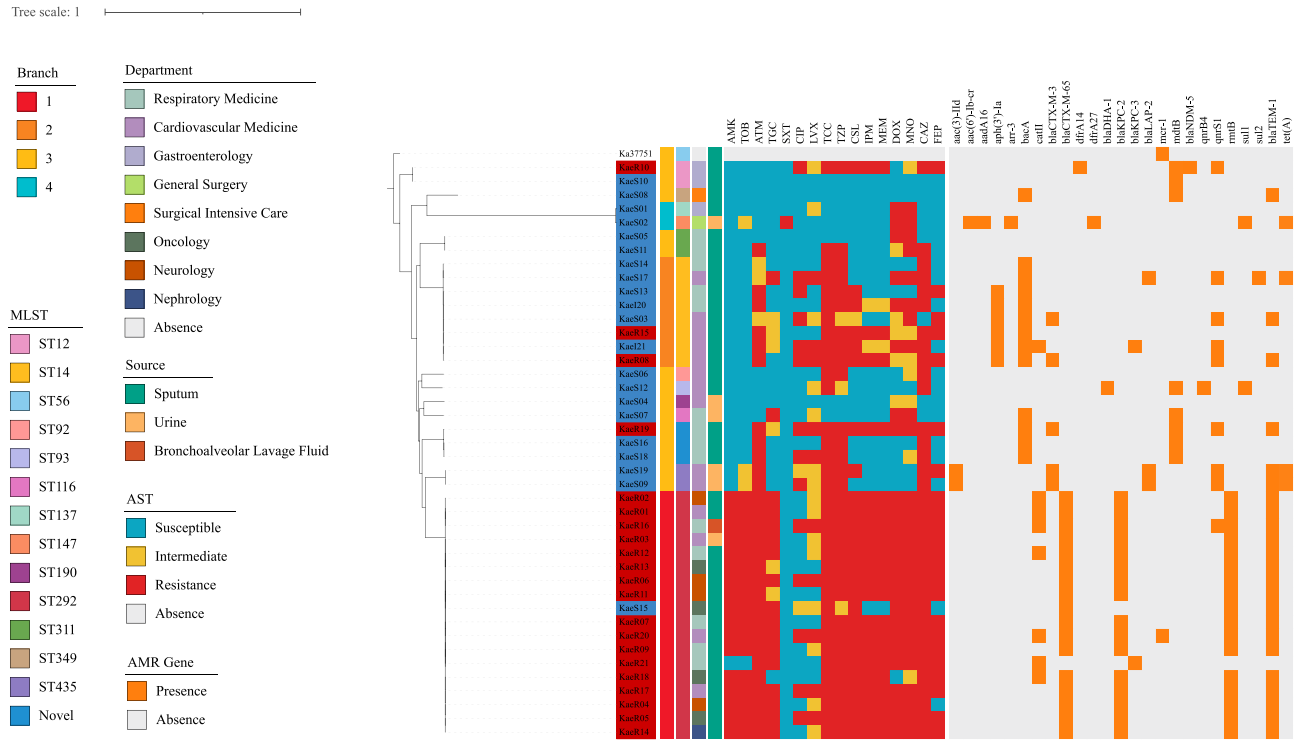

Figure 2 Phylogenetic tree of the 42 self-tested *K. aerogenes* strains with a heatmap showing the distribution of antimicrobial resistance and AMR determinants. The red text background indicates the CRKA strain, and the blue text background indicates the Non-CRKA strain. The three bands at the left of the heatmap indicate the information of the branch, MLST, department and source of the strains, respectively.

commonly carries resistance genes such as *rmtB,* $bla_{\text{CTX-M-65}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{TEM-1}}$. Among these, *rmtB* belongs to the aminoglycoside resistance gene family, while the other three genes belong to the β-lactamase family. Particularly, the presence of the $bla_{\text{KPC-2}}$ resistance gene is closely associated with carbapenem resistance.

The branch C1 strains exhibit minimal SNP variations, with discrepancies ranging from 0 to 13 SNPs (Supplementary Figure S4). They display a high degree of concordance in terms of antibiotic resistance genes and resistance patterns. Notably, with the exception of KaeR21 obtained in 2021, all of these strains were obtained in 2022, underscoring a pronounced trend of clonal dissemination among these isolates. Importantly, these strains were sourced from patients across diverse hospital departments, indicative of the prevalent cross-departmental transmission of this particular clone within the healthcare facility. Notably, KaeS15 exhibits distinct characteristics within this group. Unlike the others, it does not carry the bla_{KPC} resistance gene and displays sensitivity to carbapenem antibiotics.

Plasmid Analysis

Two CRKA strains KaeR16 and KaeR21, which carry the bla_{KPC} resistance gene, were selected for third-generation sequencing to investigate the plasmid structure. Three complete plasmids pKaeR16-2, pKaeR16-3 and pKaeR21-2 were obtained in this study. Characteristics of the plasmids are shown in Supplementary Table S3.

The plasmid structures of pKaeR16-2 and pKaeR21-2, both carrying the bla_{KPC} gene, exhibit a high degree of similarity. Furthermore, they share a high similarity with a plasmid designated pBSI057-KPC2 (NCBI accession number: MT269835), which was isolated from a blood infection in China. These three plasmids belonged to IncFII plasmid replicon. Almost all of the bla_{KPC}-carrying strains contained pKaeR16-2-like IncFII plasmid (Figure 3). From circular comparisons image of plasmids, it can be seen that the pKaeR16-2 plasmid contains a large number of insertion sequences, which is closely related to the transfer of drug resistance genes (Figure 4A). Numerous insertion sequences are found near the bla_{KPC} gene. Specifically, the IS*Kpn27* insert is situated upstream of the bla_{KPC} gene. In comparison to the pKaeR21-2 plasmid, the pKaeR16-2 plasmid carries additional resistance genes, including $bla_{\text{CTX-M-65}}$, $bla_{\text{TEM-1}}$, and

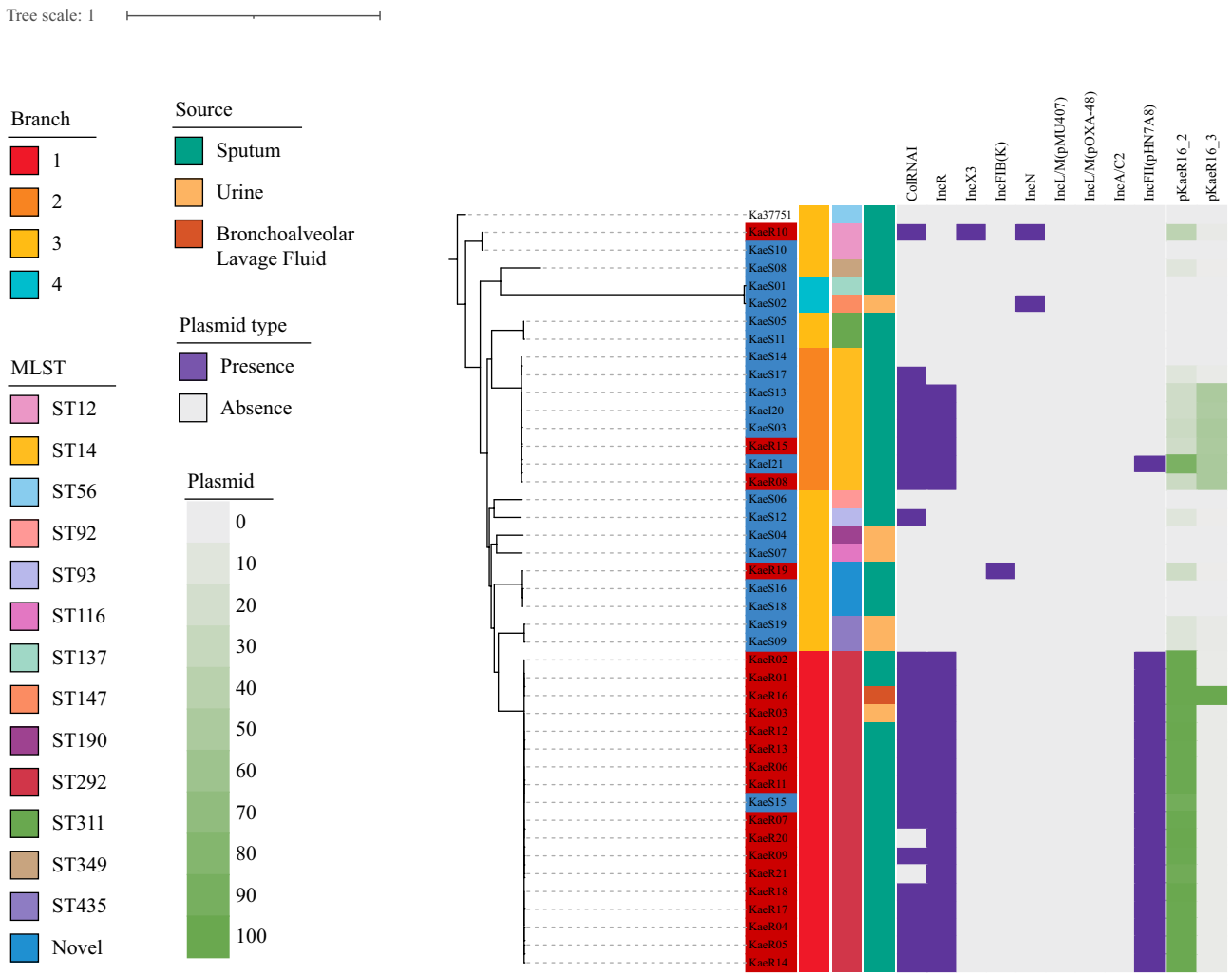

Figure 3 The plasmid replicons and the distribution of pKaeR16-2 and pKaeR16-3 in the *K. aerogenes* strains.

rmtB. The acquisition of these resistance genes is closely associated with transferable sequences. Particularly, the pKaeR16-2 plasmid contains repetitive acquisition of gene sequences: IS26-IS*Sbo1*-IS26-*tnpR*-$bla_{\text{TEM-1}}$-*rmtB*-IS26-IS903.

In the plasmid pKaeR16-3, the *qnrS1* gene was identified, which is a plasmid-mediated quinolone resistance gene (Figure 4B). The pKaeR16-3 was highly similar to pCFSAN027384 (NCBI accession number: CP074250.1). Compared with pCFSAN027384, pKaeR16-3 had an additional *ISEc63-IS26-qnrS1-hin-IS26* sequence at the position of 22,038 to 32627bp. Two additional sequences annotated as putative proteins were found at positions 78,661–78,966 and 80,355–81,053.

Discussion

The increasing global prevalence of β-Lactamase-Producing Pathogens, particularly Carbapenemase-producing *Enterobacteriaceae*, poses a serious threat to public health worldwide.[6,33] The bla_{KPC} gene, which is one of the most common carbapenemases identified globally, exemplifies this danger.[34] The bla_{KPC} resistance gene was first discovered in 1996 in a *Klebsiella pneumoniae* isolate from a patient in the eastern United States.[35] Since then, it has rapidly spread globally, affecting numerous regions, including coastal areas of China such as Zhejiang and Shanghai.[36] In China, the prevalence of bla_{KPC}-producing strains is mainly due to the $bla_{\text{KPC-2}}$ variant.[37,38] These strains are often associated with *K. pneumoniae*, a nosocomial pathogen commonly found in healthcare environments.[36] Isolates carrying the bla_{KPC} gene

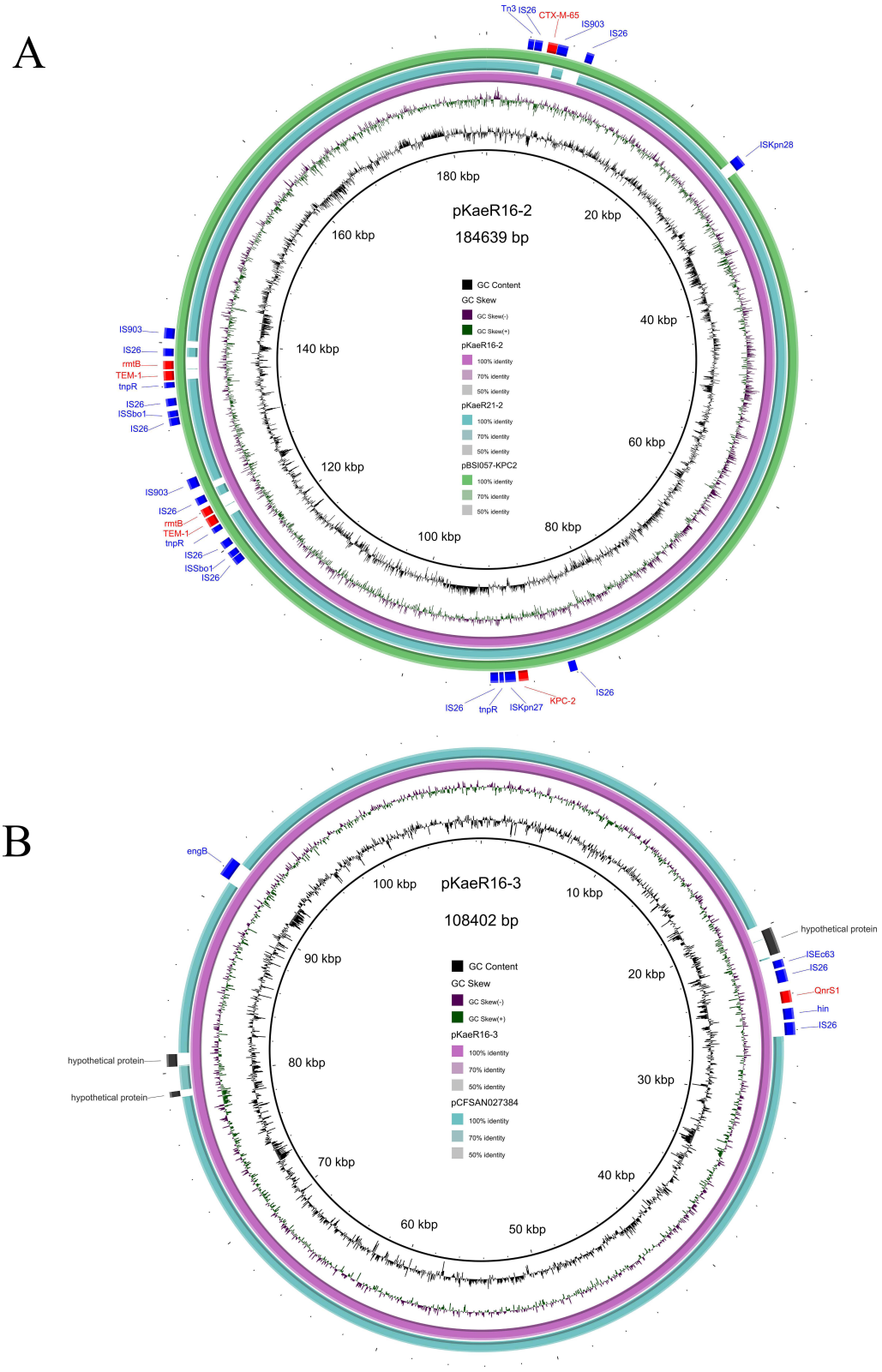

Figure 4 Circular comparisons image of plasmids. (**A**) Circular comparisons image of plasmids, pKaeR16-2, pKaeR21-2 and pBSI057-KPC2; (**B**) Circular comparisons image of plasmids, KaeR16-3 and pCFSAN027384.

typically exhibit resistance to a wide variety of drugs, including β-lactams, especially to carbapenem antibiotics. A study found that 92% (11/12) of CRKA carries the bla_{KPC-2} gene, confirming its pivotal role in carbapenem resistance mechanisms.[10] Furthermore, these pathogens often carry additional resistance determinants such as ESBLs, resulting in a highly resistant phenotype. Recent clinical surveillance efforts in China reveal a concerning trend: a rapid increase in carbapenem resistance among *K. pneumoniae* and other *Enterobacteriaceae* species, with rates exceeding 11%.[39] The increasing resistance to carbapenems not only reduces their therapeutic effectiveness but also limits the options available to clinicians, making it difficult to manage severe Gram-negative bacterial infections. The consequences of this resistance are significant, requiring immediate and coordinated global action to control the spread and impact of these dangerous pathogens.

This study reports the prevalence and transmission of CRKA carrying the bla_{KPC-2} gene within a hospital setting. Infected patients primarily originated from the Cardiovascular Internal Medicine Department (33.3%) and the Respiratory Internal Medicine Department (33.3%). The spread of strains in these departments may be associated with mechanical ventilation or invasive surgeries, particularly surgical procedures. CRKA demonstrated extensive antibiotic resistance compared to Non-CRKA, notably exhibiting complete resistance to β-lactamase inhibitors and cefotetan. This underscores the limited therapeutic options available for treating infections caused by CRKA and emphasizes the severity of the multidrug resistance exhibited by CRKA.

The antibiotic resistance of bacterial strains is closely related to the resistance genes they carry. CRKA exhibits extremely high resistance to β-lactam antibiotics such as Piperacillin/tazobactam and Cefotetan. This resistance is closely associated with the widespread presence of bla_{TEM-1} and $bla_{CTX-M-65}$ resistance genes in CRKA. bla_{KPC-2} and bla_{KPC-3} can directly lead to resistance to carbapenem antibiotics like Meropenem and Imipenem. The prevalence of bla_{KPC-2} /bla_{KPC-3} in CRKA reaches 81.0%. KaeR10 does not contain the bla_{KPC} resistance gene but carries the bla_{NDM-5} resistance gene. bla_{NDM-5} gene is a type of metallo-β-lactamase that can hydrolyze almost all β-lactam antibiotics, including carbapenems. Compared to Non-CRKA, CRKA exhibits extremely high resistance to aminoglycoside antibiotics such as Amikacin and Tobramycin. The observed difference is primarily attributed to the presence of the *rmtB* resistance gene. This gene modifies the binding site of aminoglycoside antibiotics on the bacterial 16S rRNA ribosome, resulting in bacterial resistance to aminoglycoside antibiotics.[40] Additionally, *rmtB* can be transferred horizontally among different bacteria, further complicating the issue of aminoglycoside resistance. Common hospital infection bacteria, such as *Pseudomonas aeruginosa, Escherichia coli*, and *K. pneumoniae*, are often found to carry this gene. Plasmid-mediated quinolone resistance genes (PMQR), such as *qnr* and *aac(6')-Ib-cr*, can lead to bacterial resistance to ciprofloxacin.[41] Among the 14 ciprofloxacin-resistant bacterial strains studied, 8 were found to carry the *qnrS1* resistance gene. Tigecycline, as a third-generation tetracycline, is considered the "last line of defense" against multi-drug resistant *Enterobacteriaceae*, including carbapenemase-producing strains.[42] Currently, tigecycline resistance mechanisms identified in *Enterobacteriaceae* include mutations in the ribosomal protein binding site, modifying enzyme genes, and active efflux pump transport systems. Plasmid-borne transferable modifying enzyme genes such as *tet(X)* and its variants can mediate high-level resistance to tigecycline. The efflux pumps include the RND family (*AcrAB-TolC, OqxAB*)[43] and mutations in *Tet(A)* and *Tet(L)* efflux pumps in the MFS family.[44] Efflux pumps are widespread in *Enterobacteriaceae* bacteria, and the overexpression of efflux pump-related genes is a significant mechanism contributing to bacterial multidrug resistance.[45] It is reported that the AcrAB efflux pump is a major resistance mechanism for tigecycline in *Enterobacteriaceae bacteria*.[46] In this study, four Non-CRKA strains carried the *tet(A)* resistance gene, but only one strain showed resistance to tigecycline, and no mutation was observed in the *tet(A)* gene. The presence of the AcrAB-TolC efflux pump was observed in all strains, with a higher tigecycline resistance rate (66.7%) in carbapenem-resistant strains. The underlying factors contributing to the disparity in tigecycline resistance rates between CRKA and Non-CRKA are not yet well-defined, necessitating further investigation.

Bacterial biofilms enhance the resistance of bacteria, enabling them to adapt to harsh environments and exhibit antibiotic resistance, which can lead to the emergence of multidrug-resistant, extensively drug-resistant, or even totally drug-resistant bacteria.[47,48] Bacteria can form and grow biofilms on the surface of medical devices (such as sutures, catheters, and dental implants) through the production of extracellular polymeric substances,[47] causing persistent chronic infections and posing a significant threat to human health.[49] In this study, all 42 strains of *K. aerogenes* demonstrated

strong biofilm formation capabilities, with no significant difference in biofilm strength between CRKA and Non-CRKA strains. The strong biofilm-forming ability may facilitate the long-term colonization and growth of *K. aerogenes*, especially CRKA strains, on hospital equipment and pipelines, potentially leading to further spread of these strains within the hospital environment. Moreover, the study found that some antibiotic-resistant strains, despite lacking relevant resistance genes, might have their resistance influenced by their biofilms.

The production of hemolysins by bacteria is capable of disrupting the cell membranes of host erythrocytes, resulting in the release of cellular contents, particularly hemoglobin. This feature, shared by many pathogenic bacteria such as *Streptococcus*[50] and *Staphylococcus aureus*,[51] also interferes with the host immune system by inducing an immune response and inflammation, which in turn facilitates bacterial invasion and spread.[52] *K. aerogenes* does not normally have hemolytic activity, but may develop hemolytic activity under certain specific pathogenic factors or stressful environments.[53] Although direct hemolytic events caused by *K. aerogenes* are rare in clinical practice, its potential hemolytic capacity should not be completely ignored, especially in immunosuppressed or high-risk patient populations.

Research has found that in the *Enterobacteriaceae* family, $bla_{\text{KPC-2}}$ genes are often located on IncFIIK2-type plasmids.[54] The genes expressing bla_{KPC} are frequently present in the form of the composite transposon Tn4401. In a $bla_{\text{KPC-2}}$-carrying *K. aerogenes* strain isolated from Taiwan, the $bla_{\text{KPC-2}}$ resistance gene in the pKPC-LK30 plasmid was adjacent to a disrupted Tn3 and IS*Kpn8*, followed by $bla_{\text{KPC-2}}$ and IS*Kpn6*-like elements. The region containing $bla_{\text{KPC-2}}$ and a part of the downstream IS*Kpn6*-like gene is similar to Tn4401.[55] In the pPAEC79 plasmid extracted from *Pseudomonas aeruginosa*, the $bla_{\text{KPC-2}}$ gene environment includes *korC*-ΔIS*Kpn6*-$bla_{\text{KPC-2}}$-IS*Kpn27*, where the $bla_{\text{KPC-2}}$ gene is closely related to the IS*Kpn6* family transposon and IS*Kpn27* family transposon.[56] Plasmid structures often undergo recombination during their transmission. In a multidrug-resistant *K. aerogenes* strain isolated in Guangzhou, China, it carries multiple resistance genes including *fosA3,* $bla_{\text{KPC-2}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1}}$, and *rmtB*. Detailed comparisons suggest that the pKP1034 plasmid might have evolved through recombination of the $bla_{\text{KPC-2}}$ carrying plasmid pKPC-LK30 from Taiwan and the plasmid carrying the prevalent *fosA3* gene, pHN7A8, from mainland China.[57] The pCT-KPC334 plasmid shares a highly similar gene structure with pKP1034 but has experienced slightly different recombination events.[58] These findings highlight the complexity and dynamism of plasmid evolution, especially concerning the mechanisms by which resistance genes are acquired, recombined, and propagated among various bacterial species and geographical locations. Understanding these processes is crucial for developing strategies to mitigate the spread of antibiotic resistance. In this study, the majority of carbapenem-resistant *K. aerogenes* strains carried ColRNAI, IncR, and IncFII(pHN7A8) plasmid replicon types, with the $bla_{\text{KPC-2}}$ resistance gene situated on the IncFII(pHN7A8) plasmid. The $bla_{\text{KPC-2}}$ gene structure was determined to be IS26-$bla_{\text{KPC-2}}$-ΔTn3-IS26, with the Tn3 disrupted by the inserted IS26 sequences. Plasmids pKaeR16-2 and pKPC-LK30 (93% query coverage and 99.98% nucleotide identity), as well as pKP1034 (94% query coverage and 99.98% nucleotide identity), demonstrate very high similarity, indicating a similar environment for the $bla_{\text{KPC-2}}$ gene. Additionally, on the pKaeR16-2 plasmid, there is the $bla_{\text{CTX-M-65}}$ gene mediated by Tn3, along with a complete insertion of the IS26-IS*Sbo1*-IS26-*tnpR*-$bla_{\text{TEM-1}}$-*rmtB*-IS26-IS903 segment, which further enhances the antibiotic resistance of the bacteria, including resistance to β-lactam antibiotics and ESBLs.

Fourteen sequence types (STs) were identified in this study, with ST292 being the most prevalent in CRKA. Furthermore, there was a novel ST type identified, which is closely related to ST192, indicating genetic diversity among *K. aerogenes*. Malek et al identified ST4 and ST93 as major ST clones associated with human infections of *K. aerogenes*.[59] A previous study comprehensively characterized 91 isolated strains of *K. aerogenes* using whole-genome sequencing data from GenBank. The findings indicated that the prevalent sequence types (STs) for *K. aerogenes* were ST93 and ST440.[60] To our knowledge, this is the first reported study on the epidemic spread of CRKA clones within the ST292. In light of the emergence of extensively drug-resistant strains within this ST, there is a pressing need to enhance focused surveillance and monitoring for this particular ST.

Malek's research indicated that CRKA isolates tend to form a tight monoclonal cluster, suggesting prolonged intraward transmission.[59] In our study, the clonally transmitted CRKA clustered on the C1 branch of the phylogenetic tree, with core SNP differences within 13 bp, carrying similar resistance genes, plasmids, and resistance phenotypes. These isolates originated from different departments, indicating the occurrence of a clonal spread phenomenon within the same or different wards during the same period.

In conclusion, our study sheds light on the alarming rise of MDR *K. aerogenes* strains, particularly those harboring carbapenemase genes. This clone transmission phenomenon within the hospital may lead to the further spread and dissemination of extensively drug-resistant CRKA in the population. The clonal dissemination of these strains within hospital settings underscores the pressing need for comprehensive infection control measures. Additionally, the role of excessive antibiotic use in fueling resistance highlights the importance of antibiotic stewardship to preserve the efficacy of available antibiotics. As MDR strains continue to evolve and spread, collaborative efforts between healthcare providers, researchers, and policymakers are essential to safeguard public health and address the growing threat of antibiotic resistance.

Data Sharing Statement

The sequencing data generated in this study have been deposited in the China National Microbiology Data Center (NMDC) under the BioProject number NMDC10018615. Source data are provided with this paper. Strain numbers are listed in Supplementary Table 1. The complete plasmid sequences have been deposited under the project NMDC10018615 with accessions NMDC60143167-NMDC60143169.

Ethical Statement

The clinical samples were part of the routine hospital laboratory procedure. The isolates from these samples were not specifically isolated for the present study. As all data about the clinical samples or isolates were collected and interpreted anonymously, the Ethics Committee of the Second Medical Centre of PLA General Hospital, Beijing, China waived the need for written informed consent from the participants related to the clinical samples or isolates.

Acknowledgments

Special thanks to the Chinese People's Liberation Army General Hospital for providing bacterial strains, drug sensitivity testing, and species identification. Additionally, we express gratitude to all the authors involved in this study for their significant contributions in conceptualization, research design, execution, data acquisition, analysis, and interpretation.

Funding

This work was supported by the Beijing Municipal Natural Science Foundation (NO. L234051) and the National Nature Science Foundation of China (grant numbers: no. 82173580 and 32141003).

Disclosure

The authors report no conflicts of interest in this work.

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