ORIGINAL PAPER
Polish Journal of Microbiology
2025, Vol. 74, No 1, 82–94
https://doi.org/10.33073/pjm-2025-007

Characterization of OXA232-Producing Carbapenem-Resistant *Klebsiella pneumoniae*: Genomic Analysis and Virulence Assessment

ZHOUXUN LI[1, 2], CHUNYAN WU[1], XUEMEI CAI[1], YONGLI SONG[1], XINGPING ZHENG[1, 3], YUAN HE[1] and GUIBO SONG[1]*

[1] Department of Clinical Laboratory, The First Affiliated Hospital of Kunming Medical University; Yunnan Key Laboratory of Laboratory Medicine; Yunnan Province Clinical Research Center for Laboratory Medicine; Kunming, China
[2] Department of Nuclear Medicine, The Affiliated Hospital of Yunnan University, Kunming, China
[3] Department of Transfusion Medicine, The Affiliated Hospital of Yunnan University, Kunming, China

Submitted 12 November 2024, accepted 24 January 2025, published online 25 March 2025

Abstract

Globally, the infection rate of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) producing OXA-48-like carbapenemase is increasing, posing a significant public health threat due to its high antibiotic resistance. Between December 2019 and April 2023, ten CRKP strains carrying the OXA-48-like carbapenemase were isolated from inpatients at the First Affiliated Hospital of Kunming Medical University. Whole-genome sequencing (WGS) revealed that all strains carried the OXA-232 gene, a variant of OXA-48-like, located on the non-conjugative ColKP3 plasmid. Sequence typing identified nine strains as ST231 and one as ST11. The ST231 strains carried common virulence genes, including yersiniabactin (ybtA, fyuA, irp2) and aerobactin (iucABCD, iutA), while the ST11 strain carried high-virulence genes (rmpA, rmpA2, peg-344) as well as KPC-2 and OXA-232 carbapenemase genes on separate plasmids, suggesting that CRKP can harbor multiple plasmids with carbapenemase genes. Sequence typing of 264 global ST231 CRKP isolates (n = 264) showed a distinct clonal relationship between our strains and Indian CRKP isolates, indicating potential cross-border transmission. The virulence potential and immune response of the ST231 strains were assessed using a mouse respiratory infection model. The concentrations of inflammatory factors CCL2/MCP-1, IL-6, and TNF-α in the alveolar lavage fluid and blood of the model mice were detected. Combined with the pathological analysis of lung and liver tissues, it reveals variability in virulence and immune response despite carrying identical resistance and virulence genes. This underscores the urgent need for monitoring and tailored public health strategies to combat the global spread of drug-resistant strains.

Keywords: *Klebsiella pneumoniae*, OXA-232, ST231, multidrug resistance, virulence factors

Introduction

The rise of antibiotic-resistant pathogens emerged as a critical global health challenge, complicating the treatment of common infectious diseases. Among these, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) emerged as a particularly concerning threat, given its significant role in hospital-acquired infections and its resistance to last-resort antibiotics (Lan et al. 2021). This issue was starkly highlighted by the widespread dissemination of strains harboring OXA-48-like carbapenemases, which were identified as a key driver behind the alarming rise in resistance rates observed worldwide (Aslan et al. 2022).

The identification of OXA-48-like enzymes marked a landmark in the understanding of bacterial resistance mechanisms. These enzymes were first discovered in Turkey in the early 2000s, isolated from the urethra of a 54-year-old patient suffering from a urinary tract infection and skin burns at Istanbul College Hospital (Poirel et al. 2004). Since then, OXA-48-like enzymes have been reported in various *Klebsiella* strains across multiple continents, highlighting their widespread dissemination (Chen et al. 2021; Cerón et al. 2023; Singh et al. 2023). OXA-48-like enzymes belong to a class of broad-spectrum β-lactamases capable of hydrolyzing penicillins, cephalosporins, and carbapenems, thus conferring resistance to these key antibiotics (Boyd et al. 2022).

* Corresponding author: G.Song, Department of Clinical Laboratory, The First Affiliated Hospital of Kunming Medical University; Yunnan Key Laboratory of Laboratory Medicine; Yunnan Province Clinical Research Center for Laboratory Medicine; Kunming, China; **e-mail: songgb1229@163.com**

Characterized by potent hydrolytic activity against carbapenems and diminished activity against cephalosporins like ceftriaxone, these enzymes pose unique challenges in both diagnosis and treatments (Boyd et al. 2022).

OXA-232, the third most common OXA-48-like variant globally, has garnered attention due to its enhanced activity against a broad spectrum of beta-lactams, thereby complicating therapeutic protocols. To date, eight sequence types (STs) of OXA-232-producing *K. pneumoniae* isolates reported worldwide are ST14, ST15, ST16, ST17, ST147, ST231, ST307 and ST395 (Chen et al. 2023). In China, OXA-232-producing *K. pneumoniae* identified mainly belongs to ST15, which carries a 6.1 kp unconjugated ColKP3 plasmid containing the $bla_{\text{OXA-232}}$ gene (Wu et al. 2023).

Rising antimicrobial resistance (AMR) in bacterial pathogens is mainly driven by the horizontal transfer of AMR genes and the accumulation of spontaneous mutations, with different mobile genetic elements (MGEs) playing a key role in their dissemination (Mehrotra et al. 2023). In this study, we hypothesized that OXA-232-producing *K. pneumoniae* strains isolated from a hospital in Kunming may display genomic diversity attributable to regional variations, particularly in their plasmid content and resistance gene profiles. Furthermore, the dissemination of the OXA-232 resistance gene may be further amplified by horizontal gene transfer (HGT).

Experimental

Materials and Method

Collection and isolation of strains. This study was conducted in accordance with the ethical guidelines of the First Affiliated Hospital of Kunming Medical University (Approval No. 2021-L20). All CRKP strains were isolated from patient body fluids at the First Affiliated Hospital of Kunming Medical University between December 2019 and April 2023. None of the patients reported recent international travel during this period. CRKP strains carrying the OXA-48 enzyme were initially screened using PCR. Our research team designed the primers used to detect the OXA-48-like gene. The primers were as follows: OXA-48-F: (5'-ACACCAAGTCTTTAAGTGGGATG-3'); OXA-48-R: (5'-CCCGAAATGTCCTCATTACC-3'). These primers were validated through sequencing.

Whole genome sequencing and multi-locus sequence typing. The genomic DNA of the strains was extracted from bacterial pellets, and high-quality DNA samples ($OD_{260/280} = 1.8–2.0$) were sent to Shanghai Majorbio for sequencing services (contract number: MJ20230306063). Second-generation sequencing was performed using the the Illumina® NovaSeq™ 6000 platform (Illumina, Inc., USA), while third-generation sequencing was carried out using the Oxford Nanopore platform (Oxford Nanopore Technologies plc., UK). Each sample was sequenced to a depth of 100×. The sequencing data were assembled using SOAPdenovo2 (https://github.com/aquaskyline/SOAPdenovo2) and Unicycler (https://github.com/rrwick/Unicycler/releases). Predicted genes were annotated and analyzed using six major functional databases: NR, Swiss-Prot, Pfam, COG, GO, and KEGG. The sequence type of each isolate was determined using MLST (https://bitbucket.org/genomicepidemiology/mlst/src/master). Plasmids were annotated using the PLSDB database (https://ccb-microbe.cs.uni-saarland.de/plsdb), insertion sequences were analyzed using ISEScan (https://github.com/xiezhq/ISEScan/releases), and integrons were predicted using Integron_Finder (https://github.com/gem-pasteur/Integron_Finder). K- and O-types were identified using Kleborate software.

Evolutionary tree analysis. In the PATRIC database (https://www.patricbrc.org), strains were identified using the keyword "MLST.Klebsiella_pneumoniae.231", and reference genome sequences were downloaded based on genome IDs. If unavailable, NCBI Assembly Accessions were retrieved using BioSample Accession numbers. Genome sequences were annotated using Abricate (v1.0.1, https://github.com/tseemann/abricate) against the CARD database to identify OXA-48-like genes. The presence of yersiniabactin, aerobactin, K_locus, and O_locus elements was determined with Kleborate (v2.4.1, https://github.com/klebgenomics/Kleborate). Strains without these annotations were excluded, resulting in 264 strains (including 9 in-house strains). Core SNP analysis was performed on the 264 strains using Snippy (v4.6.0, https://github.com/tseemann/snippy), with strain 573.15592 (GenBank accession: GCA_003432165.1) as the reference. Recombined regions in core SNPs were removed using Gubbins (v3.3.1, https://github.com/nickjcroucher/gubbins), and an evolutionary tree was constructed using Fasttree (v2.1.11, http://www.microbesonline.org/fasttree).

Toxicity potential analysis and inflammatory factor assay. All animal experiments were reviewed and approved by the Laboratory Animal Ethics Committee of Yunnan Besitai Biological Technology (Approval No. BST-mouse-2024037-07). A mouse respiratory infection model was developed using 6-week-old male C57 mice (three mice per group). Each mouse was inoculated with 30 μl of bacterial suspension (10^8 CFU/ml) by intranasal instillation. The control group received the same volume of physiological saline intranasally. Standard strains *K. pneumoniae* ATCC® 700603™ from the Clinical Laboratory of the First Affiliated Hospital of Kunming Medical University were used as positive controls. The health of each mouse was monitored daily.

After seven days, alveolar lavage fluid and blood samples were collected from the mice. The alveolar lavage fluid was inoculated on agar plates and incubated at 37°C for 48 hours to assess bacterial growth, followed by colony counting and bacterial identification. Inflammatory factors CCL2/MCP-1, IL-6, and TNF-α in blood were measured by ELISA to assess the inflammatory response in the mice. ELISA assays were performed using kits purchased from MultiSciences (Lianke) Biotech Co., Ltd. (China), including Mouse CCL2/MCP-1 (Catalog No. EK287/2-96), IL-6 (Catalog No. EK206/3-96), and TNF-α (Catalog No. EK282HS3-96).

Statistical analysis. Statistical analyses and data visualizations were performed using GraphPad Prism v9.0 (GraphPad Software, USA, www.graphpad.com). Categorical data were presented as counts or percentages. *T*-tests were used to compare between two groups, while Analysis of Variance (ANOVA) was applied for comparisons across multiple groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Clinical Characteristics. A total of 10 unique, non-redundant strains of OXA-48-like enzyme-producing *K. pneumoniae* are obtained from inpatients ranging in age from 37 to 78 years. The isolates were derived from six distinct clinical departments: Intensive Care Unit (ICU, three isolates), Cardiac Surgery (two isolates), Transplantation Centre (two isolates), Emergency Intensive Care Unit (EICU, one isolate), Neurology (one isolate), and Hepatobiliary Surgery (one isolate). The corresponding specimens were sourced from sputum (four isolates), blood (two isolates), drainage fluids (two isolates), and ascitic fluid two isolates), providing a comprehensive representation of the clinical diversity. These findings are summarized in Table I.

Genomic Analysis and Typing. Sequencing results identified nine isolates as sequence type ST231 and one as ST11. All ST231 isolates contained four plasmids carrying genes encoding β-lactamases (CTX-M-186, TEM-1, TEM-243, CTX-M-15, and SHV-1) and the carbapenemase OXA-232. The ST11 isolate carried seven plasmids with multiple resistance genes, including carbapenemases OXA-232 and KPC-2 (Table II). The $bla_{\text{OXA-232}}$ gene was consistently identified on a 6.1 kb ColKP3-type nonconjugated plasmid in the majority of isolates, displaying 100% sequence identity with plasmid pWSD411_7 (accession No. NZ_CP045680.1), reported in *K. pneumoniae* at Shaw Hospital, Zhejiang University, in 2019. In contrast, strain Kp8 harbored a ColKP3-type plasmid with over 99% similarity to pKPM503 (accession No. NZ_CP031737.1), described

Table I
Clinical and demographic characteristics.

Patient No./isolate	Age/ sex	Ward	Specimen	Diagnosis	Invasive procedure	Antimicrobial therapy	Prognosis
12(Kp1)	52(M)	ICU	drainage fluids	abscess of the parotid gland, septic shock	incision and drainage of an abscess	meropenem, tigecycline, fluconazole	unknown
32(Kp2)	68(M)	Cardiac Surgery	sputum	severe pneumonia, septic shock	heart surgery		died
52(Kp3)	51(M)	Transplantation Centre	blood	malignant tumor of the hilar bile duct	resection of hepatic bile duct lesions	tigecycline	recovered
55(Kp4)	45(F)	Transplantation Centre	drainage fluids	liver abscess	partial hepatic lobectomy	vancomycin, flucytosine	recovered
206(Kp6)	56(M)	EICU	ascites	abdominal infection, infectious shock	left thoracocentesis for drainage	cefoperazone sulbactam, polymyxin, meropenem, linezolid, tigecycline	unknown
209(Kp7)	78(M)	Neurology	blood	sepsis, bacteremia	non-operative	imipenem, tigecycline	recovered
255(Kp8)	50(M)	ICU	ascites	acute necrotizing pancreatitis, septic shock	incision and drainage of retro-peritoneal abscesses	vancomycin, imipenem, tigecycline, caspofungin	recovered
356(Kp9)	68(M)	ICU	sputum	severe pneumonia, septic shock	tracheal tube	vancomycin, imipenem, polymyxin, caspofungin	died
434(Kp10)	37(M)	Hepatobiliary Surgery	sputum	septic shock	abdominal explo-ratory surgery, tracheal tube	ertapenem, meropenem, imipenem	unknown
J19(Kp13)	58(M)	Cardiac Surgery	sputum	coronary atheroscle-rotic heart disease	coronary bypass graft		recovered

Table II
Genotypic profiles and resistance mechanisms of *Klebsiella pneumoniae* isolates.

Isolates	ST type	Plasmid replicons	Size of plasmid replicons	β-lactamases and carbapenemases
Kp1	ST11	IncFIB(K)/IncHI1B, IncR/IncFII, IncFIB, IncFII, ColRNAI, ColKP3	193835 bp, 133766 bp, 112792 bp, 84876 bp, 11970 bp, 5596bp, 6141 bp	SHV-11, SHV-12, CTX-M-65, LAP-2, OXA-232, KPC-2
Kp2	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	126359 bp, 71307 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp3	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127803 bp, 71307 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp4	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127803 bp, 71688 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp6	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	126406 bp, 71307 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp7	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127803 bp, 71307 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp8	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127803 bp, 71307 bp, 6101 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp9	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127806 bp, 71307 bp, 6141 bp CTX-M-15, SHV-1, OXA-232	CTX-M-186, TEM-1,
Kp10	ST231	IncFII/IncFIB, IncFIA/IncFII, ColKP3	127806bp, 71307 bp, 6141 bp	CTX-M-186, TEM-1, CTX-M-15, SHV-1, OXA-232
Kp13	ST231	IncFIB/IncFII(K), IncFIA/IncFII, ColKP3	126359 bp, 71305 bp, 6141 bp	CTX-M-186, TEM-1, CTX-M-15, SHV-1, OXA-232

in 2020 at Sungkyunkwan University, Korea. Moreover, despite this minor divergence, the $bla_{OXA-232}$ gene in Kp8 was consistently localized on the ColKP3-type plasmid, underscoring the evolutionary conservation and adaptability of this genetic element across diverse plasmid contexts.

An IncFII/FIB-type plasmid was present in all isolates except kp13, containing resistance genes such as rmtF, AAC(6')-Ib, CTX-M-186, TEM-1, TEM-243, catA1, qnrS1, and ARR-2. This plasmid shared over 99% homology with pIncFIBpQil (accession No. NZ_CP036321.1), previously isolated from *K. pneumoniae* in India. Compared to pIncFIBpQil, the nine ST231 strains exhibited two gaps in the gene sequence, bordered by mobile genetic elements and deletions of specific resistance genes (Fig. 1).

A second plasmid, an IncFIA/FII-type, was detected containing resistance genes aadA2, mphA, sul1, and dfrA12, along with the aerobactin virulence gene iuc5. This plasmid showed over 99% homology with pIncFIA (accession No. NZ_CP036329.1), also isolated from *K. pneumoniae* in India.

A third plasmid, an IncFIB/IncFII(k)-type, was exclusively identified in Kp13. It displays 100% homology with plasmid p1 (accession No. NZ_CP033947.1) from *K. pneumoniae* subspecies *pneumonia* in the United States. The resistance genes in this plasmid were consistent with those in the IncFII/FIB-type plasmid.

Additionally, the *iuc5* virulence gene in the ST11 strain was located in the IncFIB(k)/IncHI1B plasmid, which also carried high-virulence genes, including rmpA, rmpA2, and peg-344. This plasmid showed over 99% homology with pKP20194a-p1 (accession No. NZ_CP054781.1), isolated from a highly virulent ST11-type *K. pneumoniae* strain in Hunan. These findings highlight the varied genetic adaptations and spread of plasmid-encoded resistance genes, emphasizing the need for surveillance to monitor and control the dissemination of these multidrug-resistant plasmids.

The Virulence genes. The results demonstrated the distribution and diversity of key virulence genes among the strains, with aerobactin (*iucABCD*) and yersiniabactin (*ybtA*, *fyuA*, *irp2*) identified as the primary iron uptake systems. The universal presence of aerobactin highlighted its critical role in bacterial survival under iron-limited conditions, reinforcing its status as a hallmark of high-virulence *Klebsiella pneumoniae* (hvKP). In contrast, yersiniabactin, while present in most strains, is absent in some (e.g., Kp1 and Kp13), suggesting variability in virulence mechanisms. Other virulence factors, such as genes involved in immune modulation, adherence, and effector delivery systems, were also detected. Notably, Kp1 carries high-virulence genes like *rmpA*, *rmpA2*, and *peg-344*, associated with hypermucoviscosity and an enhanced pathogenic profile.

The phylogenetic tree revealed clustering patterns that align with virulence gene profiles. Kp3, Kp6, Kp7, and Kp8 cluster closely, sharing similar virulence characteristics, and indicating potential clonal dissemina-

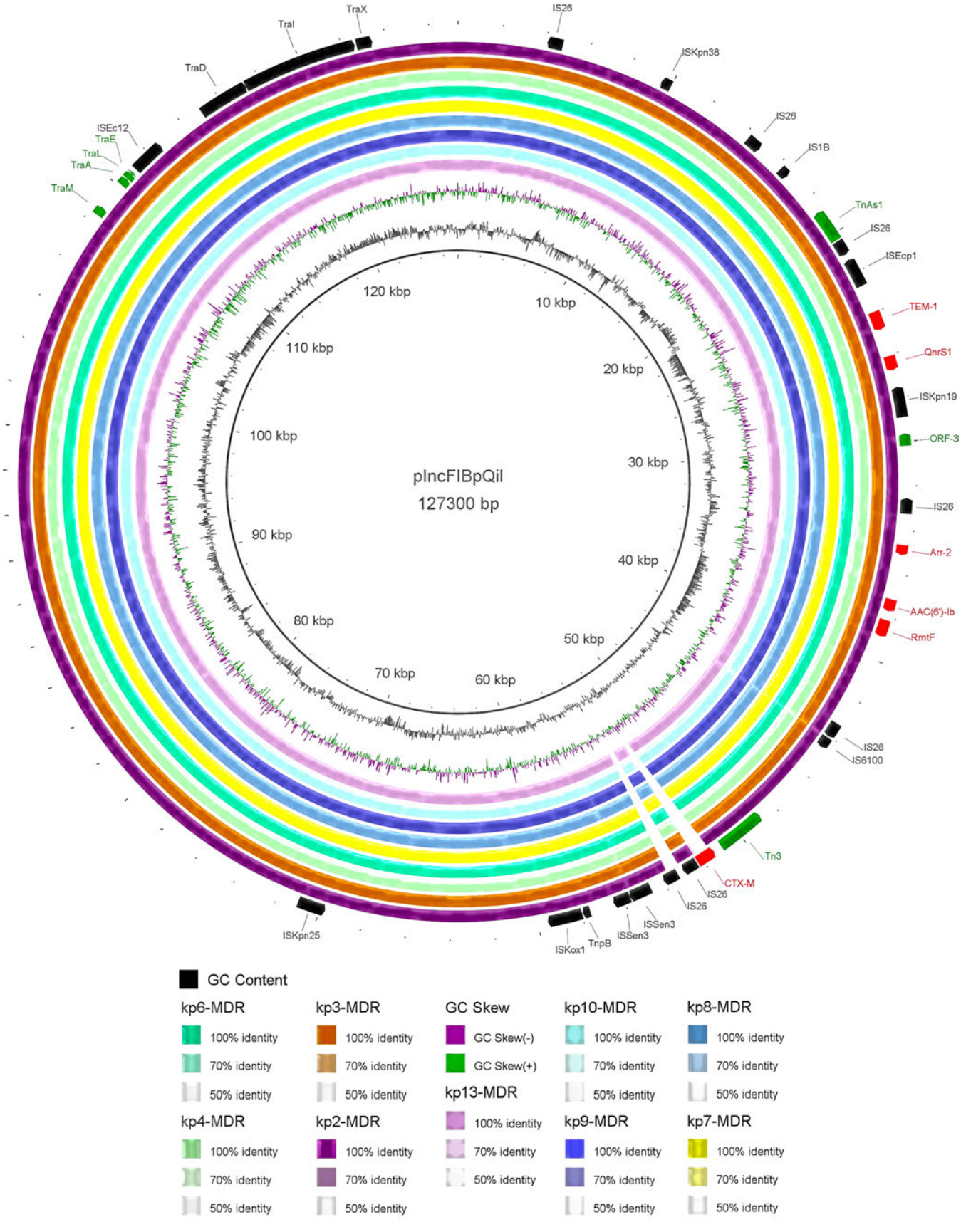

Fig. 1. Circular representation of IncFII/FIB-type plasmids in ST231 isolates.

The plasmid pIncFIB-Q1 spans 127,300 base pairs (bp). The map is divided into several tracks, each representing different genomic features. The outermost tracks mark specific genes with resistance markers (e.g., blaKPC, qnrB19), the middle tracks show genes with varying expression levels indicated by different color shades, and the innermost tracks depict GC content and GC skew. Each track uses a distinct color code, as the legend explains, to represent gene density, antibiotic resistance genes, virulence factors, and mobile genetic elements.

tion. Conversely, Kp13 is phylogenetically distinct but harbors unique virulence traits, reflecting the genetic plasticity of *K. pneumoniae* in acquiring virulence determinants through horizontal gene transfer (HGT). This strain, which combines high-virulence genes with carbapenem resistance (e.g., OXA-232), represented a significant clinical threat. The observed variability in virulence gene profiles suggested differences in pathogenic potential across strains, warranting further investigation into other virulence factors, such as enterobactin and capsule-associated genes, to provide a more comprehensive understanding of their pathogenic mechanisms.

Comparative Genomic Analysis. A comparative analysis was conducted using 264 publicly available ST231 genomes with the OXA-232 gene from different global regions to examine the genetic characteristics of the nine ST231 *K. pneumoniae* strains. A phylogenetic tree based on core SNPs (Single Nucleotide Polymorphisms) was constructed to map evolutionary relationships. The phylogenetic analysis divided the 264 ST231 genomes into four branches. The largest branch, designated clade 2, contained 215 genomes primarily from South Asia, East Asia, and Europe. Most ST231 isolates in this branch shared the podocarp gene locus KL51 and lipopolysaccharide locus O1V1, and carried the OXA-48-like variant of the resistance gene OXA-232. They also frequently harbored the aerobactin gene iuc5 and the *Yersinia pestis* mobile genetic element ICEKp5, which encodes important virulence factors. The second-largest branch, clade 3, included isolates with similar K/O gene loci to clade 2, but these typically carried OXA-type resistance genes other than OXA-48-like variants. Clade 3 isolates often showed deletions in the ICEKp3 and iuc5 genes, indicating potential variations in virulence profiles. The nine ST231 isolates in this study clustered within the clade 2 branch, primarily alongside Indian isolates, suggesting a close genetic relationship with these strains. This clustering provides key insights into the potential geographical origin of these strains and their genetic links to the global ST231 *K. pneumoniae* population. The genetic proximity to Indian isolates suggests possible spread and adaptation of these strains across regions, underscoring the need for global surveillance and coordinated efforts to control their dissemination (Fig. 3).

Virulence and pathogenicity testing. Histopathological analysis of liver, lung, and kidney tissues from infected mice was conducted to evaluate the virulence and pathogenicity of OXA-232-producing carbapenem-resistant *K. pneumoniae* (ST231 clone). Because the research focuses on the invasion capabilities of the respiratory tract and blood, four isolates from sputum and two isolates from the blood within the ST231 group were included in the testing. Mice were monitored for survival over seven days (Fig. 4), alveolar lavage fluid and blood samples were collected for bacterial colony counting (Fig. 5) and quantification of inflammatory factors, including CCL2/MCP-1, IL-6, and TNF-α (Fig. 6). During the seven-day study, mice infected with ST231-type isolates showed varying degrees of weight loss but ultimately survived, one of the isolates from the blood, Kp7, caused more significant weight loss in the model mice compared to the positive control ATCC® 700603™ (Fig. 4). The results of bacterial colony counting demonstrated that the invasive ability of each strain of bacteria on the lungs and blood was not uniform. For instance, Kp7 had a strong invasive ability on the lungs but a weak one on the blood. In contrast, Kp13 had a strong invasive ability on both (Fig. 5). Inflammatory factors, including CCL2/MCP-1, IL-6, and TNF-α, were elevated in both alveolar lavage fluid and blood, with the increase being more pronounced in alveolar fluid, likely due to initial lung invasion (Fig. 6). Histopathological assessments confirmed significant damage to lung and liver tissues, the lung tissue mainly shows pulmonary alveolar fusion, exudation of inflammatory cells, and proliferation of surface interstitial cells. In contrast, the liver tissue mainly shows cellular necrosis in the form of punctate and fragmented lesions (Fig. 7). These findings highlight the virulence of ST231 *K. pneumoniae*, demonstrating its ability to invade the bloodstream through the respiratory tract and cause substantial inflammatory damage in major organs.

Discussion

This study examined the genomic characteristics, resistance mechanisms, and virulence factors of OXA-232-producing carbapenem-resistant *K. pneumoniae*, focusing on the ST231 clone. In all ST231 isolates, the $bla_{\text{OXA-232}}$ gene was consistently located on a nonconjugative ColKP3 plasmid, aligning with previous studies identifying this plasmid as a frequent carrier of the OXA-232 gene (Potron et al. 2013; Abdul Momin et al. 2017; Mancini et al. 2018). The stable presence and dissemination potential of the $bla_{\text{OXA-232}}$ gene indicated that these strains exhibit high resistance levels with the potential for widespread distribution across various clinical settings. Notably, the detection of IncFIB(pQil) plasmids in all ST231 *K. pneumoniae* isolates supported prior findings that both ColKP3 and IncFIB(pQil) plasmids can carry $bla_{\text{OXA-232}}$ and $bla_{\text{TEM-1}}$, highlighting the essential role of these plasmids in spreading multiple resistance genes (Lutgring et al. 2018).

Our study also revealed structural changes in the IncFIB(pQil) plasmid, including deleting the antibiotic resistance gene CTX-M, which mirrors the plasmid structure of an ST231 strain previously identified in China. We found that a 8.6kb region containing $bla_{\text{TEM-181}}$

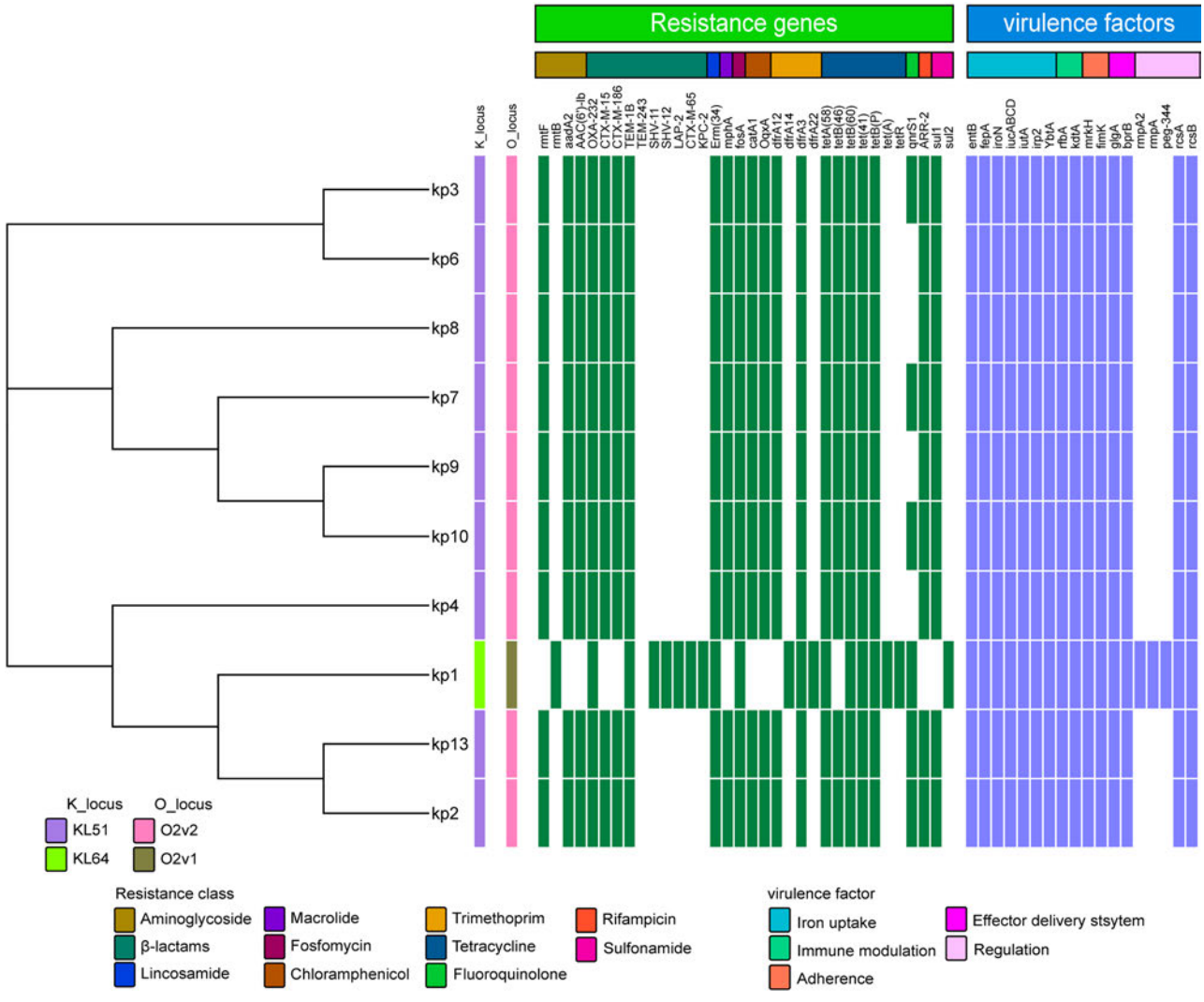

Fig. 2. Phylogenetic analysis and genotypic characterization of bacterial strains.

The phylogenetic relationships and distribution of resistance genes and virulence factors across ten bacterial strains (Kp1 to Kp13) are displayed. The phylogenetic tree (left) illustrates evolutionary distances based on whole-genome sequencing data, while the heatmap (right) indicates the presence (colored) and absence (white) of specific resistance genes (green) and virulence factors (blue).

and qnrS1 was lost from an MDR plasmid, likely due to surrounding mobile genetic elements (Chen et al. 2023). This dynamic evolution in plasmid structure reflected ongoing bacterial adaptation and survival strategies under antibiotic selective pressure. Detailed analysis of these plasmids enhances our understanding of their role in resistance gene spread, supporting the development of effective intervention strategies.

MLST typing identified ST11 and ST231 as multidrug-resistant clones primarily originating in South and Southeast Asia (Wyres et al. 2020). Phylogenetic analysis confirmed an increasing prevalence of these isolates in Europe, showing significant genetic similarities to Indian isolates (Shankar et al. 2019). This clustering suggests potential transnational dissemination, underscoring the need for strong global surveillance systems. The close genetic relationship to Indian strains implied regional transmission dynamics, likely facilitated by geographical and clinical interactions, even though the patients had no recent travel history. This finding is essential for creating region-specific public health strategies.

We found that two key iron-carrier virulence genes: yersiniabactin and aerobactin. The yersiniabactin genes, implicated in respiratory infections, promoted pneumonia development in mouse models (Bachman et al. 2012; Holt et al. 2015). Yersiniabactin was a critical siderophore system that enables pathogens to acquire iron in iron-limited environments, such as the host, enhancing bacterial fitness during infection and contributing to increased pathogenicity. Aerobactin plays a crucial role in nasopharyngeal colonization, enhancing the bacterium's ability to establish and maintain infection in this region (Bachman et al. 2009). The combined presence of these virulence factors significantly improved the ability of these isolates to acquire iron,

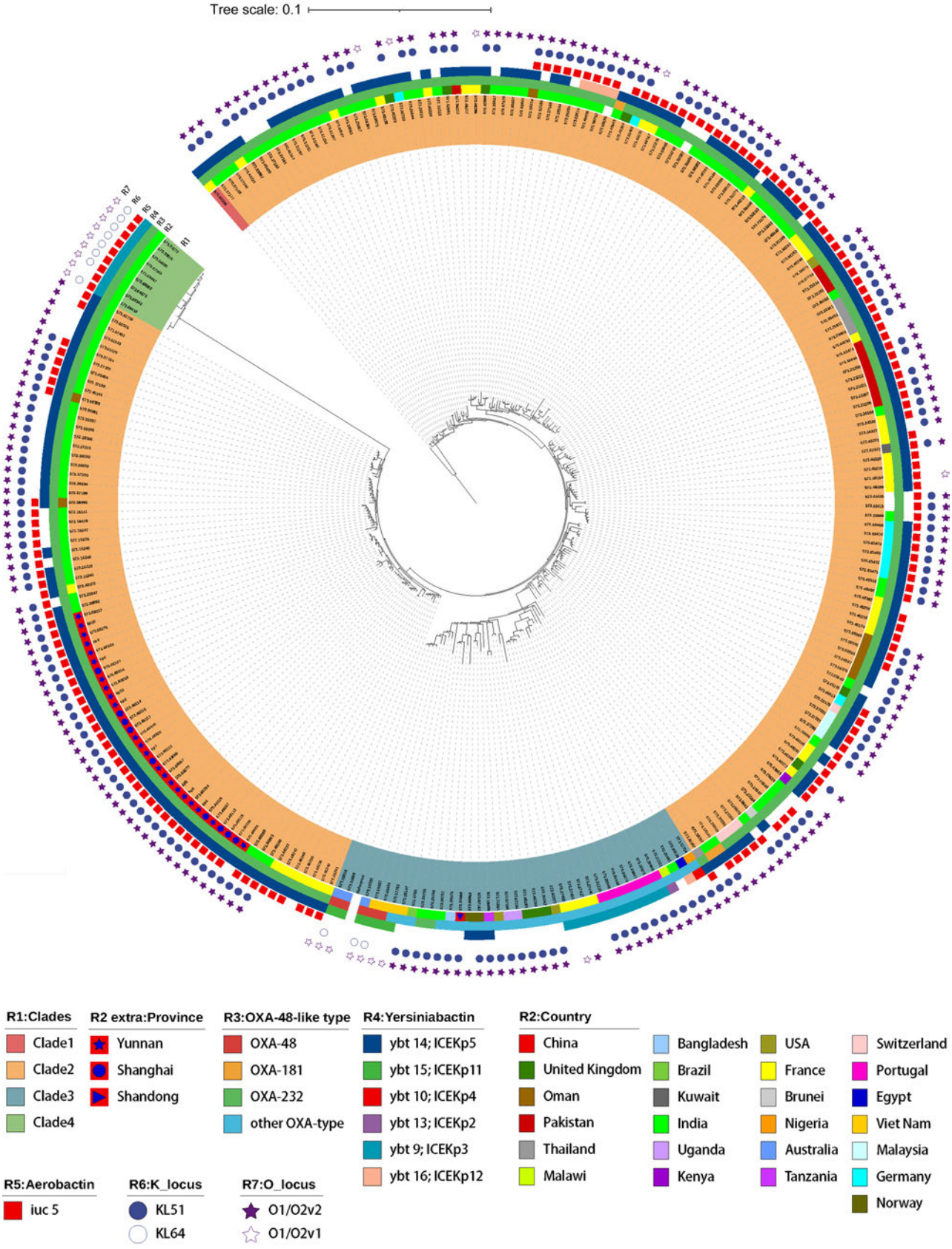

Fig. 3. Phylogenetic analysis of global ST231 *Klebsiella pneumoniae* isolates.

The phylogenetic tree constructed from core SNP analyses of 264 global ST231 isolates is color-coded to represent various clades (R1: Clades), geographical origins (R2: Country and extra: Province), and specific genetic characteristics (R3: OXA-48-like types; R4: Yersiniabactin types; R5: Aerobactin type, R6: K locus, R7: O locus). Each concentric ring corresponds to different attributes, such as antibiotic resistance genes, toxin production genes, and other relevant genetic markers. The innermost circle shows phylogenetic relationships based on whole-genome sequencing data.

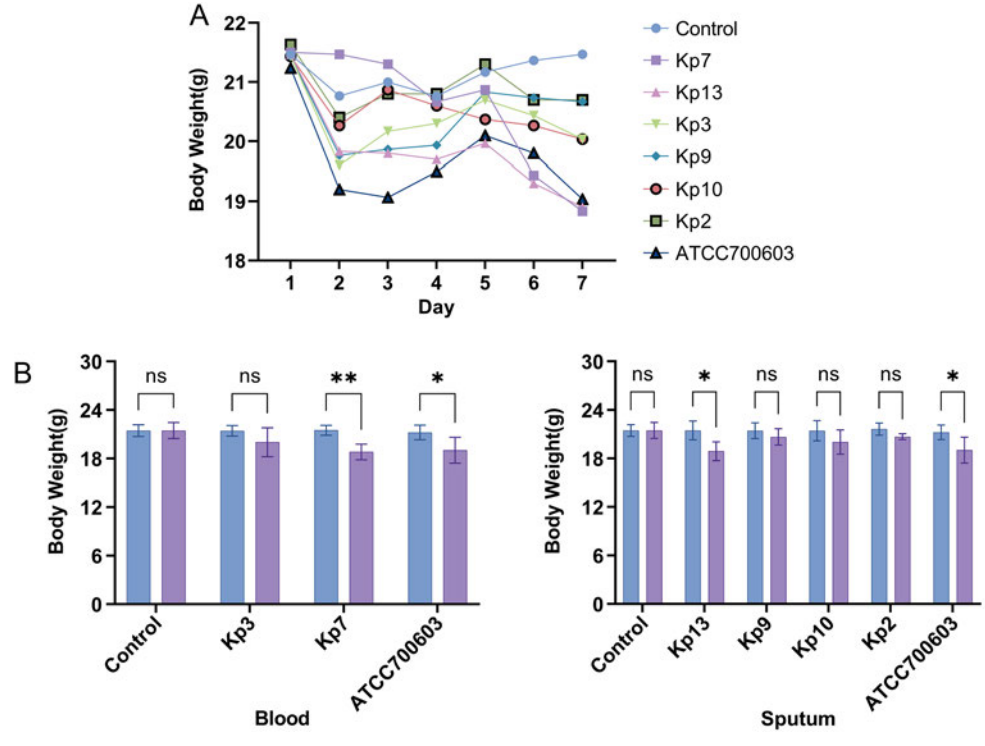

Fig. 4. Weight change and survival analysis of mice infected with ST231 *Klebsiella pneumoniae*.
Six-week-old male C57BL/6 mice, grouped in trios, were inoculated intranasally with 30 µl of 10^8 CFU/ml bacterial suspension. Health monitoring occurred daily for seven days.
a) Daily average weight fluctuations among mice infected with different ST231 *K. pneumoniae* strains;
b) comparison of mouse weight between day 1 and day 7 for different ST231 *K. pneumoniae* strains.
* – $p < 0.05$, ** – $p < 0.01$.
The standard strain ATCC® 700603™ as a positive control.

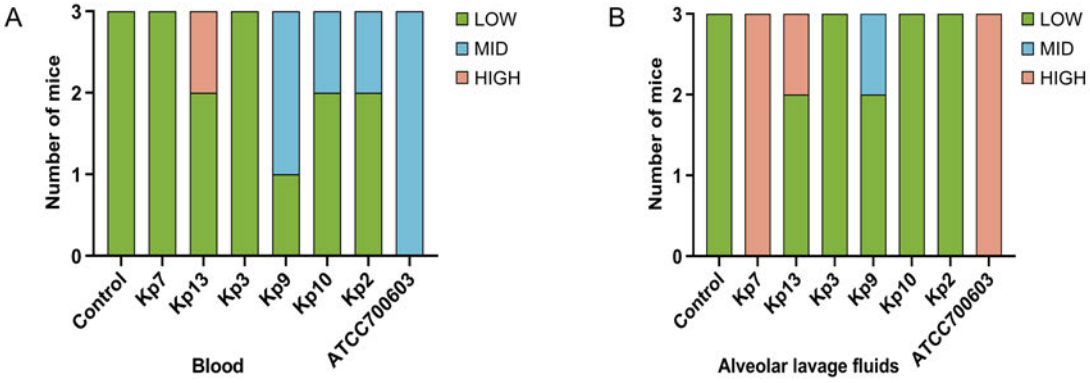

Fig. 5. Colony count analysis in mouse alveolar lavage fluids and blood samples post infection.
The growth levels, categorized into low (< 30%), medium (30–60%), and high (> 60%) based on percentage colony formation.
a) The colony counts from cultures of blood samples collected from mice;
b) the colony counts from cultures of alveolar lavage fluids obtained from mice.

a vital nutrient, allowing them to adapt to various hosts. The presence of yersiniabactin (ybtA, fyuA, irp2) and aerobactin (iucABCD, iutA) was consistent with previous research findings (Shen et al. 2023). A notable observation was the detection of an ST11 isolate carrying high-virulence genes (rmpA, rmpA2, peg-344) along with carbapenemase resistance genes (OXA-232, KPC-2). This combination represented a significant clinical challenge, highlighting *K. pneumoniae*'s capacity to accumulate multiple resistance and virulence determinants, increasing its public health threat.

A comparative analysis was conducted on 264 publicly available ST231 *K. pneumoniae* genomes to understand the global dissemination of these strains. A phylogenetic tree based on core SNPs grouped these genomes into four distinct clades. The largest clade predominantly included genomes from South Asia, East Asia, and Europe, with most ST231 isolates carrying $bla_{\text{OXA-232}}$

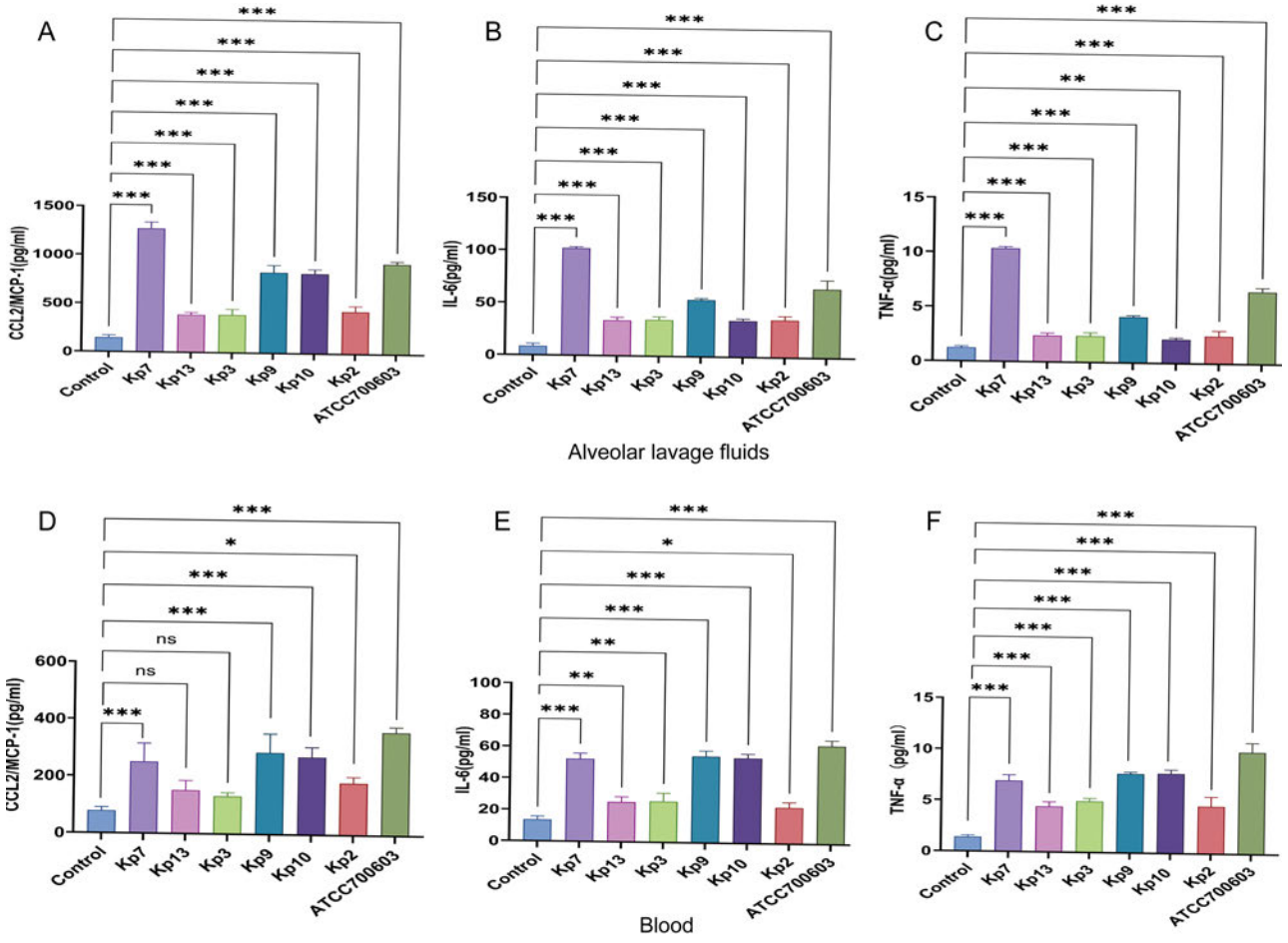

Fig. 6. Inflammatory response to ST231 infection in mice.

The levels of key inflammatory markers (CCL2/MCP-1, IL-6, and TNF-α) were quantified in the alveolar lavage fluid (a-c) and blood (d-f) of infected mice. The data from nine independent experiments.

* – $p < 0.05$, ** – $p < 0.01$. *** – $p < 0.001$. The standard strain ATCC® 700603™ as a positive control.

and other key virulence factors. Although the phylogenetic analysis revealed that these 10 isolates belonged to distinct strains with substantial divergence in their core genomes they exhibited a remarkable convergence in the number and types of plasmids and resistance genes. This striking convergence was likely driven by extensive horizontal gene transfer (HGT) of resistance determinants, facilitated by the selective pressures exerted by frequent antibiotic use in hospital environments. Such conditions created a permissive ecosystem for genetic exchange, enabling even phylogenetically unrelated strains to acquire similar resistance profiles. While the phylogenetic analysis suggested no immediate epidemiological threat, the convergence of resistance genes highlighted the latent risk of their accelerated dissemination through HGT, emphasizing the critical role of hospital settings as reservoirs and amplifiers of antibiotic resistance. These findings underscore the urgency of implementing stringent infection control measures and robust antibiotic stewardship programs to mitigate the spread of resistance genes and their far-reaching impact on clinical outcomes and public health.

CCL2/MCP-1, IL-6, and TNF-α were mediators of intercellular communication during the immune response. IL-6 was produced rapidly and transiently in response to infections and tissue injuries, promoting host defense by stimulating acute phase responses, hematopoiesis, and immune reactions (Tanaka et al. 2014). TNF-α was an inflammatory cytokine produced by macrophages/monocytes during acute inflammation, being responsible for various intracellular signaling events leading to necrosis or apoptosis (Idriss et al. 2000). CCL2/MCP-1 played a critical role in the inflammatory process by attracting and enhancing the expression of other inflammatory factors/cells. It facilitated the migration and infiltration of inflammatory cells, such as monocytes/macrophages, and other cytokines, to the inflammation site, contributing to the progression of various diseases (Singh et al. 2021). Therefore, these cytokines can serve as indicators of inflammatory responses. This enabled us to compare the intensity of inflammatory reactions elicited by different strains.

Consequently, we detected the concentrations of inflammatory factors CCL2/MCP-1, IL-6, and TNF-α

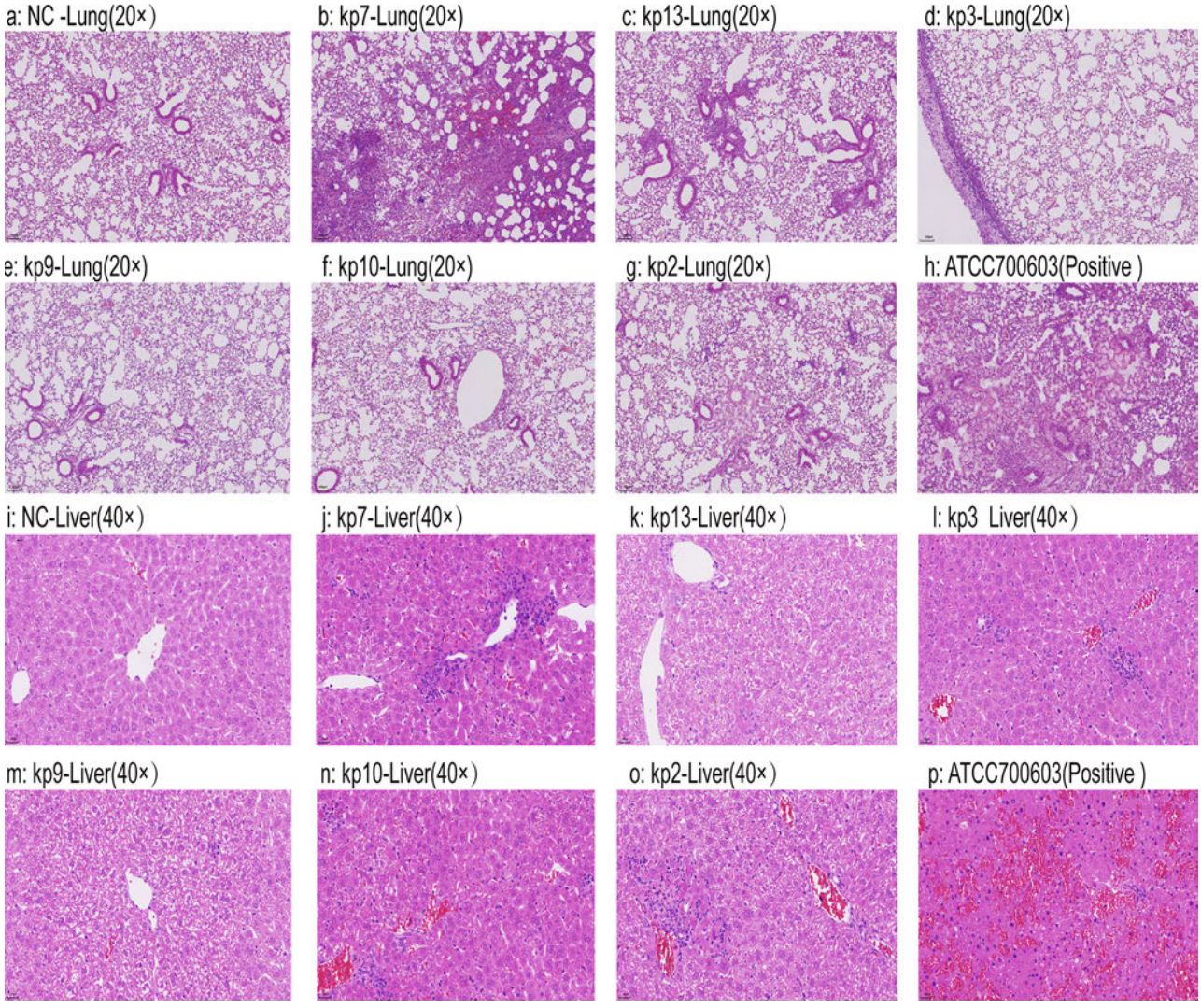

Fig. 7. Histopathological impact of ST231 isolates on mouse tissues.

The images, stained with hematoxylin and eosin, reveal significant tissue damage, particularly in the lungs (b–g) and livers (j–o), where extensive cellular infiltration and structural disruption are evident. Renal injury was not significant, and data are not shown.

in the alveolar lavage fluid and blood of mouse models. The results indicated that all strains presented statistically significant differences from the negative control, and the intensity of inflammatory reactions varied among different strains. For instance, the concentration of inflammatory factors in the alveolar lavage fluid of the Kp7 strain was higher than that of other strains, but the concentration of inflammatory factors in the blood of the Kp9 and Kp10 strains was lower than that of the Kp7 strain (Fig. 6). The lung tissue, as the primary site of respiratory infection, provided crucial information on inflammation and damage following intranasal inoculation. Previous reports on multi-drug-resistant *K. pneumoniae* isolated from patients with liver and renal abscesses have highlighted the significance of evaluating organ damage (Lee et al. 2008; Qian et al. 2023). These analyses will enhance our understanding of the systemic infection mechanisms of these highly resistant strains. Therefore, we collected the model mice's lung, liver, and kidney tissues for pathological analysis. It was found that although the strains invaded the blood of the model mice, there was no damage to the kidney tissues, while the lung and liver tissues showed apparent damage, and the degree of damage to organ tissues varied among each strain.

Despite consistent resistance profiles, the virulence of ST231 isolates showed significant variability. The presence of key iron-scavenging systems, such as the iuc cluster – a promising marker for high-virulence phenotypes (Shankar et al. 2021) – indicated their ability to adapt to iron-limited environments, increasing their pathogenic potential. However, mouse model experiments showed that while these isolates can invade the bloodstream and cause substantial inflammatory responses, their overall virulence remains moderate. This aligns with previous studies (Chen et al. 2023), suggesting that despite high resistance, the virulence of these strains may be less severe than expected. This

discrepancy could be attributed to variations in virulence gene expression, differences in regulatory pathways, or interactions with other unidentified virulence factors. Additionally, strain-specific traits, such as hypermucoviscosity (e.g., Kp13), may elicit varied host immune responses, leading to differing degrees of tissue damage. These findings highlight the need for further investigation into the interplay between virulence factors and host immunity to better understand the pathogenic mechanisms of *K. pneumoniae*.

Limitations and Future Directions

The study's limitations include a small sample size and limited geographical scope, making it difficult to draw broad conclusions. Additionally, while the respiratory infection model offered insights into virulence, it does not fully replicate the complexity of human infection. Future studies should involve more considerable, geographically diverse cohorts and use advanced genomic and proteomic analyses to clarify these isolates' transmission pathways and genetic evolution. In conclusion, this study underscores the need for active surveillance and large-scale genomic investigations to understand the distribution and genetic characteristics of OXA-232-producing *K. pneumoniae*, particularly ST231 strains. Enhanced monitoring will be essential to developing effective strategies to limit the spread of these high-risk clones and mitigate their clinical impact. In our research, the tested pathogenic factors are not the sole determinants of virulence and pathogenicity; other regulatory systems also play crucial roles in modulating these traits. Therefore, conducting further research and monitoring it is of great significance.

Availability of data and material

The complete gene sequences for all collected *K. pneumoniae* isolates are available in the GenBank database, accessible under the Accession No. PRJNA1072014.

Author contributions

All authors contributed to the study conception and design. Zhouxun Li participated in designing the study, in the collection of the isolates, in whole genome sequencing, in operating the animal experiments in data analysis and in drafting the manuscript. Chunyan Wu participated in data interpretation and in drafting the manuscript. Xuemei Cai participated in whole genome sequencing and in data analysis. Yongli Song participated in data analysis, in designing the animal experiments and operating the animal experiments. Xingping Zheng and Yuan He participated in Operating the animal experiments and in data analysis, and Guibo Song initiated the study, participated in its design, in the analysis of all data, in drafting the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by grants from Yunnan High-level Personnel training support plan (Grant No. YNWR-QNBJ-2020-261), Yunnan Fundamental Research Projects (Grant No. 202301AT070236), Funding Major Science and Technology Projects of Yunnan Province/First Affiliated Hospital of Kunming Medical University (Grant No. 2023zdpy05), and Special Foundation for National Science and Technology Basic Research Program of China (2019FY101200, 2019FY101209).

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Abdul Momin MHF, Liakopoulos A, Phee LM, Wareham DW. Emergence and nosocomial spread of carbapenem-resistant OXA-232-producing *Klebsiella pneumoniae* in Brunei Darussalam. J Glob Antimicrob Resist. 2017 Jun;9:96–99. https://doi.org/10.1016/j.jgar.2017.02.008

Aslan AT, Kırbaş E, Sancak B, Tanrıverdi ES, Otlu B, Gürsoy NC, Yılmaz YA, Tozluyurt A, Liste Ü, Bıçakcıgil A, et al; Study Group for Carbapenem Resistance (SCARE). A retrospective observational cohort study of the clinical epidemiology of bloodstream infections due to carbapenem-resistant *Klebsiella pneumoniae* in an OXA-48 endemic setting. Int J Antimicrob Agents. 2022 Apr;59(4):106554. https://doi.org/10.1016/j.ijantimicag.2022.106554

Bachman MA, Lenio S, Schmidt L, Oyler JE, Weiser JN. Interaction of lipocalin 2, transferrin, and siderophores determines the replicative niche of *Klebsiella pneumoniae* during pneumonia. mBio. 2012 Nov;3(6):e00224-11. https://doi.org/10.1128/mBio.00224-11

Bachman MA, Miller VL, Weiser JN. Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. PLoS Pathog. 2009 Oct;5(10):e1000622. https://doi.org/10.1371/journal.ppat.1000622

Boyd SE, Holmes A, Peck R, Livermore DM, Hope W. OXA-48-like β-lactamases: Global epidemiology, treatment options, and development pipeline. Antimicrob Agents Chemother. 2022 Aug;66(8):e0021622. https://doi.org/10.1128/aac.00216-22

Cerón S, Salem-Bango Z, Contreras DA, Ranson EL, Yang S. Clinical and genomic characterization of carbapenem-resistant *Klebsiella pneumoniae* with concurrent production of NDM and OXA-48-like carbapenemases in Southern California, 2016–2022. Microorganisms. 2023 Jun;11(7):1717. https://doi.org/10.3390/microorganisms11071717

Chen T, Xu H, Chen Y, Ji J, Ying C, Liu Z, Xu H, Zhou K, Xiao Y, Shen P. Identification and characterization of OXA-232-producing sequence Type 231 multidrug resistant *Klebsiella pneumoniae* strains causing bloodstream infections in China. Microbiol Spectr. 2023 Mar;11(2):e0260722. https://doi.org/10.1128/spectrum.02607-22

Chen Y, Fang L, Yang Y, Yan R, Fu Y, Shen P, Zhao D, Chen Y, Hua X, Jiang Y, et al. Emergence of carbapenem-resistant *Klebsiella pneumoniae* harbouring bla_{OXA-48}-like genes in China. J Med Microbiol. 2021 Mar;70(3):001306. https://doi.org/10.1099/jmm.0.001306

Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci USA. 2015 Jul;112(27):E3574-E3581. https://doi.org/10.1073/pnas.1501049112

Idriss HT, Naismith JH. TNFα and the TNF receptor superfamily: Structure-function relationship(s). Microsc Res Tech. 2000 Aug; 50(3):184–195. https://doi.org/10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H

Lan P, Jiang Y, Zhou J, Yu Y. A global perspective on the convergence of hypervirulence and carbapenem resistance in *Klebsiella pneumoniae*. J Glob Antimicrob Resist. 2021 Jun;25:26–34. https://doi.org/10.1016/j.jgar.2021.02.020

Lee BE, Seol HY, Kim TK, Seong EY, Song SH, Lee DW, Lee SB, Kwak IS. Recent clinical overview of renal and perirenal abscesses in 56 consecutive cases. Korean J Intern Med. 2008 Sep;23(3):140–148. https://doi.org/10.3904/kjim.2008.23.3.140

Lutgring JD, Zhu W, de Man TJB, Avillan JJ, Anderson KF, Lonsway DR, Rowe LA, Batra D, Rasheed JK, Limbago BM. Phenotypic and Genotypic characterization of *Enterobacteriaceae* producing oxacillinase-48-like carbapenemases, United States. Emerg Infect Dis. 2018 Apr;24(4):700–709. https://doi.org/10.3201/eid2404.171377

Mancini S, Poirel L, Tritten ML, Lienhard R, Bassi C, Nordmann P. Emergence of an MDR *Klebsiella pneumoniae* ST231 producing OXA-232 and RmtF in Switzerland. J Antimicrob Chemother. 2018 Mar;73(3):821–823. https://doi.org/10.1093/jac/dkx428

Mehrotra T, Konar D, Pragasam AK, Kumar S, Jana P, Babele P, Paul D, Purohit A, Tanwar S, Bakshi S, et al. Antimicrobial resistance heterogeneity among multidrug-resistant Gram-negative pathogens: Phenotypic, genotypic, and proteomic analysis. Proc Natl Acad Sci USA. 2023 Aug;120(33):e2305465120. https://doi.org/10.1073/pnas.2305465120

Poirel L, Héritier C, Tolün V, Nordmann P. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2004 Jan;48(1):15–22. https://doi.org/10.1128/aac.48.1.15-22.2004

Potron A, Rondinaud E, Poirel L, Belmonte O, Boyer S, Camiade S, Nordmann P. Genetic and biochemical characterisation of OXA-232, a carbapenem-hydrolysing class D β-lactamase from Enterobacteriaceae. Int J Antimicrob Agents. 2013 Apr;41(4):325–329. https://doi.org/10.1016/j.ijantimicag.2012.11.007

Qian C, Zhang S, Xu M, Zeng W, Chen L, Zhao Y, Zhou C, Zhang Y, Cao J, Zhou T. Genetic and phenotypic characterization of multidrug-resistant *Klebsiella pneumoniae* from liver abscess. Microbiol Spectr. 2023 Feb;11(1):e0224022. https://doi.org/10.1128/spectrum.02240-22

Shankar C, Basu S, Lal B, Shanmugam S, Vasudevan K, Mathur P, Ramaiah S, Anbarasu A, Veeraraghavan B. Aerobactin seems to be a promising marker compared with unstable RmpA2 for the identification of hypervirulent carbapenem-resistant *Klebsiella pneumoniae*: *In silico* and *in vitro* evidence. Front Cell Infect Microbiol. 2021 Sep; 11:709681. https://doi.org/10.3389/fcimb.2021.709681

Shankar C, Mathur P, Venkatesan M, Pragasam AK, Anandan S, Khurana S, Veeraraghavan B. Rapidly disseminating $bla_{OXA-232}$ carrying *Klebsiella pneumoniae* belonging to ST231 in India: Multiple and varied mobile genetic elements. BMC Microbiol. 2019 Jun; 19(1):137. https://doi.org/10.1186/s12866-019-1513-8

Shen S, Han R, Yin D, Jiang B, Ding L, Guo Y, Wu S, Wang C, Zhang H, Hu F. A nationwide genomic study of clinical *Klebsiella pneumoniae* carrying $bla_{OXA-232}$ and *rmtF* in China. Microbiol Spectr. 2023 Jun;11(3):e0386322. https://doi.org/10.1128/spectrum.03863-22

Singh S, Anshita D, Ravichandiran V. MCP-1: Function, regulation, and involvement in disease. Int Immunopharmacol. 2021 Dec; 101(Pt_B):107598. https://doi.org/10.1016/j.intimp.2021.107598

Singh S, Pathak A, Fatima N, Sahu C, Prasad KN. Characterisation of OXA-48-like carbapenemases in *Escherichia coli* and *Klebsiella pneumoniae* from North India. 3 Biotech. 2023 May;13(5):134. https://doi.org/10.1007/s13205-023-03537-8

Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014 Sep;6(10): a016295. https://doi.org/10.1101/cshperspect.a016295

Wu Y, Jiang T, He X, Shao J, Wu C, Mao W, Jia H, He F, Kong Y, Wu J, et al. Global phylogeography and genomic epidemiology of carbapenem-resistant $bla_{OXA-232}$-carrying *Klebsiella pneumoniae* sequence type 15 lineage. Emerg Infect Dis. 2023 Nov;29(11):2246–2256. https://doi.org/10.3201/eid2911.230463

Wyres KL, Nguyen TNT, Lam MMC, Judd LM, van Vinh Chau N, Dance DAB, Ip M, Karkey A, Ling CL, Miliya T, et al. Genomic surveillance for hypervirulence and multi-drug resistance in invasive *Klebsiella pneumoniae* from South and Southeast Asia. Genome Med. 2020 Jan;12(1):11. https://doi.org/10.1186/s13073-019-0706-y