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ORIGINAL RESEARCH

Genomic and Phenotypic Characterization of Extensively Drug-Resistant Clinical *Klebsiella quasipneumoniae* subsp. *similipneumoniae* ST1929 Isolates

Jiawei Ding[1,*], Muli Xu[1,*], Yuanzhi Xia[2,*], Xilin Kang[3], Yan Yu[1], Jiyong Chang[1], Zidan Hu[1], Pei He[1], Yao Yao[1], Ni Shen[1], Wenlin Tai[4], Lei Feng[1]

[1]Department of Medical Laboratory, The Affiliated Yan'an Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China; [2]Department of Clinical Laboratory, The Second Affiliated Hospital of Xiamen Medical College, Xiamen, Fujian, People's Republic of China; [3]Department of Blood Transfusion, The Affiliated Yan'an Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China; [4]Department of Medical Laboratory, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China

*These authors contributed equally to this work

Correspondence: Wenlin Tai; Lei Feng, Email taiwenlin@kmmu.edu.cn; fngj2004@163.com

Background: *Klebsiella quasipneumoniae* subsp. *similipneumoniae* is an emerging member of the *K. pneumoniae* complex that is often misidentified in routine diagnostics. Its clinical relevance and genomic characteristics remain poorly understood.

Methods: Four *K. quasipneumoniae* subsp. *similipneumoniae* isolates were collected from hospitalized patients in different wards and identified by whole-genome sequencing. Antimicrobial susceptibility testing was conducted using the broth microdilution method. Phylogenetic, comparative genomic, and plasmid transfer analyses were conducted to elucidate genetic relatedness and resistance mobility. Virulence phenotypes were assessed by measuring capsule production, biofilm formation, serum resistance, and in vivo pathogenicity in a *Galleria mellonella* infection model.

Results: All four isolates belonged to the novel ST1929-KL159 clonotype and exhibited an extensively drug-resistant phenotype, with resistance to β-lactams, carbapenems, fluoroquinolones, tigecycline, and trimethoprim-sulfamethoxazole. Amikacin was the only consistently active agent; one isolate showed high-level resistance to polymyxin B due to a premature stop mutation in *mgrB* (Gln30*). Genomic analysis revealed the coexistence of $bla_{\text{NDM-1}}$ and the tigecycline resistance cluster *tmexC2D2-toprJ2* on a IncU plasmid, which transferred efficiently to *E. coli* recipients. Comparative genomics demonstrated strong similarity to multidrug-resistant plasmids from diverse *Enterobacterales*. Phylogenetic analysis confirmed the global distribution of *K. quasipneumoniae* subsp. *similipneumoniae* but highlighted the rarity of ST1929, with close relatedness among the four isolates suggesting recent nosocomial transmission. Despite the absence of classical virulence genes, ST1929 exhibited enhanced biofilm formation, moderate capsule production, and intermediate serum resistance, but low virulence in the *G. mellonella* model.

Conclusion: This study provides the first description of *K. quasipneumoniae* subsp. *similipneumoniae* ST1929, an extensively drug-resistant lineage harboring $bla_{\text{NDM-1}}$ and the tigecycline resistance cluster *tmexC2D2-toprJ2* on a transferable IncU plasmid. The findings underscore the need for accurate detection and surveillance of this rare lineage.

Keywords: *Klebsiella quasipneumoniae* subsp. *similipneumoniae*, ST1929, resistance, *tmexCD2-toprJ2*, virulence

Introduction

Klebsiella quasipneumoniae, a member of the *K. pneumoniae* complex, has emerged as a clinically relevant but historically underrecognized pathogen.[1,2] Originally classified as phylogroup KpII within *K. pneumoniae*, this species was formally recognized as a distinct taxonomic entity in 2014 and subsequently subdivided into two subspecies: *K. quasipneumoniae* subsp. *quasipneumoniae*, and *K. quasipneumoniae* subsp. *similipneumoniae*.[3] The close

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phylogenetic relationship between *K. quasipneumoniae* and *K. pneumoniae*, coupled with overlapping phenotypic characteristics, has historically led to frequent misidentification using conventional laboratory methods,[4] which may hinder timely diagnosis, appropriate antimicrobial therapy, and effective infection control interventions in clinical settings.

K. quasipneumoniae subsp. *similipneumoniae* represents a particularly concerning subset within this species complex, exhibiting remarkable adaptability to hospital environments and an alarming propensity for acquiring multidrug resistance determinants.[1,5] Unlike hypervirulent *K. pneumoniae* strains that typically cause community-acquired infections, *K. quasipneumoniae* subsp. *similipneumoniae* is primarily associated with hospital-acquired infections in immunocompromised patients.[6] Genomic evidence indicates its broad distribution across human, animal, and environmental reservoirs, suggesting its adaptability to diverse ecological niches.[5,7] Although generally considered less virulent, this subspecies demonstrates a remarkable propensity for acquiring multidrug resistance determinants, including carbapenemases and resistance to last-resort agents such as polymyxins and tigecycline.[2,8,9] These characteristics complicate therapeutic decision-making, posing significant infection control challenges.

Plasmid-mediated resistance mechanisms are of particular concern as they compromise the efficacy of last-resort antibiotics.[10,11] Notably, the recently identified *tmexCD-toprJ* efflux pump gene cluster in *Klebsiella* spp. confers transferable tigecycline resistance and frequently coexists with carbapenemase genes on mobile plasmids, posing a risk of pan-resistance and rapid horizontal dissemination across species.[12,13] The co-occurrence of multiple resistance genes on mobile genetic elements facilitates horizontal gene transfer.[9] Epidemiological studies have revealed high genetic diversity within *K. quasipneumoniae* subsp. *similipneumoniae*, while certain sequence types (STs) such as ST138, ST334, and ST367 have achieved global distribution and are linked to healthcare-associated outbreaks.[1,14,15] However, rare STs remain poorly characterized, and their resistance gene repertoires, plasmid dynamics, and potential for nosocomial transmission are not well understood. Moreover, clinical outcomes of infections caused by MDR *K. quasipneumoniae* are seldom documented, leaving critical gaps in both infection control and therapeutic strategies.

In this study, we report four extensively drug-resistant *K. quasipneumoniae* subsp. *similipneumoniae* isolates belonging to the rarely reported sequence type ST1929 and capsule type KL159, recovered from patients with severe infections in a tertiary hospital in China. We characterized their resistomes, plasmid architectures, phylogenetic relationships, transferability of resistance plasmids, and assessed phenotypic traits and in vivo virulence.

Materials and Methods

Bacterial Isolates and Clinical Data Collection

Between February 2024 and December 2024, a total of 82 non-duplicate carbapenem-resistant isolates initially identified as *K. pneumoniae* were recovered from clinical specimens of hospitalized patients at the Department of Medical Laboratory, the Yan'an Hospital of Kunming Medical University, Yunnan Province, China. Among these, four isolates simultaneously harboring both bla_{NDM} and the *tmexCD-toprJ* efflux pump gene cluster were identified by polymerase chain reaction (PCR) followed by Sanger sequencing. These isolates were obtained from different specimens, including blood, sputum, and cerebrospinal fluid. Initial species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Clinical and demographic data of the corresponding patients were retrieved from electronic medical records, including age, sex, ward of admission, underlying comorbidities, invasive procedures, antimicrobial therapy, and clinical outcomes.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was conducted for the four *K. quasipneumoniae* subsp. *similipneumoniae* isolates co-harboring bla_{NDM} and the *tmexCD-toprJ* gene cluster. Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024). The antimicrobial agents tested included: aztreonam, amikacin, ampicillin-sulbactam, piperacillin-tazobactam, cefuroxime, ceftazidime, ceftazidime-avibactam, imipenem, meropenem, gentamicin, levofloxacin, trimethoprim-sulfamethoxazole, tigecycline, and polymyxin B. Quality control was ensured by parallel testing with *E. coli* ATCC

25922 and *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603. MIC results were interpreted according to CLSI breakpoints, except for tigecycline and polymyxin B, which were interpreted based on the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024).

Whole Genome Sequencing and Analysis

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Whole-genome sequencing was performed on the Illumina NovaSeq 6000 platform, generating 150-bp paired-end reads. One representative isolate was subjected to third-generation sequencing on the PacBio platform to explore the complete genomic and plasmid architecture. Raw Illumina reads were quality-filtered using Trimmomatic v0.39,[16] and hybrid genome assembly was carried out with Unicycler v0.5.0.[17] Assembly quality was assessed using QUAST v5.0.[18] For genotyping and genomic characterization, Kleborate v2.0.0 (http://github.com/katholt/Kleborate) was employed to determine multilocus sequence type (MLST), capsule (K) and lipopolysaccharide O-antigen serotypes and to predict acquired antimicrobial resistance genes and virulence-associated loci, including yersiniabactin, aerobactin, salmochelin, *rmpA*/*rmpA2*, and colibactin.[19] Plasmid replicon typing was performed using the PlasmidFinder database,[20] and resistance determinants were further screened with Abricate (https://github.com/tseemann/abricate) against the ResFinder database (https://bitbucket.org/genomicepidemiology/resfinder_db). Comparative genomic analyses were conducted using BRIG for circular genome visualization and Clinker for linear gene cluster comparison.[21,22]

Conjugation Assay

A conjugation experiment was performed to evaluate the transferability of resistance plasmids from ST1929 *K. quasipneumoniae* subsp. *similipneumoniae* to the recipient strain *E. coli* J53 (sodium azide-resistant). Both donor and recipient strains were cultured in Luria-Bertani (LB) broth at 37°C until reaching the logarithmic growth phase. Equal volumes of donor and recipient cultures were mixed at a 1:1 ratio and centrifuged at 8000 × g for 1 min. The resulting pellet was gently resuspended in 20 µL of 10 mM $MgSO_4$ and spotted onto LB agar plates. Plates were incubated overnight at 37°C to allow conjugation. After incubation, bacterial spots were scraped, serially diluted with sterile saline, and plated onto selective agar containing sulfamethoxazole (2 mg/L), tigecycline (2 mg/L), and sodium azide (100 mg/L) to screen for transconjugants. Conjugation frequency was calculated as the ratio of transconjugants to donor cells. All putative transconjugants were subjected to PCR amplification to verify the presence of target resistance genes, confirming successful plasmid transfer. Species identity of transconjugants was further confirmed using the VITEK 2 Compact system. Primer sequences used for PCR amplification of resistance genes are listed in Table 1.

Phylogenetic Analysis

To investigate the genetic background of sequence type ST1929 and its phylogenetic relationship with other *K. quasipneumoniae* subsp. *similipneumoniae* strains, we retrieved all available genome assemblies of this subspecies from the NCBI RefSeq database as of December 15, 2024. Genome quality was assessed using QUAST, and low-quality

Table 1 Primer Sequences Used for PCR Detection of Resistance Genes in Transconjugants

Name	Sequence
tmexC2-F	TTCCGTGATCTCCTATTTG
tmexC2-R	GATCGAGTTGCTGGATAC
tmexD2-F	TAGAGGAACTTCGGATTG
tmexD2-R	CTTCTCGGTGAACATGAT
toprJ2-F	TCAAGATAACGCAGGTAG
toprJ2-R	AAGTGACCAGCAACTATC
bla$_{NDM-1}$-F	CCAACGGTGATATTGTCA
bla$_{NDM-1}$-R	CAGCACACTTCCTATCTC

assemblies were excluded from further analyses.[18] To reduce redundancy and retain representative diversity, assemblies were dereplicated using Assembly-dereplicator (https://github.com/rrwick/Assembly-Dereplicator) with a similarity threshold of 0.001, effectively removing highly similar genomes. Multilocus sequence typing (MLST) was performed with the MLST tool (https://github.com/tseemann/mlst) to verify the sequence types across the dataset. The final dataset comprised 183 genomes, including the four isolates sequenced in this study. Core genome SNPs were identified and aligned using Snippy v4.6.0 (https://github.com/tseemann/snippy), with *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603 (RefSeq accession no. GCF_003181175.1) employed as the reference genome. To account for homologous recombination, the core SNP alignment was processed with Gubbins v3.2.1 to detect and mask recombinant regions.[23] A maximum-likelihood phylogenetic tree was constructed with IQ-TREE2,[24] and the final tree was visualized and annotated using the Interactive Tree of Life (iTOL) web platform.[25]

Capsule Quantification

Capsular polysaccharide production of *K. quasipneumoniae* subsp. *similipneumoniae* was quantified by uronic acid extraction and measurement, following previously described protocols.[26] Briefly, overnight cultures grown in LB broth were diluted 1:100 into fresh medium and incubated at 37°C for 6 h. A 500 µL aliquot of culture was mixed with 100 µL of 1% Zwittergent 3–12 detergent and heated at 50°C for 20 min. The mixture was centrifuged at 13,000 × g for 5 min, after which 300 µL of the supernatant was combined with 1.2 mL of absolute ethanol and centrifuged again at 13,000 × g for 5 min. The resulting pellet was air-dried and resuspended in 200 µL of sterile water, followed by the addition of 1.2 mL of tetraborate solution (12.5 mM sodium tetraborate in concentrated sulfuric acid). Samples were incubated at 100°C for 5 min and rapidly cooled on ice for at least 10 min. Subsequently, 20 µL of m-hydroxydiphenyl reagent was added, and the mixture was incubated at room temperature for 5 min. Absorbance was measured at 520 nm using a spectrophotometer to determine uronic acid content.

Serum Killing Assay

A serum killing assay was performed to evaluate the survival of *K. quasipneumoniae* subsp. *similipneumoniae* strains in human serum and their resistance to complement-mediated killing. Human serum was obtained from a healthy volunteer and separated by centrifugation. Mid-logarithmic phase bacterial cultures were prepared, and 50 µL of bacterial suspension (approximately 1×10^6 CFU) was mixed with 150 µL of pooled human serum in sterile microtubes. The mixtures were incubated at 37°C, and aliquots were collected at 0, 1, 2, and 3 h to assess bacterial survival. Viable counts were determined by serial dilution and plating onto LB agar, followed by incubation at 37°C for 24 h. Each assay was performed in triplicate for every strain tested.

Galleria mellonella Infection Model

G. mellonella larvae were randomly assigned to experimental groups. Each larva was injected with 10 µL of bacterial suspension containing approximately 1×10^6 CFU using a precision Hamilton syringe (Hamilton Company, Reno, NV, USA). Injections were administered into the last left proleg. Control larvae were injected with 10 µL of sterile 0.9% (w/v) sodium chloride solution. Following injection, larvae were incubated at 37°C in the dark. Survival was monitored every 12 h for 72 h. Larvae were considered dead when they showed complete melanization and no response to gentle stimulation. Each group consisted of 10 larvae, and all experiments were performed in triplicate.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Differences among groups were assessed by one-way analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

Results

Clinical Characteristics of the Patients

During the study period, four multidrug-resistant *K. quasipneumoniae* subsp. *similipneumoniae* isolates were recovered from blood, sputum, and cerebrospinal fluid specimens of four hospitalized patients. Clinical and demographic data are

Table 2 Clinical Characteristics of Four Patients Infected with *K. quasipneumoniae* subsp. *similipneumoniae* ST1929-KL159 Isolates

No.	Isolates	Sex	Age	Ward	Specimen	Diagnosis	Invasive Procedures	Underlying Disease(s)	Clinical Outcome	Hospital Stay (Days)
1	KPN28	M	67	ICU	Sputum	Hospital-acquired pneumonia (HAP)	None	COPD	Recovered	28
2	KPN32	F	54	ICU	Blood	Severe pneumonia with resp. failure	Mechanical ventilation	Type 2 diabetes mellitus	Recovered	46
3	KPN43	M	73	Respiratory ward	Sputum	Pulmonary infection	Central venous catheterization	Hypertension, CKD	Deceased	35
4	KPN59	M	45	Hematology ward	Cerebrospinal fluid	Pneumonia with bacteremia	Arterial catheterization, endotracheal intubation	Acute myeloid leukemia	Treatment abandoned	52

Abbreviations: BALF, bronchoalveolar lavage fluid; CSF, cerebrospinal fluid; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; ICU, intensive care unit.

summarized in Table 2. The cases occurred sporadically across different wards, including the respiratory department, intensive care unit, and hematology department. The affected patients were middle-aged to elderly (45–73 years), predominantly male, and all presented with severe pulmonary infections. Most patients had underlying comorbidities such as chronic obstructive pulmonary disease, diabetes mellitus, chronic kidney disease, or hematological malignancy. Three patients underwent invasive procedures. Clinical outcomes were variable: two patients improved after prolonged hospitalization, one patient abandoned therapy due to hematological disease, and one elderly patient with multiple comorbidities died of progressive respiratory failure. All patients had received broad-spectrum antimicrobial therapy prior to culture positivity. Initial empirical regimens typically included carbapenems (imipenem or meropenem) and β-lactam/β-lactamase inhibitor combinations (piperacillin–tazobactam or cefoperazone–sulbactam). After confirmation of multidrug resistance, polymyxin B was administered in three cases. Patient 1 responded to polymyxin B and was discharged after 28 days of hospitalization. Patient 2, with multiple comorbidities, showed no improvement despite polymyxin B therapy and died of respiratory failure. Patient 3 required prolonged mechanical ventilation but eventually improved with meropenem plus tigecycline combination therapy. Patient 4, diagnosed with acute myeloid leukemia, received successive courses of ceftazidime–avibactam, tigecycline, and polymyxin B, but persistent infection led to treatment abandonment.

Identification of ST1929-KL159 K. quasipneumoniae subsp. similipneumoniae Isolates

All four isolates exhibited a non-hypermucoviscous phenotype, as indicated by negative string tests. Initial species identification using MALDI-TOF MS misclassified the isolates as *K. pneumoniae*. However, subsequent whole-genome sequencing confirmed that they belonged to *K. quasipneumoniae* subsp. *similipneumoniae*, a closely related member within the *K. pneumoniae* complex. Genomic analysis revealed genome sizes ranging from 5.3 to 5.4 Mb, with GC contents of 57.3–57.4%. MLST assigned all isolates to sequence type ST1929, with the following allelic profile: *gapA* (18), *infB* (22), *mdh* (26), *pgi* (110), *phoE* (234), *rpoB* (20), and *tonB* (314). Capsule locus typing identified the KL159 capsule type, consistent with *wzi* allele 301. In silico O-antigen typing further classified all isolates as OL13. The resistome was highly conserved, with each genome harboring 14 resistance genes spanning nine antimicrobial classes. These included *aac(6')-Ib*, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV-12}}$ (variants with substitutions 238S, 240K, 35Q), *bla*$_{\text{TEM-1B}}$, *fosA, fosA3, mph(E), mph(A), catB8, qnrS1, oqxB, oqxA, sul1, tmexC2, tmexD2, toprJ2* and the chromosomal *bla*$_{\text{OKP-B-7}}$. Notably, KPN32 uniquely carried a nonsense mutation in *mgrB* (c.88C > T; p.Gln30*).

Resistance Characteristics of ST1929-KL159 K. quasipneumoniae subsp. similipneumoniae Isolates

Antimicrobial susceptibility testing demonstrated that all four ST1929-KL159 isolates exhibited an extensively drug-resistant phenotype (Table 3). The strains were uniformly resistant to β-lactam/β-lactamase inhibitor combinations (ampicillin–sulbactam, piperacillin–tazobactam, and ceftazidime–avibactam), cephalosporins (cefuroxime and

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Table 3 Antimicrobial Susceptibility Profiles of Four ST1929-KL159 *K. quasipneumoniae* subsp. *similipneumoniae* Isolates

Antimicrobial Agent	KPN28	KPN32	KPN43	KPN59
Aztreonam	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Amikacin	16 (S)	16 (S)	16 (S)	16 (S)
Ampicillin-sulbactam	>32/16 (R)	>32/16 (R)	>32/16 (R)	>32/16 (R)
Piperacillin-tazobactam	32/4 (R)	64/4 (R)	64/4 (R)	32/4 (R)
Cefuroxime	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Ceftazidime	>128 (R)	>128 (R)	>128 (R)	>128 (R)
Ceftazidime-avibactam	>16/4 (R)	>16/4 (R)	>16/4 (R)	>16/4 (R)
Imipenem	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Meropenem	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Gentamicin	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Levofloxacin	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Trimethoprim-sulfamethoxazole	>4/76 (R)	>4/76 (R)	>4/76 (R)	>4/76 (R)
Tigecycline	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Polymyxin B	1 (S)	32 (R)	1 (S)	1 (S)

Note: MICs are expressed in mg/L.
Abbreviations: R, resistant; I, intermediate; S, sensitive.

ceftazidime), the monobactam aztreonam, and carbapenems (imipenem and meropenem), indicating broad resistance to β-lactam agents, including third-generation cephalosporins and carbapenems. Beyond β-lactams, all isolates were resistant to gentamicin, levofloxacin, tigecycline, and trimethoprim–sulfamethoxazole, further limiting available therapeutic options. Amikacin remained the only agent within the susceptible range. Polymyxin B retained activity against three isolates (KPN28, KPN43, and KPN59; MIC = 1 mg/L), whereas KPN32 exhibited high-level resistance (MIC = 32 mg/L).

Genomic and Plasmid Characteristics of ST1929-KL159 K. quasipneumoniae subsp. similipneumoniae KPN28

Long-read sequencing of KPN28 yielded a complete genome comprising a 5.17 Mb chromosome and two plasmids, pKPN28-1 (313 kb, IncU, rep_cluster_1254) and pKPN28-2 (104 kb, IncFIB/IncFII) (Table 4). The chromosome encoded the intrinsic β-lactamase *bla*$_{\text{OKP-B-7}}$, along with *fosA* and the efflux pump genes *oqxA*/*oqxB*. The large plasmid pKPN28-1 harbored a broad array of resistance determinants, including *aac(6')-Ib, bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-1B}}$, *fosA3, mph(E), mph(A), catB8, qnrS1, sul1* and the tigecycline efflux cluster *tmexC2D2-toprJ2*. pKPN28-2 did not carry any resistance or virulence genes.

As shown in Figure 1A, comparative genomic analysis revealed that the large IncU-type plasmid pKPN28-1 (313,512 bp) shared extensive sequence similarity with previously reported multidrug-resistant plasmids. It exhibited the highest homology with *K. pneumoniae* plasmid pNB5_NDM (99% query cover, 99.71% identity; CP092653.1) and *K. quasipneumoniae* plasmid pNUITM-VK10 (95% cover, 99.47% identity; AP025166.1). Substantial similarity was

Table 4 Genomic and Plasmid Characteristics ST1929-KL159 *K. quasipneumoniae* subsp. *similipneumoniae* KPN28

Characteristic	KPN28		
	Chromosome	**pKPN28-1**	**pKPN28-2**
Size (bp)	5,168,234	313,512	104,153
GC content (%)	58.05	47.95	49.89
Incompatibility group	/	IncU, rep_cluster_1254	IncFIB/IncFII
Resistance genes	*bla*$_{\text{OKP-B-7}}$, *fosA, oqxB, oqxA*	*aac(6')-Ib, bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-1B}}$, *fosA3, mph(E), mph(A), catB8, qnrS1, sul1, tmexC2, tmexD2, toprJ2*	/
Virulence genes	/	/	/

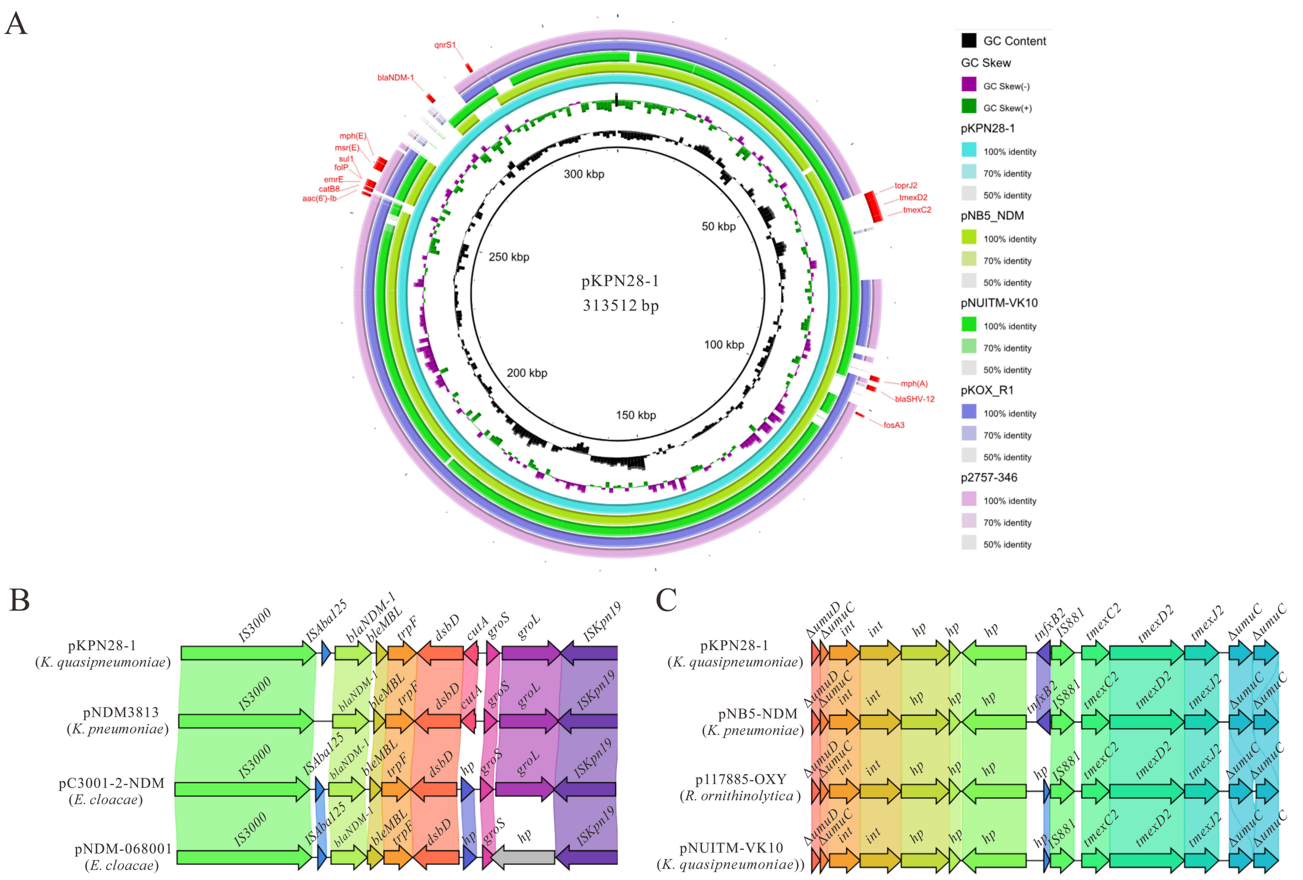

Figure 1 Comparative genomic analysis of the multidrug-resistant plasmid pKPN28-1 from ST1929 *K. quasipneumoniae* subsp. *similipneumoniae* isolate KPN28. (**A**) Circular comparison of IncU-type plasmid pKPN28-1 (313 kb, IncU). (**B**) Linear alignment of the *bla*$_{\mathrm{NDM-1}}$-containing resistance island in pKPN28-1 compared with homologous regions in plasmids from *K. pneumoniae* (pNDM3813, CP138523.1), *K. quasipneumoniae* (pC3001-2-NDM, CP039792.1), and *E. cloacae* (pNDM-068001, MZ156799.1). (**C**) Linear alignment of the *tmexC2D2-toprJ2* tigecycline resistance operon in pKPN28-1 and related plasmids from *K. pneumoniae* (pNB5_NDM, CP092653.1), *K. quasipneumoniae* (pNUITM-VK10, AP025166.1), and *R. ornithinolytica* (p117885-OXY, MT549900.1).

also detected with plasmids from *K. michiganensis* (78% cover, 99.98% identity; CP003684.1) and *K. variicola* (74% cover, 100% identity; CP060810.1).

Structural comparison of resistance regions provided further insights into their genetic context. As shown in Figure 1B, the *bla*$_{\mathrm{NDM-1}}$ locus in pKPN28-1 was embedded within a conserved resistance island flanked by ISCR1 and IS5075, with the structure: *ISKpn19-groL-groS-cutA-dsbD-trpF-ble*$_{\mathrm{MBL}}$*-bla*$_{\mathrm{NDM-1}}$*-ISAba125-IS3000*. This configuration was highly conserved among plasmids from *K. pneumoniae* (pNDM3813, CP138523.1), *K. quasipneumoniae* (pC3001-2-NDM, CP039792.1), and *E. cloacae* (pNDM-068001, MZ156799.1). In addition, the *tmexC2D2-toprJ2* cluster in pKPN28-1 displayed a conserved structural context (Figure 1C). The operon was flanked by *umuC*/*umuD*, forming the arrangement *IS881-tmexC2-tmexD2-toprJ2-umuC*/*umuD*. This structure was nearly identical to that observed in *K. pneumoniae* plasmid pNB5_NDM (CP092653.1), *K. quasipneumoniae* plasmid pNUITM-VK10 (AP025166.1), and *R. ornithinolytica* plasmid p117885-OXY (MT549900.1), suggesting the horizontal transfer of this tigecycline resistance determinant across diverse Enterobacterales species.

Efficient Conjugative Transfer of Resistance Plasmids in ST1929-KL159 K. quasipneumoniae subsp. similipneumoniae

To assess the transferability of resistance plasmids, conjugation assays were performed using E. coli J53 as the recipient. Donor strain KPN28 successfully transferred its large IncU plasmid (pKPN28-1), which co-harbored *bla*$_{\mathrm{NDM-1}}$ and the *tmexCD2-toprJ2* tigecycline resistance cluster. Transconjugants were obtained on selective plates containing meropenem, tigecycline, and sodium azide, and the presence of resistance genes was confirmed by PCR. The conjugation frequency ranged from 1.57×10^{-6} to 4.35×10^{-6} per donor cell. These results demonstrate that IncU plasmids can mediate the co-transfer of carbapenem and tigecycline resistance, underscoring their critical role in the horizontal dissemination of last-line antimicrobial resistance determinants.

Phylogenetic Analysis of ST1929 K. quasipneumoniae subsp. similipneumoniae Strains

To investigate the evolutionary relationships and genetic diversity within *K. quasipneumoniae* subsp. *similipneumoniae*, a core-genome SNP-based phylogenetic analysis was performed using 179 publicly available genomes retrieved from the NCBI database together with the four ST1929-KL159 isolates characterized in this study (KPN28, KPN32, KPN43, and KPN59). As shown in Figure 2, the isolates exhibited a broad geographic and host distribution. Most isolates were recovered from human clinical samples (n = 146), whereas a smaller fraction was derived from animal sources (n = 27). The majority of strains originated from China (n = 48), followed by Japan (n = 23), Singapore (n = 18), the United States (n = 18), Thailand (n = 9), Brazil (n = 8), Norway (n = 6), Pakistan (n = 6), Australia (n = 4), and the United Kingdom (n = 4), indicating that this subspecies is globally disseminated. MLST revealed 10 distinct STs among the 183 genomes analyzed. The most prevalent STs were ST138 (n = 38), ST334 (n = 30), and ST367 (n = 20), each widely distributed across multiple geographic regions. In contrast, ST1929 was rarely reported. In total, five ST1929 isolates were identified, including the four sequenced in this study and one additional genome from the public database. The four

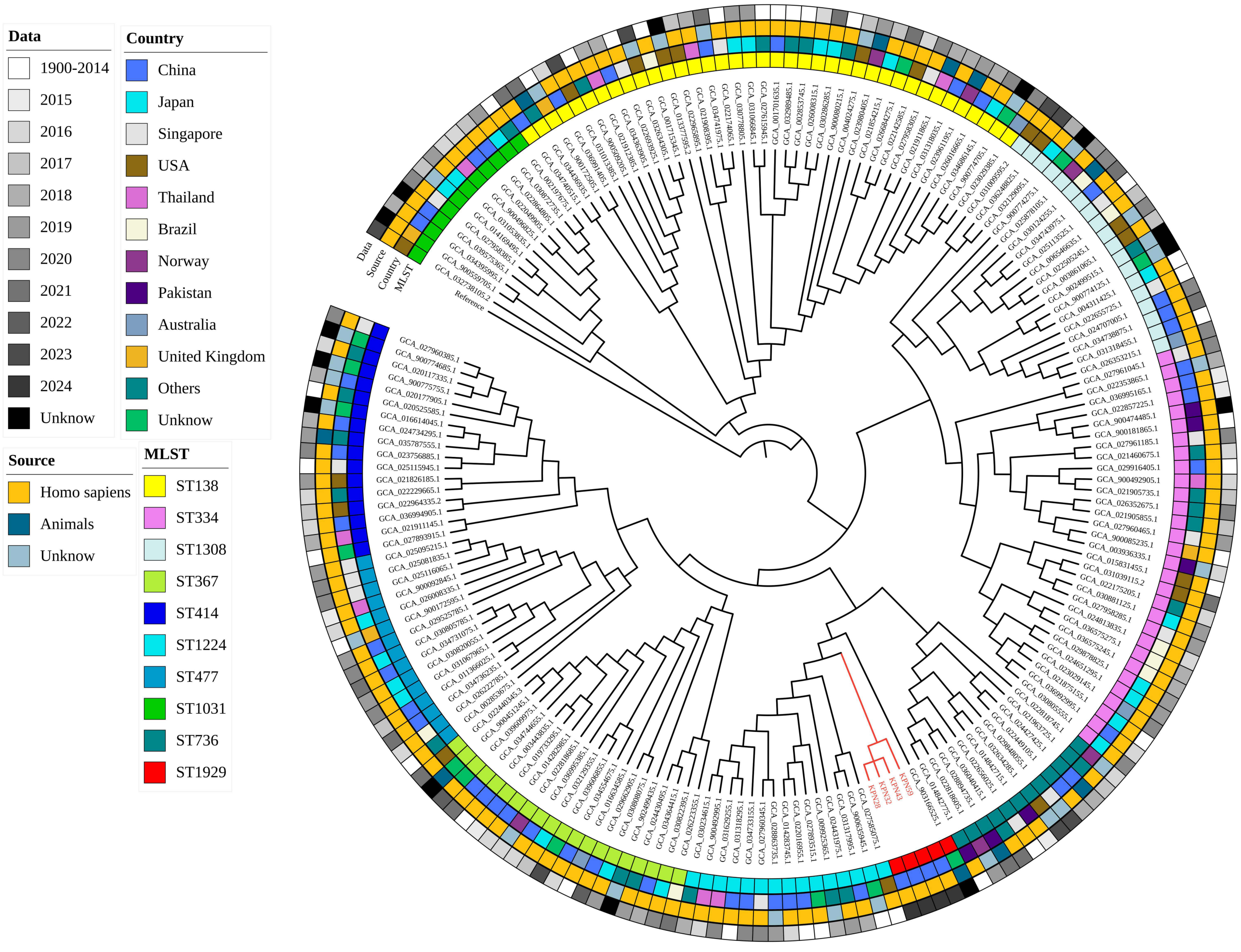

Figure 2 Global phylogenetic distribution of *K. quasipneumoniae* subsp. *similipneumoniae*. Core-genome SNP phylogeny reconstructed from 179 publicly available genomes together with the four ST1929-KL159 isolates sequenced in this study (KPN28, KPN32, KPN43, and KPN59; highlighted in red). Outer colored rings indicate year of isolation, source (host), country of origin, and MLST type.

isolates sequenced in this study formed a distinct monophyletic clade (highlighted in red in Figure 3) that was clearly separated from other STs, indicating a close genetic relationship. Pairwise SNP distances among these isolates ranged from 4 to 22 SNPs (median, 14). Notably, isolates KPN28 and KPN32 differed by only 4 SNPs, suggesting very recent common ancestry and raising the possibility of nosocomial transmission. The earliest known ST1929 genome was recorded in 2020 in Senegal (GCA_903166525.1), isolated from wild chimpanzees and termites.

Virulence Characteristics of ST1929-KL159 K. quasipneumoniae subsp. similipneumoniae Strains

To assess the pathogenic potential of the ST1929-KL159 isolates (KPN28, KPN32, KPN43, and KPN59), a series of phenotypic assays were conducted, including capsule production, biofilm formation, serum resistance, and virulence in the *G. mellonella* infection model. As shown in Figure 3A, quantification of uronic acid indicated that all four ST1929-KL159 isolates produced significantly more capsular polysaccharide than the classical reference strain ATCC 700603 ($P < 0.01$). However, their capsule levels remained markedly lower than those of the hypervirulent control strain NTUH-K2044 ($P < 0.0001$). Notably, all four ST1929-KL159 isolates exhibited strong biofilm-forming capacity compared with the controls. As shown in Figure 3B, crystal violet staining revealed mean OD_{600} values of 1.013 ± 0.072 (KPN28), 1.261 ± 0.092 (KPN32), 1.128 ± 0.064 (KPN43), and 1.073 ± 0.058 (KPN59). These levels were significantly higher than that of the classical reference strain ATCC 700603 (0.903 ± 0.078, $P < 0.05$), and markedly exceeded the hypervirulent control NTUH-K2044 (0.373 ± 0.055, $P < 0.0001$). Among the four isolates, KPN32 demonstrated the strongest biofilm production. Serum resistance assays demonstrated that all four ST1929-KL159 isolates exhibited enhanced survival

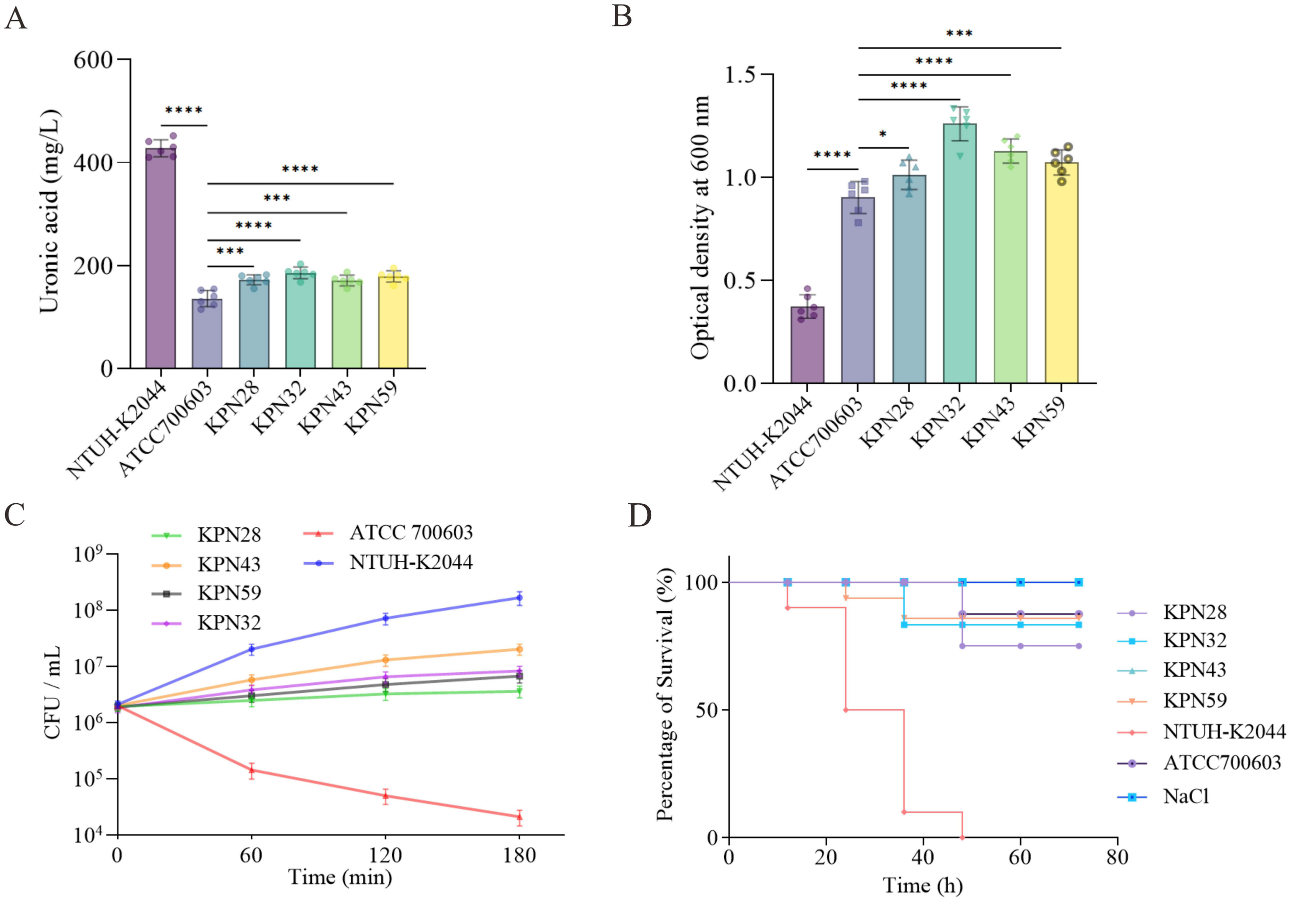

Figure 3 Virulence-associated phenotypes of ST1929-KL159 isolates. (**A**) Quantification of capsular polysaccharide production compared with classical control ATCC 700603 and hypervirulent control NTUH-K2044. (**B**) Biofilm formation measured by crystal violet staining at OD_{600}. (**C**) Serum survival assays. (**D**) *Galleria mellonella* infection model. Statistical significance was determined as follows: $P < 0.05$; $P < 0.01$; $P < 0.001$; $P < 0.0001$.

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compared with the serum-sensitive reference strain ATCC 700603, yet remained significantly more susceptible than the hypervirulent control NTUH-K2044 (Figure 3C). In the *G. mellonella* infection model (Figure 3D), larvae infected with the ST1929-KL159 isolates exhibited high survival, with ~80–90% of larvae remaining alive at 72 h post-infection. These survival rates were comparable to those observed in the ATCC 700603 and saline control groups. In contrast, infection with the hypervirulent control strain NTUH-K2044 caused rapid lethality.

Discussion

This study presents the characterization of the rarely reported *K. quasipneumoniae* subsp. *similipneumoniae* sequence type ST1929 and documents the emergence of extensively drug-resistant (XDR) isolates. Detection of this clone in multiple patients admitted to different wards illustrates the ongoing challenges in controlling nosocomial infections caused by members of the *K. pneumoniae* complex.

The clinical presentation of ST1929-KL159 infections in this study is consistent with previous descriptions of *K. quasipneumoniae* subsp. *similipneumoniae* as a healthcare-associated pathogen predominantly affecting immunocompromised or elderly patients.[6,27] The predominance of severe pulmonary infections in patients with multiple comorbidities reflects the opportunistic nature of this organism, which relies on host susceptibility rather than the hypervirulent phenotype observed in certain *K. pneumoniae* lineages.[28] The variable clinical outcomes, ranging from recovery to death or treatment discontinuation, highlight the therapeutic difficulties posed by XDR infections and the importance of timely and appropriate antimicrobial therapy.

A notable feature of the ST1929 isolates was the coexistence of multiple resistance determinants. All four carried *bla*$_{\text{NDM-1}}$, the plasmid-mediated tigecycline resistance cluster *tmexC2D2-toprJ2*, and additional genes conferring resistance to several antimicrobial classes. This combination severely restricted treatment options, with amikacin and polymyxin B remaining as the only active agents. Of concern, one isolate (KPN32) exhibited high-level polymyxin resistance due to a nonsense mutation in *mgrB*, leading to premature termination of the protein. Inactivation of *mgrB* is known to activate the PmrAB two-component system and induce lipopolysaccharide modifications in *Klebsiella* species.[29–31] Such mutations frequently emerge during or after polymyxin exposure, reflecting an adaptive response under antimicrobial pressure.[32]

Long-read sequencing provided further insight into the genomic context of resistance determinants. In KPN28, *bla*$_{\text{NDM-1}}$ was embedded within a conserved resistance island flanked by insertion sequences, a structural feature similar to plasmids described in *K. pneumoniae* and *Enterobacter spp*.[33,34] The *tmexC2D2-toprJ2* cluster was also located within a conserved operon structure shared across diverse Enterobacterales plasmids.[35] These findings suggest that *K. quasipneumoniae* can act both as a clinical pathogen and as a reservoir for high-risk plasmids within hospital environments.

The phylogenetic analysis confirmed the global dissemination of *K. quasipneumoniae* subsp. *similipneumoniae* and highlighted the rarity of ST1929. The close genetic relatedness of the four ST1929 isolates, particularly the minimal SNP differences observed between KPN28 and KPN32, is consistent with recent common ancestry and may indicate nosocomial transmission. Notably, the earliest reported ST1929 isolate originated from a non-human source in Senegal, pointing to a broader ecological distribution of this lineage. These findings raise questions regarding the evolutionary origins of ST1929 and the potential role of plasmid acquisition in facilitating adaptation to both environmental and clinical niches.

Although the isolates lacked the hypermucoviscous phenotype typically associated with hypervirulent *K. pneumoniae*,[36] they exhibited traits likely to promote persistence in healthcare environments. Enhanced biofilm formation, intermediate serum resistance, and moderate capsule production indicate the ability to survive on abiotic surfaces, resist host defenses, and colonize invasive devices. Notably, KPN32 combined high-level biofilm production with polymyxin resistance caused by a truncating mutation in *mgrB*. In the *G. mellonella* model, the ST1929 isolates showed low virulence compared with a hypervirulent control, consistent with their opportunistic behavior in immunocompromised hosts. Nevertheless, as demonstrated for other MDR organisms,[37] reduced intrinsic virulence does not preclude severe outcomes, particularly when therapeutic options are limited, as illustrated by the fatal case in this study.

The initial misidentification of the isolates as *K. pneumoniae* by MALDI-TOF MS underscores the limitations of conventional identification methods and the need for genomic approaches for accurate species assignment.[4,7] Such misclassification may obscure the epidemiology of *K. quasipneumoniae* and hinder appropriate infection control measures. The emergence of ST1929-KL159 as an extensively drug-resistant lineage with enhanced biofilm-forming capacity is therefore of concern. Of particular importance, we demonstrated efficient conjugative transfer of an IncU plasmid co-harboring $bla_{\mathrm{NDM-1}}$ and *tmexC2D2-toprJ2*. While IncF and IncHI plasmids are frequently associated with carbapenemase dissemination,[38,39] the involvement of IncU plasmids has been less well documented. Their broad host range raises the possibility of interspecies gene transfer across human, animal, and environmental reservoirs.[40–42]

Several limitations of this study should be noted. The small number of isolates restricts the generalizability of our findings, and larger-scale epidemiological investigations are needed to better define the prevalence and clinical impact of *K. quasipneumoniae* subsp. *similipneumoniae*. In addition, further in vivo studies would help to clarify the pathogenic potential of this subspecies. Finally, because epidemiological tracing of patient contacts and hospital environments was not performed, the possibility of unrecognized transmission events cannot be excluded.

In conclusion, this study provides the detailed description of *K. quasipneumoniae* subsp. *similipneumoniae* ST1929, revealing its extensive drug resistance, plasmid-mediated dissemination potential, and capacity for persistence in healthcare environments. The presence of carbapenem and tigecycline resistance determinants on a transferable IncU plasmid underscores its pivotal role in facilitating horizontal gene transfer and the spread of multidrug resistance. Although intrinsically less virulent than hypervirulent *K. pneumoniae* lineages, the emergence of this extensively drug-resistant clone highlights an urgent need for enhanced infection control surveillance and antimicrobial stewardship to prevent further transmission in hospital settings.

Data Sharing Statement

Genome sequence data from this study are available in the NCBI GenBank database under accession number PRJNA1310023.

Ethics Statement

The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All patient information was anonymized prior to analysis. The study was approved by the Institutional Ethics Committee of Yan'an Hospital of Kunming City, Yunnan Province, China (approval number: 2025-253-01). The requirement for informed consent from patients was waived by the Committee because the study was retrospective in design and only involved analysis of bacterial isolates that were collected as part of routine hospital laboratory procedures. In addition, human serum obtained from a healthy volunteer was used for the serum killing assay. Written informed consent was obtained prior to sample collection.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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